

**A CYTOTOXIC EVALUATION OF AFLATOXIN B₁,
ZEARALENONE AND THEIR EPOXIDE DERIVATIVES USING
HUMAN CELL LINES**

BY

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In memory of my grandmother

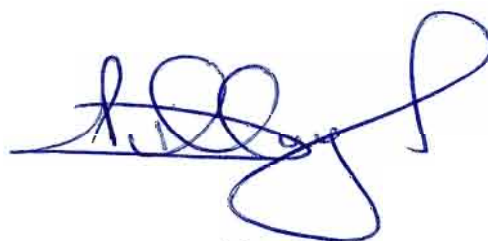
ABSTRACT

Since the discovery of mycotoxins in food, the thrust of biochemical and toxicological research has been carried out on animals which has proven to be uncoordinated and not easily extrapolated to humans. Over the last decade, there have been increasing pressures to review and reduce the use of animals in experimental toxicological studies. Consequently in this study aflatoxin B₁ (AFB₁), zearalenone (Zea) and their epoxide derivatives have been evaluated using *in vitro* assays. The HepG2, A549 and Hela cell lines were used for assessing the cytotoxicity, effects on cellular metabolism and sites of action of AFB₁, Zea and their derivatives. The cytotoxicity of these mycotoxins was evaluated using the methylthiazol tetrazolium (MTT) reduction assay. Cells, treated with mycotoxins were prepared for transmission electron microscopy (TEM), immunocytochemistry (ICC), scanning electron microscopy (SEM), confocal and light microscopy. From the cytotoxicity assay it was found that the epoxide derivatives were more toxic than the parent toxin when exposed to HepG2 cells with no significant differences in toxicity levels in A549 and Hela treated cells. Both epoxide derivatives displayed a regression of hepatoma cell proliferation at high doses (25µg/ml) while lower concentrations (<12.5µg/ml) enhanced cell growth. Microscopy analyses showed distinct cellular alterations. When exposed to AFB₁ (12.5µg/ml) hepatoma cells showed prominent ultrastructural alterations such as areas of cytoplasmic lysis and increased numbers of secondary lysosomes while cells exposed to Zea (12.5µg/ml) displayed numerous ovoid mitochondria and proliferation of rough endoplasmic reticulum which is indicative of enhanced protein synthesis. The presence of label in toxin treated cells is suggestive of the effects of these mycotoxins. Such cellular changes may lead to altered metabolism and cell function.

PREFACE

This study represents the original work by the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Department of Physiology, Faculty of Medicine, University of Natal, Durban, under the supervision of Mr A.A Chuturgoon and Professor M.F. Dutton.

A handwritten signature in blue ink, appearing to read 'D. Pillay', with a large loop at the end.

D. PILLAY

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Chapter 1

TOXICOLOGY

1.1 INTRODUCTION

As life began to evolve, the first macromolecules occurred as complex arrays with the basic attributes to life. This was accompanied by environmental factors that represented disruptive forces. These forces were not only physical but chemical as well. Chemicals existed in the early milieu of life that interacted with these life forms, disrupting the delicate balances through which they maintained their integrity (deBethizy and Hayes, 1989). Those life forms that developed protective mechanisms such as cell membranes and the ability to store energy were able to survive. These early life forms manufactured molecules capable of reacting with environmental chemicals and thus decreased their biological activity (deBethizy and Hayes, 1989). The next step in the evolution of protective mechanisms may have been the development of macromolecular catalysis to chemically alter disruptive chemicals. Today cellular mechanisms exist for metabolising the thousands of naturally occurring and synthetic toxins to which cells and cellular systems are exposed. Certainly not all mechanisms help to protect the living system and therefore toxicology has become an integral part of understanding the cellular environment.

1.2 The science of toxicology

The science of toxicology is a unification of a number of scientific disciplines (biochemistry, pharmacology and pathology) directed towards a common goal of identification, quantification and mechanistic explanation of adverse effects between a chemical substance and a living organism or biological system.

A major concern of individuals and societies has been and is the attainment of sufficient food to provide a healthful and productive life. Food is a complex chemical mixture containing nutrients and a vast diversity of other substances. Because food is of a biologic origin, it contains molecules important to the survival of the particular plant or animal species from which it was obtained. These molecules are xenobiotic to human biochemistry. They are more likely to produce adverse effects in humans. This study focuses on food-borne toxicants that are present as contaminants, secondary to microbial invasion of foods. These toxicants are referred to as mycotoxins and are produced in food ingredients that cause adverse biological effects when consumed in sufficient quantities.

Many adverse human health effects have been linked to the dietary contamination of foods with mycotoxins. Despite intensive research on mycotoxins the mode of their toxic action on humans is largely unknown. Currently there is great interest in the effects of mycotoxins on humans, in order to assess the dangers and likely results of populations being exposed to dietary mycotoxins in both first and third world countries.

1.3 Objectives

The three mycotoxins selected for investigation in this study were aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂) and zearalenone (Zea). The aflatoxins which are the most prominent mycotoxins in South Africa have been widely studied with AFB₁ being the focus of interest. Its carcinogenic properties are reputed to be related to its ability to form an epoxide in the liver. Consequently in this study the cytotoxic effects and mechanisms of action of these mycotoxins of importance in the South African context have been investigated using three human cell lines; namely, HepG2 (liver carcinoma cells); A549 (lung carcinoma cells) and HeLa (cervical carcinoma cells).

Chapter 2

LITERATURE REVIEW

2.1 MYCOTOXINS AND MYCOTOXICOSES

2.1.1 Introduction

“Wailing and writhing men collapsed in the street; others fell over and foamed in epileptic fits whilst some vomited and showed signs of insanity. Many of them shouted, “ Fire! I’m burning”. It was an invisible fire that separated the flesh from the bones and consumed it. Men, women and children died in unbearable agonising pain.”

These were the words used by a tenth century chronicler to describe a disease which affected many parts of Europe in 943 AD (Coker, 1995). The disease became known as “St Anthony’s fire” because of the burning sensation experienced by the victims, many of whom visited the shrine of St Anthony in France in the hope of being cured (Coker, 1995). “St Anthony’s fire” was caused by the consumption of rye contaminated with ergot alkaloids, compounds produced by moulds as secondary metabolites and referred to as mycotoxins (Coker, 1995).

Living organisms are continuously being exposed to an increasing number and variety of potentially harmful toxic compounds. The exposure of humans to fungal metabolites has become a major concern since the discovery of aflatoxins, a fungal metabolite produced by *Aspergillus flavus* and *A. parasiticus*, common and widely distributed food spoilage fungi (Groopman *et al.*, 1988). The discovery of the aflatoxin metabolites led to more intensive studies of mycotoxins and to the identification of a variety of compounds linked with adverse human health effects.

2.1.2 Mycotoxins

Mycotoxins are secondary metabolites which are products of metabolism whose formation is induced by the abnormal stress which results from limitations to normal growth patterns in the microbial cell (Weinberg, 1971). Several hypotheses have been postulated to explain the production of secondary metabolites in microorganisms. The suggestion that they have no function and are produced as a result of the breakdown in the regulation of the cells metabolism is considered unlikely in view of the intense pressure of natural selection which can be expected to occur. It has also been postulated that the specific products of secondary metabolism are not important but the process of secondary metabolism is of selective advantage to the organism. It is considered to provide a mechanism by which excess intermediates can be metabolised during adverse growth conditions. Such a mechanism would serve to maintain the cell in a functional state during conditions which prevented growth (Bu'lock, 1975). These adverse growth conditions occur when some environmental factor (nutrient availability, temperature and water activity) becomes limiting (Smith and Moss, 1985).

2.1.3 Conditions promoting fungal growth

Fungi are a major cause of deterioration and spoilage in stored crops. Spoilage fungi attack food and feed crops after harvest whenever environmental conditions become favourable for their proliferation (Smith & Moss, 1985). Moisture content of the seed and grain, its viability and physical state, ambient temperature, length of storage and activity of stored product insects are the main factors that determine both the initiation and extent of mould growth (Smith & Moss, 1985). The sources of contamination of any food commodity varies. Often the origin of fungi in food is the very buildings used for storage and ripening. The moulds may proliferate on the walls, ceilings and floors of these buildings. Spoilage of a product can manifest itself after several months of storage. Storage facilities include underground pits and storage bins.

The factors affecting fungal growth include; substrate composition, temperature, water availability and pH. Substrates differ in their ability to support fungal growth due to differences in their physical and chemical characteristics (Lebars 1982, as cited by Gqaleni, 1996). Physical characteristics include water activity, oxygen availability and surface area; while chemical characteristics include carbohydrates, fat and protein content, trace elements and amino acid composition. Moisture determines whether microbes can colonise a substrate or not (Gqaleni, 1996). It has been suggested that the most important factor in growth and aflatoxin production is the moisture surrounding the natural substrate (Smith and Moss, 1985). Although pH is usually easy to determine it is more difficult to control since the growth of fungi change the pH of the substrate (Gqaleni, 1996). Most fungi grow over a wide range of pH.

The proliferation of moulds on food may simply result in it having an unacceptable appearance. Mouldy foods are consumed throughout the world during times of famine, as a matter of taste and through ignorance of the adverse health effects. Modern toxicology has evolved rapidly during the past hundred years. The exponential growth of this discipline can be traced to the world war two era which marked the increase in the production of drugs, pesticides and chemicals. The threat of mycotoxins to human and animal health has become more important as the demand on the available food supply increases. The presence of mycotoxins is potentially the most serious quality problem which faces producers, manufacturers and handlers of food. The United Nations Food and Agricultural Organisation has estimated that up to 25% of the worlds foods are contaminated with mycotoxins (Smith and Moss, 1985). There is therefore this urgency to minimise their presence in foods.

The following diagram illustrates the factors affecting mycotoxin production

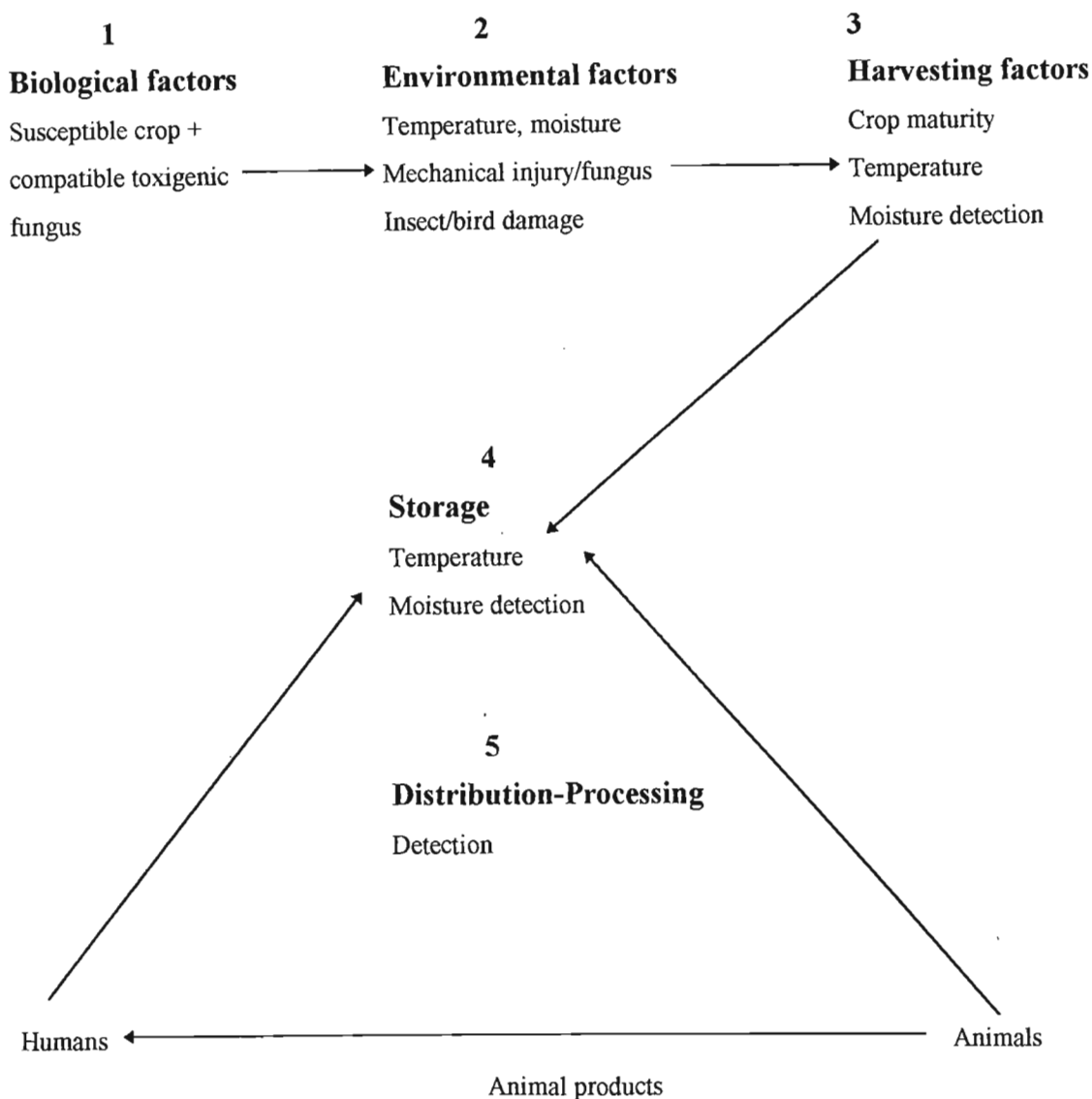


Fig 2.1 Factors affecting the occurrence of mycotoxins in the food chain (Smith *et al.*, 1994).

2.1.4 Mycotoxicoses

Mycotoxicoses has been defined as those illnesses of man and animals caused by toxigenic fungi (Moreau, 1970). In most instances toxigenic moulds that grow and proliferate on food products diffuse into it which is followed by the ingestion of these toxin contaminated foods and subsequent toxin-related diseases in man and animals. Mycotoxicoses are often associated with effects on the liver and kidney, and thus the fungal toxins are frequently hepatotoxic and nephrotoxic. A mycotoxicosis is essentially

characterised by being neither contagious nor infectious but by the fact that toxins are responsible. There exists three forms of mycotoxicoses; acute primary mycotoxicoses, chronic primary mycotoxicoses and secondary mycotoxin diseases.

Acute primary mycotoxicoses

Animals have been found to show marked signs of disease and death. Observed acute disease syndromes include hepatitis, haemorrhage and nephritis. Fortunately, natural contamination levels of mycotoxins are not high to allow for acute primary mycotoxicoses but instead promotes chronic primary mycotoxicoses (Smith *et al.*, 1994). Humans would normally avoid foods that are visually contaminated by moulds and thus human health problems resulting from exposure to acutely toxic levels are relatively rare (Smith *et al.*, 1994).

Chronic primary mycotoxicoses

There are no clearly defined macroscopic changes in the infected individual. Symptoms would appear in groups of animals as reduced productivity in the form of reduced egg production and reproductive efficiency.

Secondary mycotoxin disease

The biological effects of mycotoxins are chronic due to their wide variety of chemical structures. Some of these effects would be mutagenic, oestrogenic, carcinogenic and embryotoxic. This may also be accompanied by suppressed immunoresponses. Mycotoxins may enter the human dietary system by direct or indirect contamination of foods. Direct contamination occurs when the food becomes infected with a toxigenic fungus and subsequently produces the toxin. On the other hand indirect contamination occurs when an ingredient of a process is already contaminated with a toxin-producing fungus and while the fungus may be removed during processing, the mycotoxins still remain in the final product. The human body copes with these toxicants via a variety of metabolic processes essential in maintaining cell integrity.

2.1.5 Absorption, distribution and excretion of toxicants

The toxicity of any substance depends on the dose. Ultimately it is not the dose but the concentration of the toxicant at the site(s) of action that determines cytotoxicity. The same dose of two or more chemicals may lead to vastly different concentrations in a particular target organ of toxicity. This differential pattern may be due to differences in the disposition (absorption, distribution, biotransformation and excretion) of the xenobiotics. The various factors affecting disposition are depicted in figure 2.2 below.

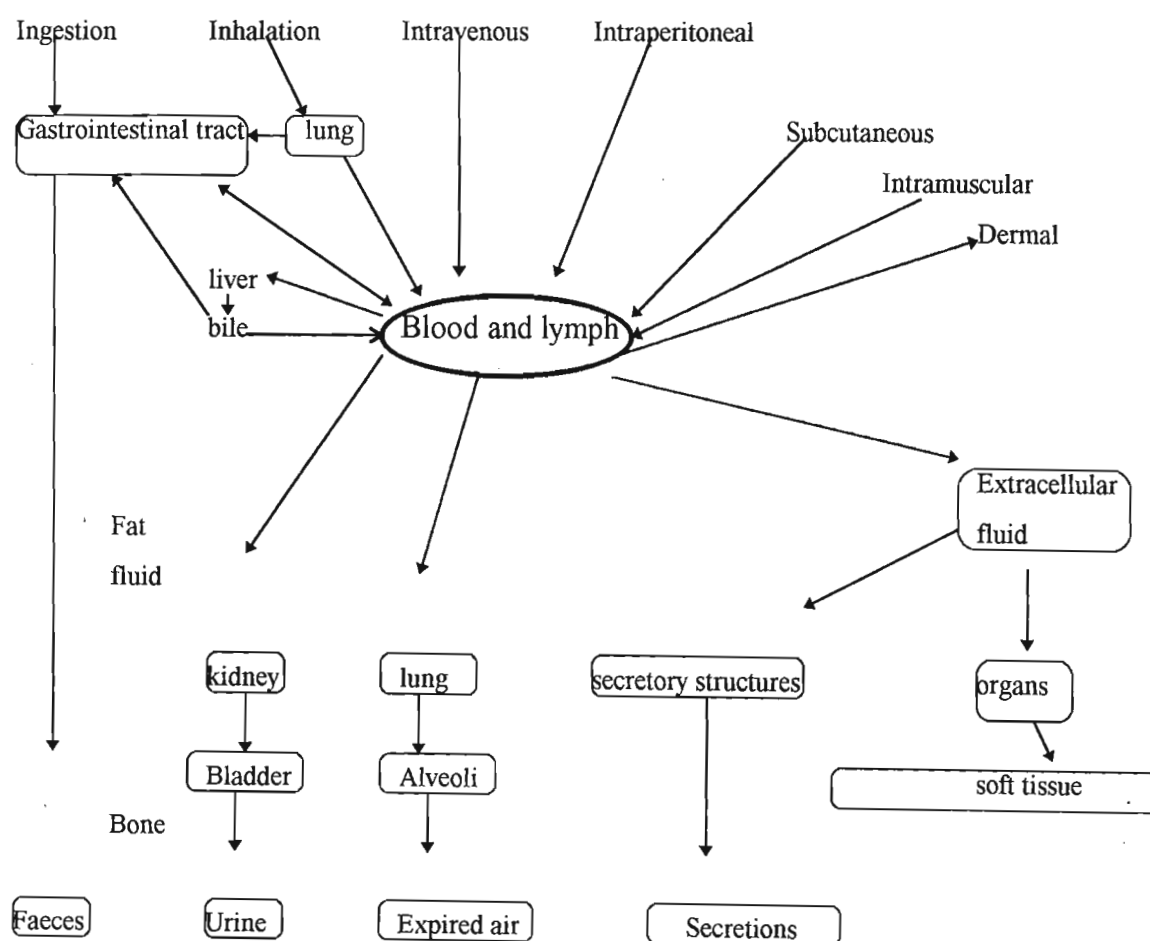


Fig.2.2 Routes of absorption, distribution and excretion of toxicants in the body (Klaasen and Rozman, 1991).

Any or all of these factors may have a minor or major effect on the concentration and thus the toxicity of a chemical in a target organ. If the amount of chemical absorbed is small then it may never reach sufficiently high concentrations at a

potential site of action to cause toxicity or the biotransformation may result in the production of a less or more toxic metabolite (Klaasen and Rozman, 1991). All of these processes are interrelated. The rate of excretion of a chemical may depend on its distribution and or biotransformation. If a chemical is distributed to and stored in fat, its elimination is likely to be slow.

Absorption

Absorption occurs when toxicants cross body membranes and enter the blood stream. The main sites of absorption are the gastrointestinal tract (GIT), lungs and the skin. The GIT is one of the most important sites where toxins are absorbed. Many environmental toxicants are absorbed together with food from the GIT. This tract is viewed as a tube traversing the body although in the body, its contents are considered exterior to it. The mammalian GIT has specialised transport systems (carrier-mediated) for the absorption of nutrients and electrolytes. After entering the blood, a toxicant is available for distribution.

Distribution

The rate of distribution to organs or tissues is primarily determined by blood flow and the rate of diffusion out of the capillary bed to the cells of the target organ (Klaasen and Rozman, 1991). The penetration of toxicants into the cells occurs by passive diffusion or special transport processes. Some toxicants accumulate in certain parts of the body due to protein binding, active transport or high solubility in fat. Several plasma proteins bind xenobiotics and some physiological constituents in the body. Albumin, the most abundant plasma protein, binds a large number of different compounds. Albumin serves as a depot and transport protein for both endogenous and exogenous compounds. Transferrin, a β_1 -globulin is important for the transport of iron in the body. The α and β lipoproteins are important for the transport of lipid soluble compounds such as vitamins and steroid hormones (Klaasen and Rozman, 1991). Liver and kidney have a high capacity to bind a multitude of chemicals. Many organic compounds are highly lipophilic and thus rapidly penetrate cell membranes and concentrate in body fat. The binding of compounds to plasma proteins, particularly albumin, is of special importance to toxicologists because severe toxic reactions can arise if a toxicant is displaced from plasma proteins by another agent thus increasing the free fraction of the former in the plasma (Klaasen and Rozman, 1991).

Excretion

The kidney is probably the most important organ for the excretion of xenobiotics. Many of the xenobiotics have to be biotransformed into more water-soluble products before they can be excreted into the urine. Other routes of elimination are via the faeces and the lungs.

Conclusion

When the rate of absorption exceeds the rate of elimination, toxic compounds may accumulate to a critical concentration at a certain target site and toxicity may result. Many chemicals have very low inherent toxic effects and need to be activated by biotransformation into toxic metabolites to elicit its effects as seen in figure 2.3 (Klaasen and Rozman, 1991). On the other hand a very potent toxicant may be detoxified rapidly by biotransformation. Thus a toxic response produced by a xenobiotic is critically influenced by the rate of absorption, distribution, biotransformation and excretion.

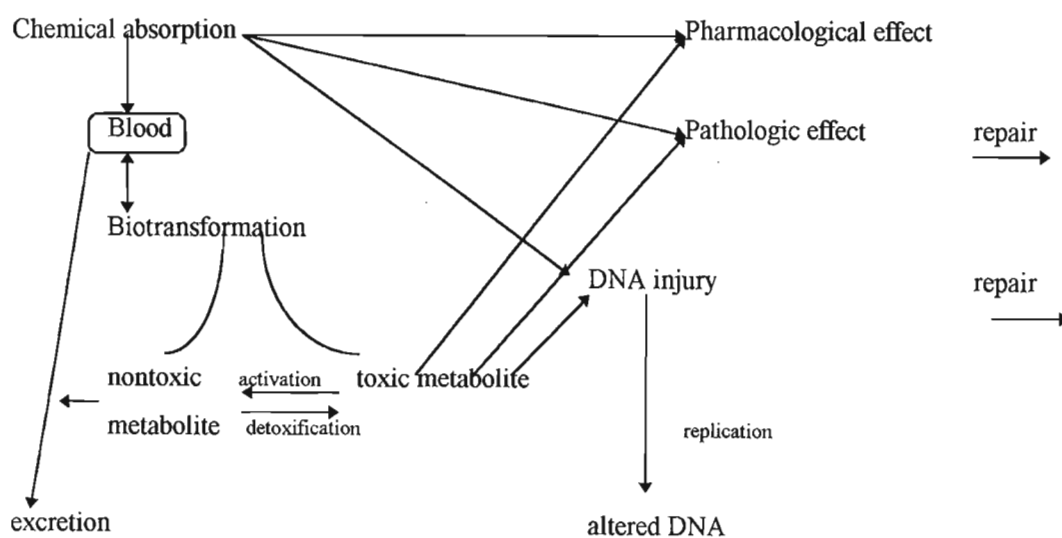


Fig 2.3 Schematic representation of the disposition and toxic effects produced by chemicals (Klaasen and Rozmann, 1991).

2.1.6 Biotransformation of toxicants

2.1.6.1 Introduction

The rate at which compounds are eliminated from the body, via the kidney depends on their water solubility. Biotransformation is the sum of the biochemical processes involved in converting lipophilic compounds to more hydrophilic metabolites (Sipes and Gandolfi, 1991). The metabolites produced are more hydrophilic than the parent compound. This increased water solubility reduces the ability of the metabolite to partition into biological membranes and therefore hinders this distribution to the various tissues and ultimately promotes the excretion of the chemicals. Since the liver is the most biochemically active organ in the metabolism of xenobiotics, the detoxification of chemical substances is frequently assessed using liver derived systems. Ultimately, the sensitivity of a biological system to toxic insult is defined and characterised by the presence of critical molecular targets. Of these macromolecules, proteins possess highly specific functional characteristics. In the case of nucleic acids, DNA is a critical toxicological target, through covalent binding of reactive chemical toxicants because of the known associations between chemical modification of DNA and mutagenicity and carcinogenicity (Sipes and Gandolfi, 1991). The biotransformation of foreign compounds within the liver is accomplished by its biphasic character involving several enzyme systems.

2.1.6.2 Phase 1 and phase 2 biotransformation

Various enzymes can biotransform lipid-soluble xenobiotics into water soluble compounds via phase 1 and phase 2 enzyme reactions. The former involves oxidation, reduction and hydrolysis whilst the latter consists of conjugation or synthetic reactions where the foreign compound or phase 1-derived metabolite is covalently linked to an endogenous molecule (e.g. glucuronic acid) producing a conjugate which is more water soluble (Sipes and Gandolfi, 1991). These conjugating moieties are added to the endogenous products to promote their secretion. These reactions are biosynthetic and require energy which are achieved by activating the cofactors or the substrates to high energy intermediates (Sipes and Gandolfi, 1991). Phase 1 reactions allow for the addition or removal of functional groups (e.g. -OH, -SH, NH₂, -COOH) (Sipes and Gandolfi, 1991). These functional groups allow the compound to undergo phase 2

reactions. The relationship between phase 1 and phase 2 reactions is illustrated in figure 2.4. Volatile organic compounds may be eliminated via the lungs with no biotransformation. Those compounds with functional groups may be conjugated directly while others undergo phase 1 reactions before conjugation. Biotransformation is complex and integrated.

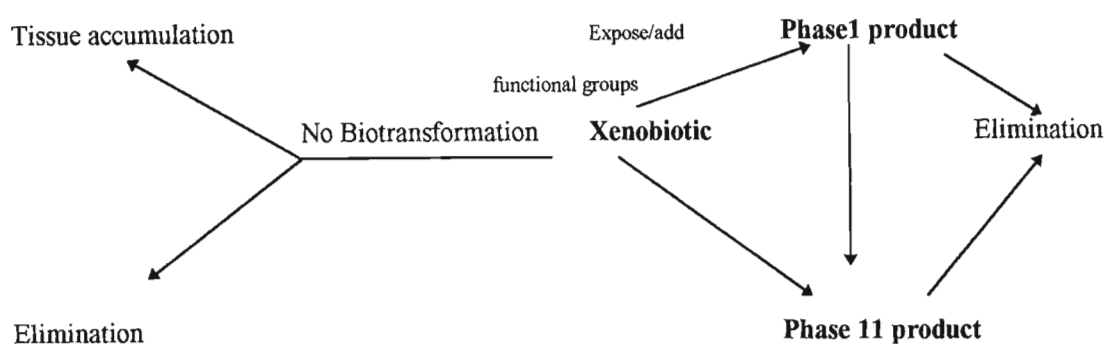


Fig 2.4 The integration of phase 1 and phase 2 biotransformation reactions (Sipes and Gandolfi, 1991).

The enzyme systems which catalyse the biotransformation of foreign compounds are localised primarily in the liver (Sipes and Gandolfi, 1991). The liver receives all the blood that has perfused the splanchnic area which contains nutrients and foreign substances, and thus it has developed the capacity to extract the nutrients and xenobiotic compounds readily from the blood and to chemically modify many of these substances before they are stored, secreted into bile or released into circulation (Sipes and Gandolfi, 1991). The phase 1 enzymes are located primarily in the endoplasmic reticulum (ER) and are membrane bound (deBethizy and Hayes, 1989). This implies that they are within a lipoprotein matrix which is essential since the lipophilic substrates will part into the lipid membranes, the site of biotransformation.

a) Phase 1 enzyme reactions

The most important enzyme systems involved in phase 1 reactions are the cytochrome P-450 (CP₄₅₀) containing monooxygenases (deBethizy and Hayes, 1989). The CP₄₅₀ system is a coupled enzyme system containing two enzymes: NADPH-CP₄₅₀ reductase and a haem-containing enzyme, CP₄₅₀. These enzymes are embedded in the phospholipid matrix of the ER as seen in figure 2.5) (Sipes and Gandolfi, 1991).

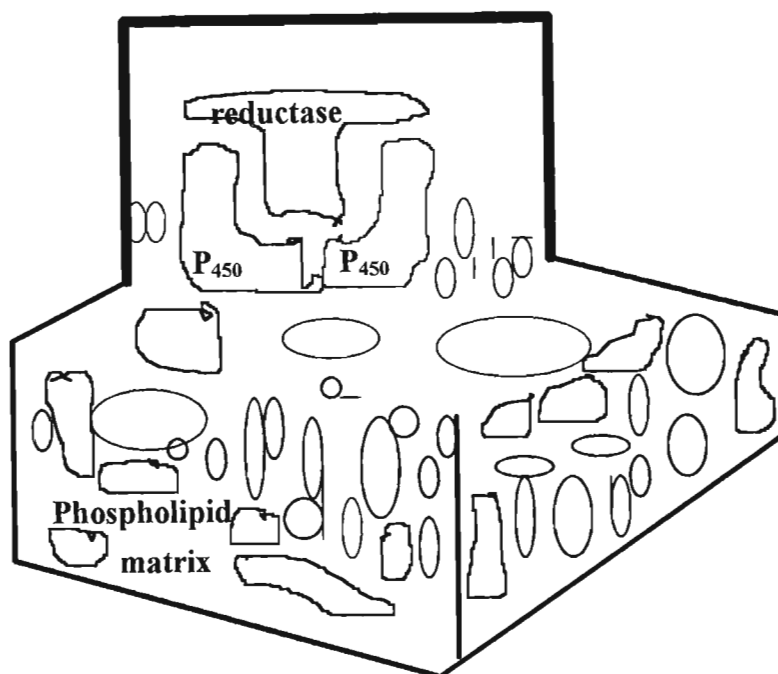


Fig 2.5. A schematic representation of the interaction of CP₄₅₀ reductase and lipid (adapted from Sipes and Gandolfi, 1991).

In reactions catalysed by CP₄₅₀, the substrate combines with the oxidised form of CP₄₅₀ (Fe³⁺) and forms a substrate-CP₄₅₀ complex (deBethizy and Hayes, 1989). This complex then accepts an electron from NADPH via NADPH CP₄₅₀ reductase which then reduces the iron in CP₄₅₀ haem molecule to the Fe²⁺ state. The reduced (Fe²⁺) substrate-CP₄₅₀ complex combines with molecular oxygen, which then accepts another electron from NADPH. The resulting oxygen species is highly reactive and unstable. One atom of this reactive oxygen is introduced into the substrate, while the other is reduced to water. The oxygenated substrate then dissociates, regenerating the oxidised form of CP₄₅₀ as seen in figure 2.6 (Sipes and Gandolfi, 1991).

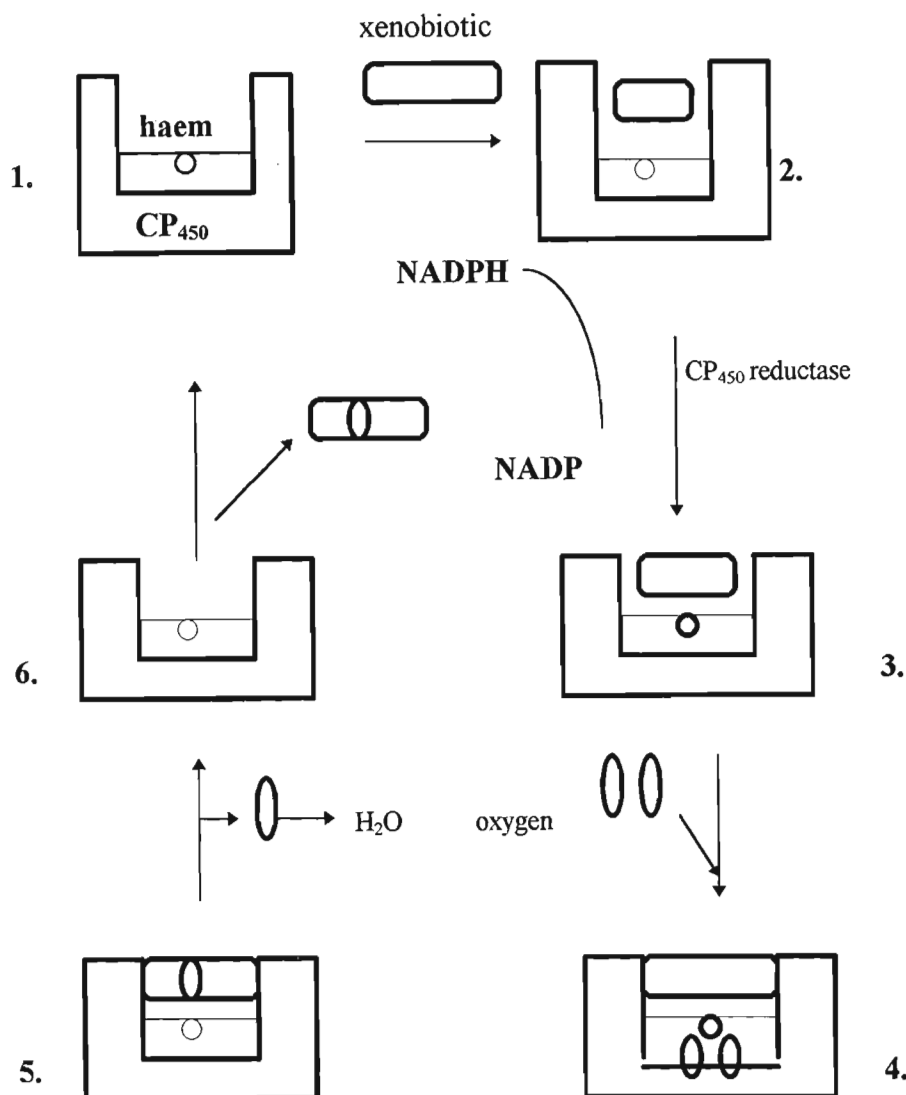


Fig 2.6 Cytochrome P₄₅₀ electron transport and oxidation of a xenobiotic (Sipes and Gandolfi, 1991).

The glucuronosyltransferase is the principal phase 2 enzyme. Glucuronidation represents an important phase 2 conjugation reaction in the conversion of monooxygenase products to form glucuronides. The general mechanism of conjugating enzymes involves the activation of an endogenous molecule to yield a high energy form. Subsequent reaction of this activated form of the endogenous molecule with the xenobiotic produces the conjugate (deBethizy and Hayes, 1989). Uridine diphosphate (UDP) glucuronosyltransferase is the enzyme that carries out the reaction. It catalyses the interaction between the high energy nucleotide UDP-glucuronic acid and the functional group on the acceptor molecule (substrate) (Sipes and Gandolfi, 1991). Similar to the monooxygenase, the glucuronosyltransferase is an integral part of the ER

and deeply embedded. Its interaction with the phospholipids of the membrane has a strong influence on its activity (deBethizy and Hayes, 1989). These enzymes also have a low order of substrate specificity which makes them ideally suited as detoxification enzymes.

The Glutathione S-transferase enzymes are capable of conjugating relatively hydrophobic electrophilic molecules. This conjugate is less lipophilic, more water soluble and likely to be excreted in the urine. The transferases facilitate the nucleophilic attack of glutathione thiolate ion on the electron deficient atom of the relatively hydrophobic electrophilic xenobiotic. These enzymes catalyse the initial step in the formation of N-acetyl cysteine (mercapturic acid) derivatives of a diverse group of xenobiotics (Sipes and Gandolfi, 1991). The cofactor for the reactions catalysed by these enzymes is the tripeptide glutathione (GSH) which is composed of glycine, glutamic acid and cysteine. The enzyme catalyses the reaction of the nucleophilic sulfhydryl of glutathione with compounds containing electrophilic carbon atoms (deBethizy and Hayes, 1989). Conjugation decreases the reaction of these compounds with essential constituents of the cell. Much evidence indicates that glutathione S-transferase acts to detoxify reactive intermediates produced by the CP₄₅₀ system (Sipes and Gandolfi, 1991). There is however a delicate balance between the rate of formation of the reactive metabolites and their inactivation by GSH. Factors which affect this balance can dramatically alter the toxic potential of chemicals that produce toxicity via reactive intermediates.

A literary review of zearalenone (Zea) and aflatoxin B₁ (AFB₁)

2.2 FUSARIOTOXICOSES

2.2.1 Introduction

Species of *Fusarium* cover a wide spectrum of activities ranging from fairly specific plant pathogens to those which are saprophytic on senescent plant materials. Due to their close association with plants and their relatively high water activity requirements for growth, fungi will have become established on a crop before harvest and may cause lots of problems in cereals following a late harvest after a wet summer (Smith and Moss, 1985). *Fusarium* species have been important for a number of years but in the last twenty years they have been extensively studied because the mycotoxins they produce can be a threat to animal and human health (Nelson *et al.*, 1995). The production of these natural products (mycotoxins) depends on environmental conditions such as temperature and the availability of nutrients. In many areas of the world the major cereal crops such as corn and wheat are particularly susceptible to infection by these *Fusarium* species. The effects of consumption will ultimately depend on the types and concentrations of the mycotoxins present. The fungi produce a large number of metabolites such as zearalenone and the tricothecenes. These singly or in combination are thought to be responsible for numerous mycotoxicoses of farm animals and humans. Thus world-wide efforts have been directed towards the detection, isolation, quantification and assessing the biochemical effects of these toxins produced in grains and other commodities.

To date, various *Fusarium* species have been analysed and their toxic properties investigated. Zearalenone (Zea), one of the less studied mycotoxins because of its speculative role in human diseases has now come to the forefront of investigations because of its oestrogenic properties such as anabolic and uterotrophic activities in cattle and lambs fed contaminated mouldy corn (Kiang *et al.*, 1978). There is now a growing public concern about the toxicological impact of Zea on humans since the recent occurrence of oestrogenic substances in food products, either as natural constituents or as contaminants (Goodman *et al.*, 1987). Their possible aetiological role in outbreaks of certain diseases is being evaluated. The presence of Zea, a secondary metabolite produced by *Fusaria*, in certain edible plants could constitute another potential source of oestrogen exposure to both animals and man (Goodman *et al.*, 1987).

2.2.2 Zearalenone

In 1928, McNutt and co-workers discovered cases of vaginal prolapse in sows in Wisconsin (Christensen *et al.*, 1967). The same phenomenon was later described in other parts of the United States of America, Australia, France and Canada. Pigs have been reported to be most affected because their feed is often rich in maize. Several fungi have been isolated from mouldy feeds causing such oestrogenic disorders. The most common fungus was found to be *Fusarium graminearum*. In 1967, Christensen, Nelson and Mirocha isolated an oestrogenic compound which they called F-2 toxin and which is now referred to as zearalenone. It has been found to display the highest oestrogenic activity.

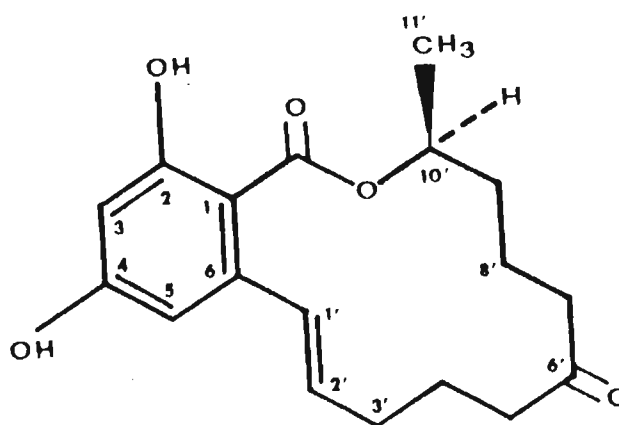


Fig. 2.7 Zearalenone, [6-(10'-hydroxy-6'-oxotrans-1-undecenyl)-b-resorcylic acid lactone], a non steroidal fungal oestrogen (Cox and Cole, 1981).

Due to its frequent occurrence and frequent detection, Zea has been studied in more detail than many of the other *Fusarium* toxins. It is slightly soluble in water (0.002g/ml) but freely soluble in methanol, ethanol and acetone (Cox and Cole, 1981) Because it is a phenol with weak acidic properties, its solubility is greatly enhanced in aqueous alkali.

2.2.3 A survey of *in vitro* studies assessing the toxicity of zearalenone

This toxin has been implicated in serious reproductive and toxicological problems related to farm animals fed contaminated mouldy corn, causing hyperoestrogenic syndrome and subsequently a large economic loss (El-Sharkawy, 1991). *Zea* and some of its metabolites have been found to exhibit anabolic and growth promoting activities in cattle (Katzenellenbogen *et al.*, 1978). This led many investigators to undertake a wide range of *in vivo* and *in vitro* metabolic studies. Its toxicological properties are still unclear but it has been reported to be potentially carcinogenic. It has however been suggested that *Zea* was a contributing factor in cases of precocious sexual development of human infants in Puerto Rico (Goodman *et al.*, 1987). Consequently there is increasing concern about the potential risk of *Zea* to humans not only from direct exposure to contaminated cereals but also through foods from animals exposed to *Zea*-contaminated feeds (Prelusky *et al.*, 1989). Any health risks to humans of the resorcylic lactones will obviously depend on the combination of their potency and the magnitude of exposure to them (Goodman *et al.*, 1987). This review aims to look at the *in vitro* studies conducted in order to assess the toxicity of *Zea* and its derivatives.

2.2.4 Zearalenone vs oestrogen

An oestrogenic syndrome involving marked changes in the reproductive tract of swine has long been recognised in animals that consumed mould-infected corn. In 1962 Stob and co-workers isolated a compound from infected corn which appeared to be responsible for the oestrogenic syndrome which resulted in vulva hypertrophy, vaginal eversion and enlarged mammary glands and growth promoting activity. This compound was identified as *Zea*.

The biological effect of oestrogens in target tissues depends on the presence of cytoplasmic oestrogen receptors. The steroid-receptor complex translocates into the nucleus, binds to DNA template and in turn stimulates cellular RNA and protein synthesis. The binding affinity of the receptor for oestrogenic molecules is extremely strong and steroid specific. However many nonsteroidal compounds can bind to oestrogen receptors and exhibit either agonistic or antagonistic effects of oestrogenic function (Kiang *et al.*, 1978). Investigations carried out by Kiang and co-workers (1978) showed that *Zea* derivatives can compete with oestradiol for binding with

cytosol oestrogen receptors. Zea was found to initiate the translocation of cytosol receptors into the nuclei and thus increase the number of nuclear binding sites. It was also found to increase rat uterine weight and increase the protein content as seen by the figure below.

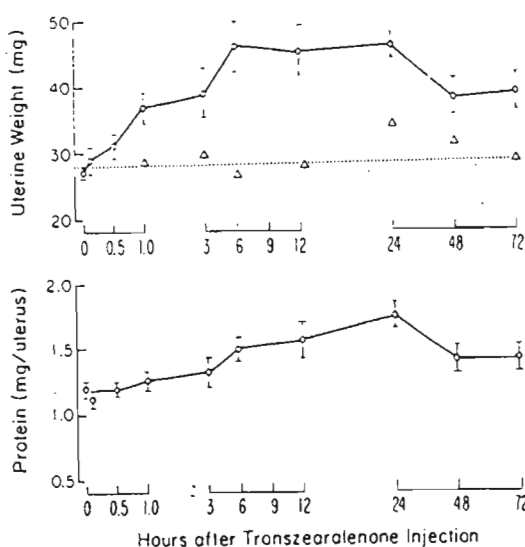


Fig 2.8 The change of wet weight and protein content of rat uteri following a dose of trans-Zea (200 μ g/ml) (Kiang *et al.*, 1978).

This biochemical characteristic of Zea derivatives could explain the infertility observed in animals after the ingestion of a large quantity of zearalenone from contaminated feed (Kiang *et al.*, 1978). The binding of Zea to oestrogen receptors is structurally specific. Steroid hormones such as progesterone and testosterone do not bind to oestrogen receptors which suggests that steroid structure itself is not solely responsible for specific binding (Kiang *et al.*, 1978).

In mice Zea was found to increase the permeability of the uterine to uridine and amino acids and was accompanied by the subsequent increase in protein and nucleic acid synthesis in a manner similar to the effects of natural oestrogen E_2 . In rats Zea binds with oestrogen receptors of both the hypothalamus and hypophysis. This would suggest that Zea affects the oestrogen feedback system through the oestrogen receptor of the rat brain.

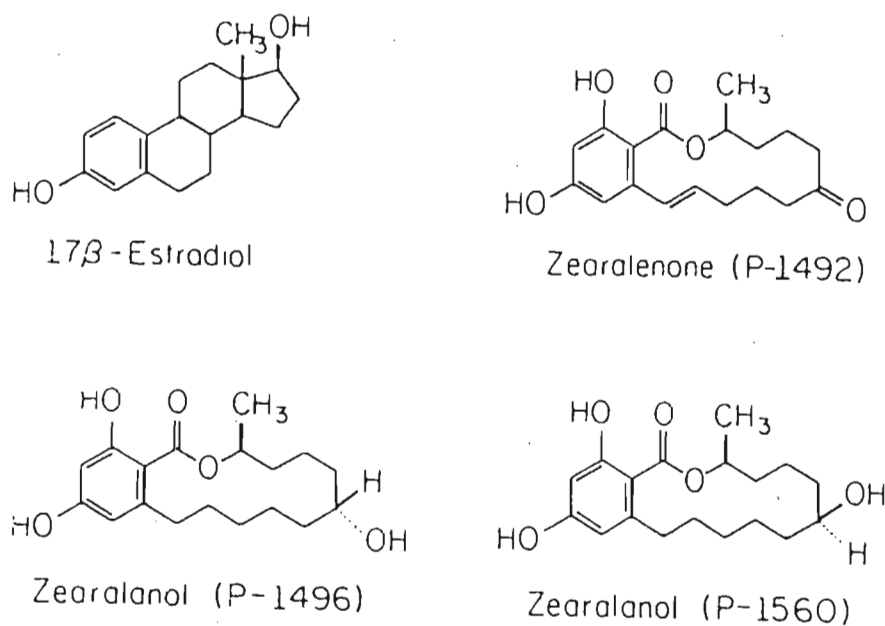


Fig 2.9 From Zea over three hundred derivatives have been synthesised including zearalenols P-1560 and P-1496 (Katzenellenbogen *et al.*, 1978).

Zearalanol is produced commercially as an anabolic agent to improve growth rate and feed efficiency in feedlot cattle (Katzenellenbogen *et al.*, 1978). Katzenellenbogen and co-workers (1978) compared the interactions of these derivatives to that of oestrogen.

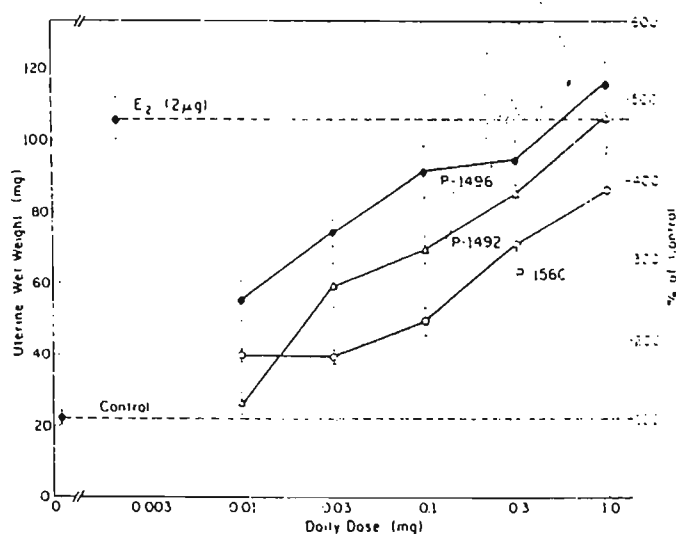


Fig 2.10 Dose response curves showing the uterotrophic activities of oestrogen (E_2) and Zea derivatives (P-1496 & P-1492 & P-1560) (Katzenellenbogen *et al.*, 1978).

From the results it was concluded that much higher doses of these compounds are required to achieve maximal uterotrophic response. It was found that uterotrophic potency increases in the order of P-1496 > P-1492 > P-1560. In the rat uterus these compounds induce the synthesis of uterine growth. In mammary tumour cells these derivatives stimulate cell proliferation and processing of nuclear oestrogen receptors.

2.2.5 Metabolism

Ueno (1993) suggested that Zea is reduced to α -zearalenol by zearalenone reductase which is about ten times more oestrogenic than the parent compound. Kiessling and co-workers (1978) investigated the metabolism of Zea in rat liver. They found that Zea was metabolised along two main pathways. One is conjugation by glucuronic acid and the other mechanism is a reduction of Zea to an isomer of zearalenol possibly catalysed by a hydroxysteroid dehydrogenase which is normally involved in the metabolism of steroids. This may lead to a disturbance of steroid

metabolism during a prolonged intake of Zea. Nogowski and co-workers (1984) also investigated the metabolic properties of Zea. They investigated the effect of Zea on liver and muscle glycogen content. Zea (200 μ g/day) was found to increase glycogen content in both liver and muscle.

Table 1 The effect of Zea on uterus weight, pancreatic hormones and carbohydrates of ovariectomized female rats (Nogowski *et al.*, 1984).

Group	Control	Zea (20 μ g/ml)	Zea (200 μ g/ml)
Uterus weight (mg/100g b.w)	39.0 \pm 3.0	42.8 \pm 4.3	86.4 \pm 5.9
Insulin (μ U/ml)	22.5 \pm 2.6	24.1 \pm 2.2	34.3 \pm 3.9
Glucagon (pg/ml)	464 \pm 53	363 \pm 33	337 \pm 14
Insulin/glucagon molar ration	1.27 \pm 0.20	1.50 \pm 0.18	2.48 \pm 0.23
Glucose(mmol/l)	5.04 \pm 0.27	4.68 \pm 0.32	5.04 \pm 0.22
Liver glycogen (mg/g)	54.5 \pm 2.5	59.6 \pm 3.4	64.1 \pm 1.9
Muscle glycogen(mg/g)	2.05 \pm 0.08	2.45 \pm 0.13	2.52 \pm 0.13

2.2.6 Zearalenone related diseases

Zearalenone and related compounds may have a useful role in pharmacy such as regulating problems during menopause. β -zearalenone a derivative of zearalenone, has been considered as a potential treatment for menopausal syndrome in women (Goodman *et al.*, 1987). Some derivatives may act as contraceptives while others, added to animal feed may increase the growth of farm animals (Mirocha, 1970 as cited by Moreau, 1979). There have however been suggestions that Zea may account for the high incidence of cervical cancer in certain parts of South Africa (Smith *et al.*, 1994).

In assessing the risk of Zea to humans, some measure of its oestrogenic potency was investigated. Typical signs of hyperoestrogenism are prolonged oestrus, changes in libido, infertility, increased incidence of pseudopregnancy, increased mammary gland development and abnormal lactation (Goodman *et al.*, 1987). In a recent outbreak of precocious puberty changes in thousands of young children in Puerto Rico, Zea and zearalenol were considered as possible causative agents (Rodríguez, 1984 as cited by Goodman *et al.*, 1987). The effects seen included premature telarche, premature pubarche, prepubertal gynecomastia and precocious pseudopuberty. It was further reported that Zea or a derivative was present in the blood of some of the patients.

It was locally found that a young African boy showed signs of hyperoestrogenic activity when his chest resembled well developed breasts (Proceedings of 10th South African Biochemistry Symposium, 1987) (Fig 2.11).



Fig 2.11 Enlargement of male breasts due to the consumption of Zea.

2.3 AFLATOXICOSIS

2.3.1 Introduction

After extensive investigations, it was concluded that aflatoxins are the most potent secondary metabolites produced by the fungal moulds, *A. flavus* and *A. parasiticus*. Many adverse human health effects have been linked to the dietary contamination of foods with this particular mycotoxin. The relationship between aflatoxin ingestion and liver cancer has been well established (Smith & Moss, 1985). Throughout the world, aflatoxins have been considered to be a major public health hazard. It has been found that developing countries show a high percentage of aflatoxin related diseases due to the inadequate storage facilities which encourages the growth of moulds (See 2.2.2). An IARC-convened Working Group in 1992 concluded that "naturally occurring aflatoxins are carcinogenic to humans" (Wild, 1993). This conclusion was based on the hepatocarcinogenicity of aflatoxins in animals and epidemiological evidence linking aflatoxin exposure to a high risk of hepatocellular carcinoma. Although over 100 fungal toxins have been identified and characterised during the "mycotoxin gold rush era", aflatoxins still remain the most important human and veterinary health hazard and their study dominates mycotoxin research (Wild *et al.*, 1986).

2.3.2 Aflatoxin B₁ and Aflatoxin B₂

The discovery of the Turkey- X disease in England in 1960, initiated the resurgence of interest in mycotoxins. The X was used to denote the unknown origin of the disease. Subsequently the etiological agent was found to be the Brazilian peanut meal which was contaminated with toxins produced by *A. flavus*, an ubiquitous mould (Wogan, 1966). The aflatoxins constitute a variety of structurally related metabolites which differ in their biological effects. The major naturally occurring aflatoxins are aflatoxin B₁, B₂, G₁ and G₂. Aflatoxin B₁ occurs most widely in the greatest amounts and has the greatest biological activity of this group of toxins

Work done on the effects of AFB₂ (dihydro-AFB₁) in rats showed that its potency is 150 times reduced compared to that of AFB₁ (Wogan, 1966). However it is more active in other systems such as duck. Roebuck and co-workers (1979) showed that duck liver metabolises AFB₂ much more actively than do mammalian species and a

significant pathway in this transformation is 2,3-desaturation to form AFB₁. The latter compound would then be available for activation through the epoxidation pathway as in other species.

2.3.3 Biochemical effects

The aflatoxins are primarily metabolised by the microsomal mixed function oxygenase system, a complex organisation of cytochrome-coupled, oxygen and NADPH dependent enzymes localised predominantly in the ER of liver cells, but also present in the kidney and lungs. These enzymes catalyse the oxidative metabolism of AFB₁ resulting in the formation of various hydroxylated derivatives and the highly reactive epoxide metabolite. The detoxification of AFB₁ is done by the enzymatic conjugation of the hydroxylated metabolites with sulphate or glucuronic acid to form water soluble sulphate or glucuronic esters which are ultimately excreted in urine or bile. The alternative route for the removal of AFB₁ involves the enzyme - catalysed reaction of the epoxide metabolite with glutathione. A few of the known detoxification pathways of AFB₁ metabolism are depicted in Fig. 2.12. It has been extensively documented that during the course of AFB₁ metabolism the reactive metabolite (AFB₁-8,9 epoxide) can react with various nucleophilic centres in cellular macromolecules such as DNA, RNA and protein with the consequences being opposite to that of detoxification, posing a biological hazard to the cell. The possible pathways by which AFB₁ is metabolised into macromolecule-binding species is illustrated in fig 2.12.

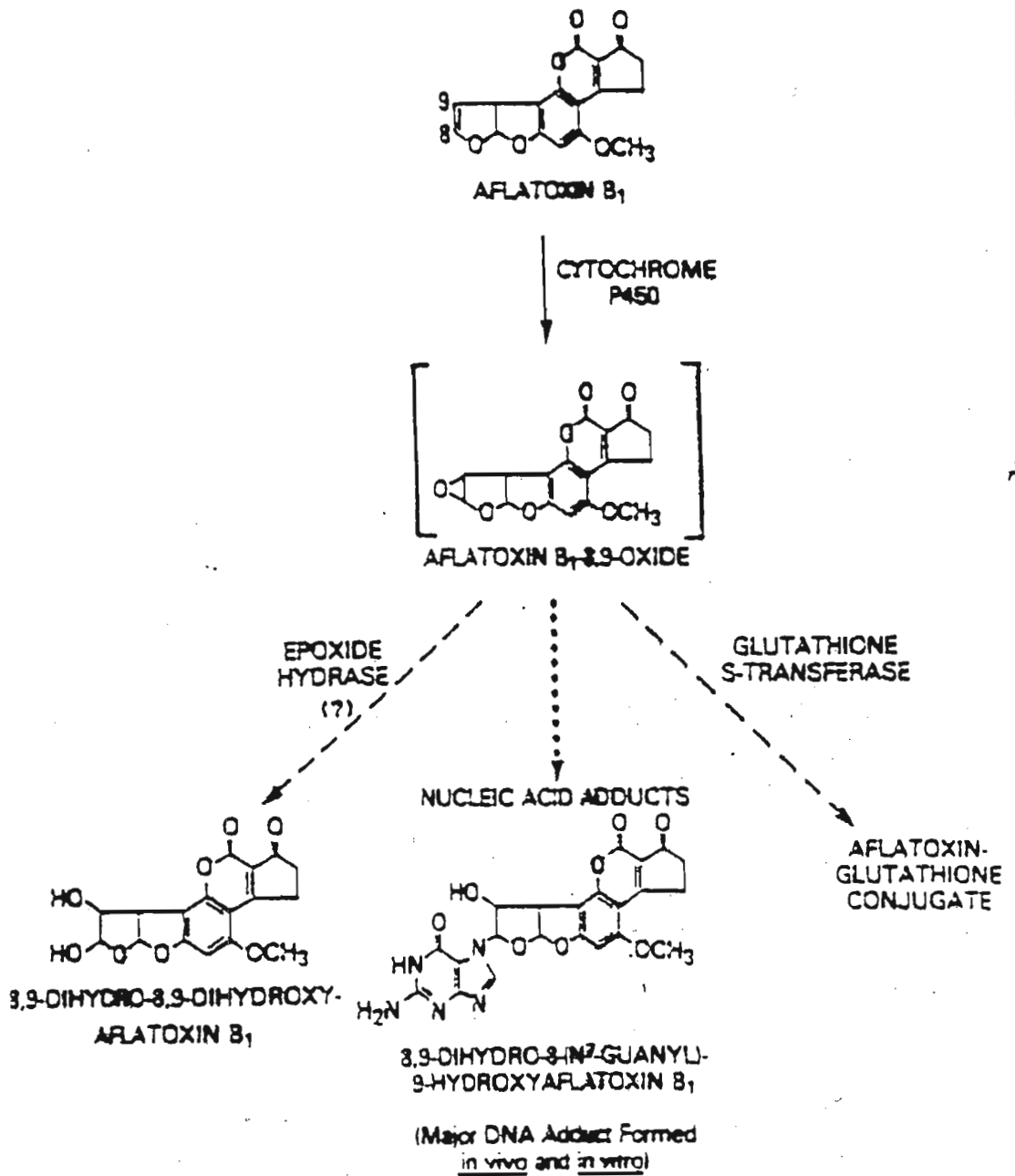


Fig 2.12 The metabolic transformation of AFB₁ (Adapted from Groopman *et al.*, 1988).

2.3.4 A survey of *in vitro* studies assessing the toxicity of AFB₁

Much effort has been made by researchers involved in mycotoxicological studies to investigate the association between aflatoxin exposure and related health effects in people (Table 2).

Table 2 Aflatoxin ingestion and liver cancer incidence in humans (Groopman *et al.*, 1988)

Population	Dietary aflatoxin intake (ng/kg body weight/day)	Cases of liver cancer in men. No./100,000 population per year	Incidence in men	Cases of liver cancer in women. No./100,000 per year	Incidence in woman
Kenya					
High altitude	3-5	1	3.1	0	0
Medium altitude	6-8	13	10.8	6	3.3
Low altitude	10-15	16	12.9	9	5.4
Swaziland					
Highveld	5-9	9	7.0	2	1.4
Middleveld	9-14	24	14.8	5	2.2
Lebombo	15-20	4	18.7	0	0
Lowveld	43-53	35	26.7	7	5.6
Thailand					
Songkhala	5-8	-	-	-	-
Ratburi	45-77	-	-	-	-
Mozambique	222	-	35.0	-	15.7

Classical epidemiological studies have been hindered by the lack of adequate dosimetry data on aflatoxin intake, excretion and metabolism (Groopman *et al.*, 1988). The carcinogenic potency of AFB₁ has been well established in many species of animals including rodents, primates and fish. Such experiments have often examined dose - response characteristics, route of administration, size and frequency of dose, sex and

age of test animal. The effects of various factors on carcinogenic responses have been investigated, including the diet, liver injury, hormonal status and microsomal enzyme activity (Groopman *et al.*, 1988). Many studies have also examined the potency and structure activity relationships of aflatoxin congeners, structural analogues and metabolites as inducers of liver tumours. This review will attempt to collate and evaluate what is known about aflatoxin metabolism, together with the relationship it may have with toxic lesions observed, using *in vitro* models.

2.3.4.1 Investigations using rat hepatocytes and human cell lines

Most of the published information on AFB₁ carcinogenicity has been obtained from studies on rats which are highly susceptible to the toxin. Cultured rat hepatocytes form an integral part of a series of *in vitro* test systems which have been developed during the last few years. Reports from several laboratories indicate wide interest in the use of rat hepatocytes in primary monolayer culture for the study of xenobiotic metabolism and carcinogenic detection, while much work in the last twenty years has been in assessing human exposure to aflatoxins via a number of systemic epidemiological studies (Groopman *et al.*, 1988).

2.3.4.2 The formation of the AFB₁ 8,9 epoxide

The effect of chemical carcinogens and other xenobiotics on hepatic microsomal enzymes involved in the metabolism of foreign compounds is becoming increasingly important since it is these "drug metabolising enzymes" which affect the resulting physiological properties of the parent compound. Gurtoo and Chandrakant (1975) utilised an *in vitro* microsomal-mixed function oxygenase system (MFO) to convert AFB₁ into reactive metabolites that bind covalently to macromolecules such as proteins and RNA. Gurtoo (1973), showed that AFB₁ binds noncovalently to liver microsomes while its metabolite binds tightly and possibly covalently to liver microsomes. Pre-treated microsomal preparations with various combinations of AFB₁, NADPH and SKF-525A (an inhibitor of MFO) were passed through Sephadex G-25 columns.

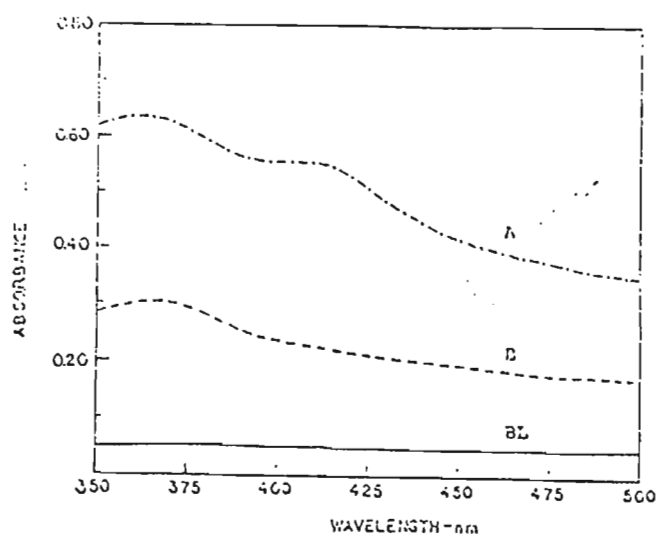


Fig 2.13 The spectral difference between two sets of microsomes pre-treated with NADPH and AFB₁ (Spectrum A) while spectrum B includes SKF-525A (Gurtoo, 1973).

Spectrum A in the figure is the spectral difference between two sets of microsomes pre-treated with NADPH in the presence and absence of AFB₁. This spectrum shows two peaks, one at 363nm due to bound AFB₁ and the other at 412nm due to a bound metabolite. Spectrum B indicates the difference between microsomes pre-treated with AFB₁, NADPH and SKF-525A and those treated with NADPH only. Under these conditions the formation of the metabolite is prevented and only a peak at 363nm is detected due to bound unmetabolized AFB₁.

Jen-Kun Lin and co-workers (1978) showed the formation of 2,3-Dihydro-2,3-dihydroxyafatoxin B₁ (dihydrodiol) as a major metabolite in the incubation of AFB₁ with rat and hamster liver microsomes. The formation of this product was cytochrome P₄₅₀ and reduced NADPH dependent.

The metabolic formation of the epoxide is supported by the release of the dihydrodiol and 2,3-dihydro-2-(guan-7-yl)-3-dihydroxyafatoxin B₁ as products of the weak hydrolysis of nucleic acid AFB₁ adducts formed in rat liver microsomes *in vitro*. From this one can deduce that the capacity of microsomal enzymes to form the 8,9

epoxide is one of the factors that determine the hepatocarcinogenicity of AFB₁. Neal and Colley (1978) examined the metabolism of AFB₁ *in vitro* in rat liver microsomal preparations using high Pressure Liquid Chromatography (Hplc). It is metabolised by rat liver to form a variety of metabolites (phenols, hydroxy compounds and epoxides). The only major non microsomal route of metabolism reported is the reduction to aflatoxicol which is mediated by the cytosolic fraction (scheme 1 below).

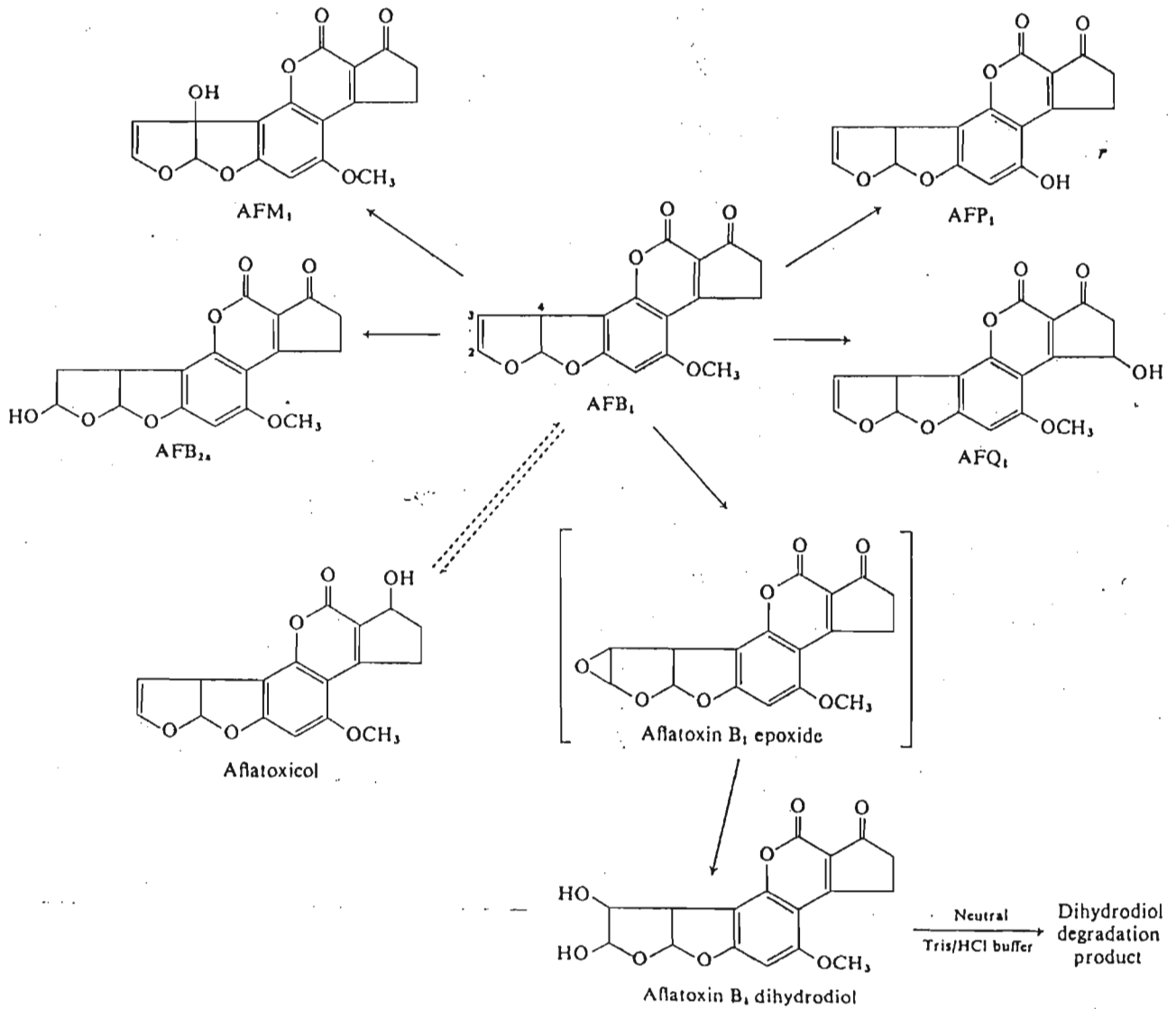


Fig 2.14 The metabolism of AFB₁ (Neal and Colley, 1978).

The biological potencies of the non polar chloroform soluble metabolites all appear to be less than that of AFB₁ except for AFM₁ (Campbell, 1970). The production of the dihydrodiol could reflect a detoxifying hydrazase reaction subsequent to the primary activation of AFB₁ by epoxidation. The formation of the dihydrodiol may therefore indicate the AFB₁ activating capacity of the microsomal preparation. Neal and Colley (1978), found AFM₁, AFQ₁ and the dihydrodiol as principal metabolites of AFB₁ using Hplc. They further found that AFM₁ underwent very limited microsomal metabolism to more polar compounds. AFP₁ was metabolised in the absence of NADPH to an unidentified metabolite. As discussed earlier, CP₄₅₀ is the principal enzyme system involved in the oxidative biotransformation of AFB₁. Human microsomes show a rapid NADPH dependent metabolism of AFB₁. It was shown that AFQ₁ appears to be the major detectable metabolite produced by human microsomal metabolism in contrast to other laboratory animals in which AFB₁-8,9,-dihydrodiol is the major soluble metabolite (Moss, 1985). Moss and Neal (1985) showed that human liver metabolises AFB₁ rapidly to AFQ₁ and possibly to a lesser extent to AFB₁-8,9-epoxide (Table 3). The apparent lack of capacity for AFB₁-GSH production by human post microsomal supernatant suggests that any AFB₁-8,9-epoxide produced is likely to undergo macromolecule binding rather than detoxification.

Table 3 The production of AFB₁ metabolites (Moss & Neal, 1985).

Sample	Sex	Rate of AFB ₁ metabolism (nmoles/mg protein/min)	Recovery of soluble aflatoxins after 30 min (% initial AFB ₁)	AFB ₁ diol production (%)	AFQ ₁ production (%)	AFM ₁ production (%)
1	M	2.7	35	27	73	trace
1	M	2.14	45	10	90	not detected
1	M	1.5	50	18	68	14
2	F	0.63	59	20	77	not detected
2	F	0.73	65	26	74	not detected
3	M	1.027	46	26	65	trace
3	M	1.44	42	26	72	

Ramsdell and co-workers (1991) showed that the proportion of AFB₁ converted to the highly reactive epoxide in microsomal incubations varies with substrate concentrations and investigated the effects of cP450 111A4 as the principle system responsible for the epoxidation of AFB₁ by human liver microsomes. They found that P450111A4 enzymes are capable of AFB₁ epoxidation at relatively high substrate concentrations (124 μ M) and at lower substrate concentrations (16 μ M) other enzymes are involved which would be typical for dietary exposures as shown by the inhibitory effect of an antibody against rat cP450111A4 on AFB₁ bioactivation (Fig 2.15 below).

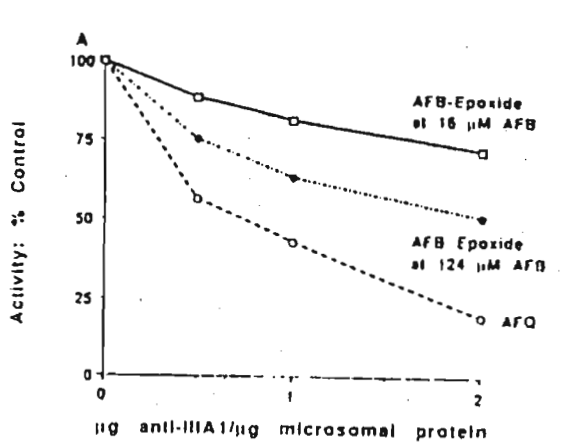


Fig 2.15 The inhibitory effect of an antibody against rat cP450111A4 on AFB₁ activation (Ramsdell *et al.*, 1991).

Geurgerich and co-workers (1992) used a number of different approaches to determine the catalytic selectivity of individual human enzymes towards procarcinogens. They found that at low AFB₁ concentrations, the results of immunoinhibition, chemical inhibition and correlation experiments are consistent with the view that both AFB₁ 8,9-epoxidation and hydroxylation are catalysed primarily by P450111A4.

2.3.4.3 Aflatoxin B₁ -protein complexes

Aflatoxin B₁ has been found to act as a hepatocarcinogen at extremely low levels. It has been suggested that metabolites of AFB₁ may interact mainly with a few critical macromolecules to bring about this malignant transformation. Several studies have therefore been directed to the study of aflatoxin macromolecule adducts formed in

liver during hepatocarcinogenesis, in order to identify important interactions in the oncogenic process. Mainigi and co-workers (1977) described different kinds and amounts of aflatoxin-protein complexes present in liver cytosol throughout hepatocarcinogenesis produced from AFB₁. Liver cytosols were incubated with [³H] AFB₁ at a concentration of 1.26 to 1.54 μCi/ml cytosol for 2 hours at 1 to 4°C with stirring. Cytosols were then subjected to molecular sieving through Sephadex G-25 gel. The isolated macromolecules were resolved either according to molecular size by filtration through Sephadex G-200 gel or according to a combination of molecular charges and sizes. In this particular study 8 classes of aflatoxin-protein complexes were found in the liver cytosol according to molecular size and 6 according to charge size. Swenson *et al.* (1974) reported that AFB₁ gives rise to more adducts of nucleic acids in rat liver than of proteins which has been offered as evidence to support the possible role of DNA as the crucial target of this hepatocarcinogen. The unusual potency of AFB₁ may result from a capacity to inflict multiple molecular insults on target cells. The table below indicates the molecular weights and amounts of various molecular size species of aflatoxin-protein complexes found in rat liver cytosol.

Table 4 Molecular weights and amounts of various molecular size species of aflatoxin-protein complexes in rat liver cytosol (Swenson *et al.*, 1974).

Component	Molecular weight	<i>In vivo</i> 18hours	<i>In vivo</i> 48hours	<i>In vitro</i>
15-20S	>300,000	6	10	6
10-15S	2-3 X 10 ⁵	5	5	4
7-5S	180,000	18	13	16
6S	120,000	18	9	6
5S	80,000	18	16	8
4S	45,000-50,000	26	21	59
3S	22,000-26,000	18	19	12
2S	12,000-15,000	8	7	5

An interesting 4S complex formed may originate from the binding of labelled toxin to several glutathione-s-transferases, which have that molecular weight in common. Mainigi and Sorof (1974) have provided evidence in support of a model in

which the target protein acts as a cytoplasmic receptor that protects activated carcinogen and carries it to the cell nucleus during carcinogenesis. From these studies it was concluded that the specific, weakly basic carcinogen-protein complexes containing activated carcinogens may act to direct or restrict the interactions of the activated carcinogens to specific sites on DNA. The formation of AFB₁-DNA adducts is implicated in tumour formation but the mechanism by which AFB₁ or its derivative is translocated into the nucleus is still unclear. The existence of cytosolic Ah receptor and binding proteins of lipophilic carcinogens involved in the metabolism of xenobiotics has been documented. Ch'ih and co-workers (1993) utilised equilibrium dialysis to prepare a number of AFB₁-protein complexes and studied their translocation into the nucleus. Results indicated that the ligand protein complex is selectively translocated into the nucleus. The highest binding was exhibited by pyruvate kinase (Table 5).

Table 5 The formation ligand protein complexes (Ch'ih, 1993).

Protein	AFB ₁ -DNA adducts (fmol/mg DNA)
Albumin	1.77± 0.88
Albumin-NLS	13.69 ± 0.20
Histones	5.51± 1.16
Pyruvate kinase	0.28± 0.10

Since AFB₁ is lipophilic and known to bind to a number of proteins it is unlikely that it enters the nucleus unbound. It is possible that it translocates across the nuclear pore as a ligand-protein complex by binding to proteins destined for the nucleus (histones), thus increasing its uptake, activation and damage to DNA, leading to an increased risk of developing cancer.

2.3.4.4 DNA adduct formation

Rodent cell cultures have been developed for the study of the mechanism of malignant transformation by chemical and physical carcinogens. It has been demonstrated that AFB₁ binds to both native and denatured DNA and lowers the RNA content. It has therefore been suggested that the extreme toxicity and carcinogenicity of AFB₁ may be the direct result of the affinity of this toxin for DNA. DNA adducts

are considered to be the promutagenic lesions which give rise to initiated cells required for transformation and carcinogenesis. The principal product of DNA binding in the liver of rats and other systems is the N7-guanine derivative (8,9-dihydro-8-(n7-guanyl)-9-hydroxyAFB₁). There exists a correlation between the level of AFB₁-DNA adducts formed and the likelihood and extent of tumourigenesis. Rechtel as cited by Eaton (1994), showed a highly linear relationship between DNA adduct formation and tumour response.

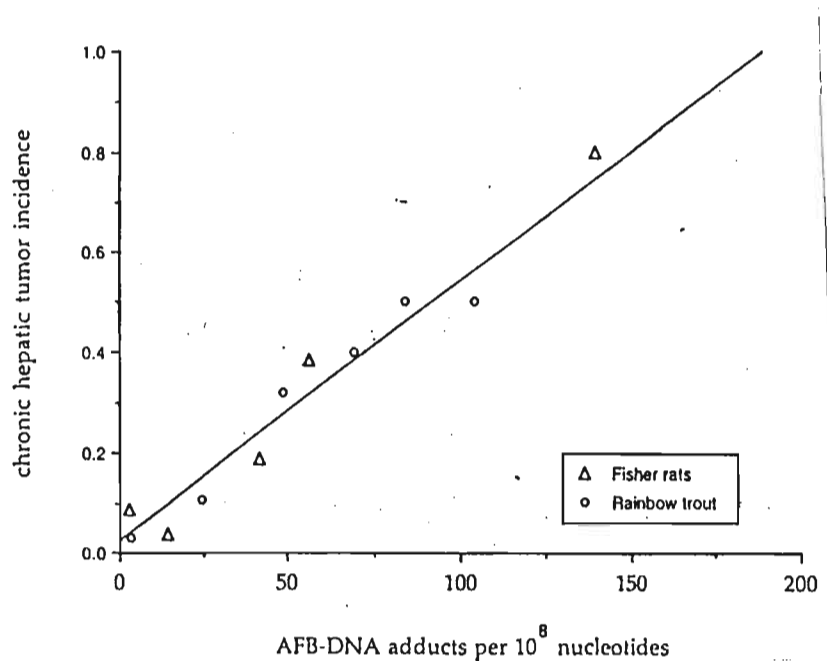


Fig 2.16 A direct relationship between AFB₁ concentration and DNA adduct formation (Eaton, 1994).

Hertzog (1980) suggested that in rat liver DNA two competing reactions occur, both of which may be important in carcinogenesis. Firstly, the release of AFB₁-guanine adducts from DNA would give rise to apurinic sites which would eventually lead to DNA strand breaks and secondly, AFB₁-guanine is attacked at the 8' position of guanine by -OH ions. The production of apurinic sites and possible apurinic endonuclease activity with the production of strand breaks might be a mechanism for mutation induction or tumour initiation. Essigman and co-workers (1976) used perfused rat liver to study carcinogen-DNA adduct formation from AFB₁. It was found that binding levels obtained were similar to those demonstrated in DNA from animals receiving similar doses. The perfusion of rat livers with AFB₁ under controlled conditions resulted in the dose dependent covalent binding of the carcinogen to DNA. The relationship between AFB₁ dose and binding to liver DNA is shown in figure 2.17.

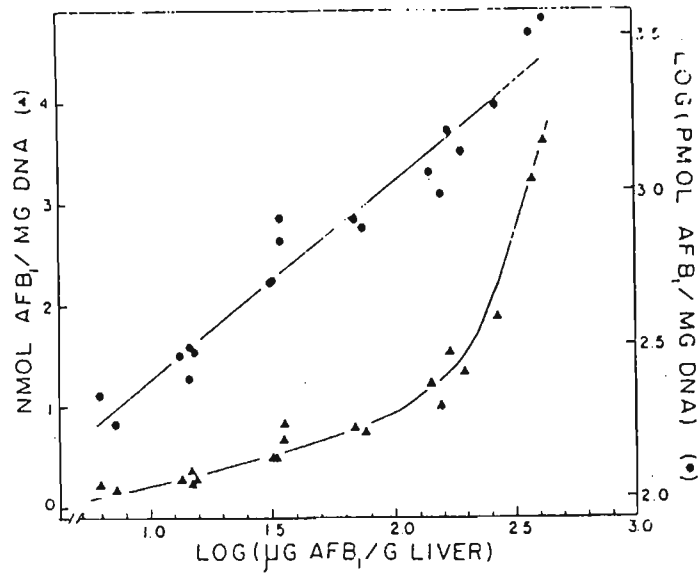


Fig 2.17 The relationship between AFB₁ dose and binding to liver DNA (Essigmann *et al.*, 1976).

The mouse embryo fibroblast line C3H/10T1/2 clone 8, was found to be able to activate AFB₁ and form covalent AFB₁:DNA adducts which are identical to adducts formed in human tissue (lungs, bronchi, colon). Wang and Cerutti (1980) aimed to relate the concentration of individual adducts to the cytotoxic potency of AFB₁.

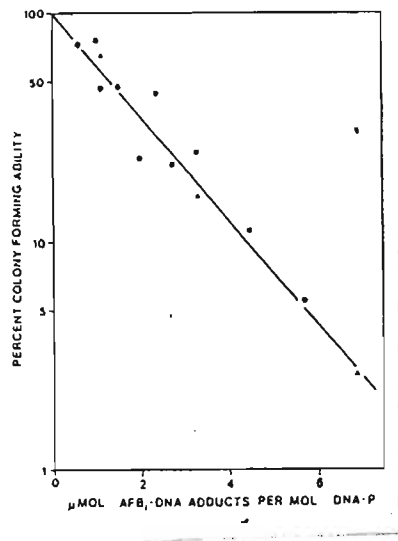


Fig 2.18 A linear relationship between the log of the colony-forming ability of 10T1/2 cells and the initial concentration of AFB₁-DNA adducts (Wang and Cerutti, 1980).

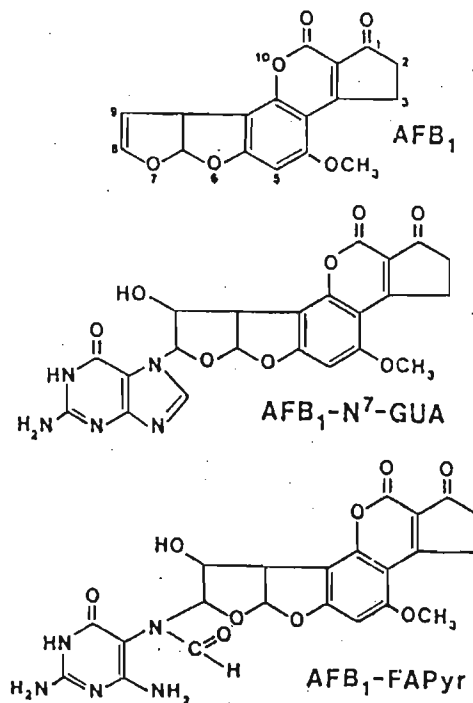


Fig 2.19 AFB₁ adduct formation accounted for as two components :structure 1 (AFB₁-N₇-Gua) and structure 11 (AFB₁-FAPyr) (Autrup *et al.*, 1979).

Extensive reviews have shown that AFB₁ can be activated by cultured human tissues and the major adduct formed between DNA and AFB₁ is chromatographically

identical to the adduct formed in rat liver. Autrup and co-workers (1979), showed that two different human tissues (bronchus and colon) can metabolise AFB₁. The adducts formed between AFB₁ and DNA in both the human bronchus and colon were qualitatively similar to the adducts formed in rat liver. They found that over 70% of the AFB₁ binding in bronchus DNA was accounted for as two components, 1 and 11 which were chromatographically identical to AFB₁-N7-Gua (structure 1) and an adduct identified as 2,3-dihydro-2-(N5-formyl-2',5',6'-triamino-4'-oxo-N5-pyrimidyl)-3-hydroxyafatoxin B₁ (AFB₁-FAPyr)(11) (Fig 2.19). They have proposed that the formation of adduct 1 induces a positive change in the imidazole ring of guanine and this possibly alters the primary structure of DNA in the vicinity of the adduct.

This substitution weakens the covalent bond between the adduct and the DNA backbone which may facilitate its rapid chemical or biochemical removal. Structure 11 would not carry a positive charge in DNA and its glycoside bond would be more chemically stable than that of structure 1. These observations add AFB₁ to the list of chemical procarcinogens metabolised by cultured human tissues and in which the carcinogen-DNA adducts are similar to the adducts formed in animal tissue susceptible to the carcinogenic action of AFB₁.

In 1976, Grant reported the occurrence of colon carcinogenesis in two men who had worked with purified aflatoxin. Patient 1, a 42 year old biochemist developed abdominal pain and bowel obstruction. He died in 1972. Ten years previously he began research into the chemical nature of aflatoxins. For several years he purified the chemical by scraping it from chromatographic plates accumulating 1 mg lots for research. Patient 2, a 28 year old student developed lower quadrant and periumbilical cramping pain. A grade ten adenocarcinoma was resected. He spent twelve months purifying aflatoxins. There was no ventilation hood where he worked but he recalled some dust exposure as a potential source of contamination.

Even though in most species the carcinogenic effects of AFB₁ is expressed in the liver, the extreme toxicity and carcinogenicity of AFB₁ suggests that in human exposure, other organs may also be effected. Stoner and co-workers (1982), studied DNA binding and adduct formation of AFB₁ in cultured bladder and tracheobronchial explants from human, monkey, dog, hamster and rat (Fig 2.20). Explants were exposed

to [^3H] AFB₁. After 24 hours, epithelial cell DNA was isolated. Binding was found to be higher in tracheobronchial tissues than in bladder tissues.

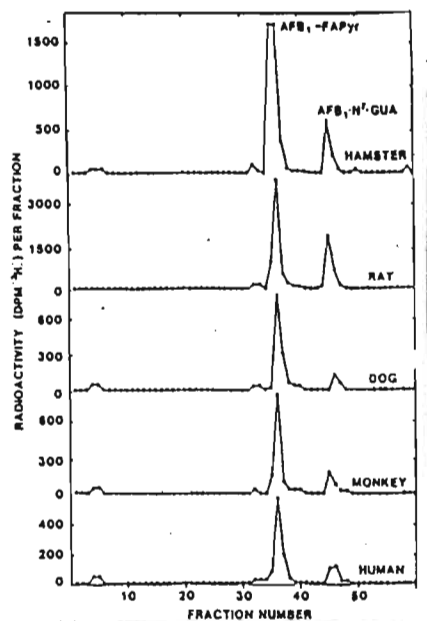


Fig 2.20 Elution profiles of AFB₁-DNA adducts formed by bladder explants of human, monkey, dog, rat and hamster. The ring-opened adduct elutes in fractions 36-38 while the closed ring structure elutes in fractions 45-47 (Stoner *et al.*, 1982).

They have however explicitly stated that *in vitro* exposure to extrahepatic tissues to increased AFB₁ concentrations (0.1 μM) represents an artificial situation, especially since *in vivo* the liver is both the target organ and the primary site of metabolism of AFB₁. Although AFB₁ has been detected in human urine and lung tumour tissue, the *in vitro* demonstration of AFB₁-DNA adduct formation in extrahepatic tissue is insufficient evidence for postulating a role of AFB₁ in the pathogenesis of respiration and bladder cancer. In both tissues from all species, two major adducts were identified. In all cases the major adduct formed representing 40 to 79% of the total amount of [^3H]AFB₁-DNA was AFB₁-FAPyr while the AFB₁-N⁷-Gua adduct represented 4 to 28% of the total. McCormick and co-workers (1985) looked at the cytotoxic and mutagenic effects of AFB₁-DNA adducts in diploid human fibroblasts. They aimed to investigate the biological effects of AFB₁ in human fibroblasts as a function of concentration and initial number of DNA adducts. Because AFB₁ is activated into its highly reactive 8,9 epoxide, they substituted AFB₁-dichloride (AFB₁-C₁₂) a direct carcinogen which is a model for the proposed ultimate reactive metabolite of the epoxide. The Hplc results indicated that the major primary adducts

formed in the human cell line and in isolated DNA was the chemically unstable guanine derivative which could undergo a change in structure with time to form a more stable secondary adduct. The cytotoxic effect of AFB₁-C₁₂ was highly correlated with the presence of either of these guanine adducts.

Leadon and colleagues (1981) investigated excision repair of AFB₁-DNA adducts produced in normal fibroblasts (NF). Results showed that 40 to 60% of the adducts formed represented AFB₁-N₇-Gua which they suggested is formed almost exclusively as primary adducts in all tissues. It was also concluded that AFB₁-N₇-Gua adducts are spontaneously and enzymatically removed in NF. The AFB₁-FAPyr formed in a secondary reaction from AFB₁-N₇-Gua represents the major lesion in NF and therefore transforms a repairable lesion into a non repairable one.

Carcinogenesis in man has been correlated circumstantially with a deficiency in DNA repair in the hereditary disease Xeroderma Pigmentosum (XP3) (Sarasin, 1977). It was found that cultured fibroblasts from XP patients were defective in the repair of UV induced damage to their DNA and the unrepaired lesions might be responsible for skin cancer in these patients. It was therefore suggested that carcinogens may be tested by assaying DNA repair following administration to cultured cells. AFB₁ has been shown to induce error-prone repair in *Escherichia Coli*, but requires metabolic activation in order to elicit its effects (via microsomes). It was found that repair deficient human cells (XP) are more sensitive to the mycotoxin than its wild type counterpart. Sarasin *et al.*(1977) demonstrated that AFB₁ activated by rat liver microsomal enzymes induces DNA repair replication in human W138 cells while AFB₁ itself does not. He also stated that a general toxic effect on cell metabolism rather than a selective effect on DNA repair may be responsible for the activity of procarcinogens and tumours promoters.

Legator and Childs (1966) looked at the effects of AFB₁ on thymidine kinase levels in a tissue culture environment knowing that the initiation DNA replication and cell proliferation is dependent on the activity of thymidine kinase. Results obtained showed the appearance of increased thymidine kinase which has been correlated with cell replication.

Table 6 The activity of thymidine kinase/ μg DNA (Legator & Childs, 1966).

	24 hours	48 hours	72 hours
control	1.07	1.24	1.18
0.5ppm aflatoxin	2.49	3.34	21.1
1.0ppm aflatoxin	1.59	6.78	31.7

2.3.4.5 Effects on protein synthesis

Among several biochemical effects observed in the rat liver, rapid and extensive inhibition of precursor incorporation into nuclear and cytoplasmic RNA follows the introduction of AFB₁ into the system. Dissociation of ribosomes from hepatic rough endoplasmic reticulum (rER) resulting from aflatoxin treatment of rats has been reported (Svoboda, 1966). AFB₁ affects rat liver protein synthesis by its suppression of amino acid incorporation into rat liver slices and isolated perfused rat liver. Harley and Rees (1969) investigated the effects of AFB₁ on cellular and protein synthesis using Hela cells. This cell line was chosen for this purpose since the site and detailed processes of RNA formation in this cell line has been extensively investigated. It was found that 20 to 40 μg of AFB₁ introduced to the cells showed marked inhibition of protein and nucleic acid synthesis. Measurements were made by comparing the incorporation of [³H] uridine, [³H] thymidine and [¹⁴C] leucine into RNA, DNA and protein. The table below shows that the incorporation of all these precursors was inhibited.

Table 7 Exposure of monolayers to AFB₁ and the percentage radioactivity incorporated (Harley & Rees, 1969).

Conc of aflatoxin ($\mu\text{g}/\text{ml}$)	% RNA inhibition	% DNA inhibition	% Protein inhibition
20	34.5	11.4	11.0
40	48.3	13.8	25.8

It was shown that the maturation of the 45S ribosomal-RNA precursor was inhibited by AFB₁, suggesting that the rate of ribosomal RNA synthesis is controlled by the maturation of the 45S RNA. It was deduced that the interaction of the toxin with the RNA component of the polyribosomes results in polysomal disaggregation. Moul'e (1973) looked at the action of the toxin on post-transcriptional events that convert 45S RNA into subribosomal particles in the rat liver cell. It was found that AFB₁ allowed 18S RNA to be excised and transferred to the cytoplasm where it appears in newly synthesised 40S subunits but impairs the subsequent emergence of the 60S particles. It has been postulated that AFB₁ or a derivative induces some alteration in the properties of the 60S particle. A specific defect in their stability could induce their immediate degradation inside the nucleus. It was concluded in this study that AFB₁ does indeed inhibit RNA synthesis by defecting some step in the transcriptional mechanism. Studies were also made on the acute effects of AFB₁ on nucleolar RNA (nRNA) synthesis. Sedimentation profiles obtained from density gradient centrifugation of nRNA showed that AFB₁ inhibited the synthesis of rapidly sedimenting nRNA. Electron microscopic studies showed the segregation of nucleolar granular and fibrillar elements (Floyd, 1968).

In tissue culture studies, Legator (1960) used a heteroploid human embryonic lung cell line (L-132) and a diploid human embryonic lung cell strain to determine the effects of aflatoxins on cell growth, morphology and mitosis. Tissue culture allows one to construct a probable sequence of events to account for the effect of aflatoxin in this biological system. Legator (1960) found that the earliest effect of this toxicant is the suppression of DNA synthesis and mitosis, with the subsequent survival of the cell in a nondividing stage which is indicative of the presence of giant cells. This effect correlates with that of alkylating agents which are known to be mutagenic, carcinogenic and antineoplastic. Scaife (1970) used established cell lines of human kidney T cells, HeLa and Chang liver cells to investigate the effects of AFB₁. He showed that AFB₁ rapidly inhibits RNA synthesis in rat liver slices while the 3 human cell lines were more slowly effected. He attributed the difference in susceptibility of the cells in culture to the cytotoxic action of AFB₁ by variations in cell permeability.

2.3.4.6 Effects on Mitochondria

Although the cells most affected in aflatoxin poisoning are those around the portal vein and bile duct, it has been reported that the mitochondria of rat liver cells are vulnerable to the toxin (Bababummi & Bassir, 1972). They also showed the markedly different susceptibilities of mitochondria from liver, kidney, heart and testis. It was found that the addition of ATP completely reversed the swelling of mitochondria to which AFB₁ was added.

Table 8 Degree of mitochondrial swelling following the exposure to AFB₁ (Bababummi and Bassir, 1972).

Tissue	Degree of mitochondrial swelling	ATPase activity (μmoles) Control	ATPase activity (μmoles) Aflatoxin 20 x10 ⁻⁵ moles
Liver	42	3.37	6.25
Kidney	18	3.49	5.58
Heart	3	2.56	2.60
Testis	2	2.38	2.31

These findings suggested that toxic doses of aflatoxin may uncouple oxidative phosphorylation. As discussed earlier, the metabolic conversion of active carcinogens into electrophilic reactive forms is an important step in the induction of cancer with the CP₄₅₀ type mixed function oxygenases being the predominant enzyme systems responsible for the activation process. A CP₄₅₀ system has been localised in hepatic mitochondria. Niranjana and Avadhan (1980) performed experiments on the *in vitro* activation of hepatic carcinogen AFB₁ by rat liver mitochondrial and submitochondrial fractions. Their results show that intact mitoplasts free of detectable microsomal contamination can effectively metabolise AFB₁ into active components which subsequently bind to mitochondrial DNA, RNA and proteins.

In 1982, both co-workers showed that the administration of AFB₁ to experimental animals resulted in the covalent binding to liver mitochondrial DNA at

concentrations 3 to 4 times higher than nuclear DNA which is accompanied by pronounced inhibition of mRNA and protein synthetic activities.

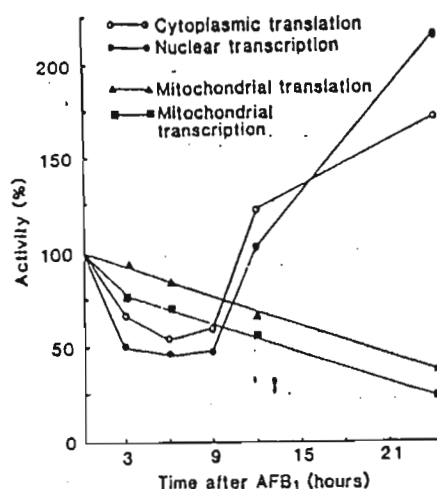


Fig 2.21 The effect of AFB₁ modification of template DNA on the transcription and translation processes using hepatocytes and mitoplast systems (Niranjan and Avadhan, 1980).

Both nuclear transcription and cytoplasmic translation are inhibited by 70 to 80%. They concluded that mitochondria are the direct and possibly one of the major cellular targets for AFB₁ during carcinogenesis. Ramachandra and co-workers (1975) studied the *in vitro* effects of AFM₁, AFB₁ and AFG₁ on oxidative phosphorylation by rat liver with succinate as the substrate. They found that all these toxins inhibit the electron transport chain at 1×10^{-4} M concentration and the site of inhibition is between cytochrome band c. It was further discovered that AFB₁ acts as an uncoupler at low concentrations and as inhibitors at high concentrations.

2.3.4.7 The role of the glutathione system

As in the case of the formation of electrophilic metabolites of many xenobiotics, conjugation with glutathione S transferase (GST) is believed to play an important role in the detoxification of AFB₁ in the liver. The GST are a group of isoenzymes catalysing the conjugation of reduced glutathione (GSH) with a wide spectrum of compounds possessing an electrophilic centre. The epoxide formed following AFB₁ metabolism is capable of GST mediated conjugation with GSH. Species vary in their

sensitivity to aflatoxin toxicity which is in part dependent on their capacities to activate the toxin and their abilities to detoxify the reactive metabolite, by conjugation with GSH (Neal *et al.*, 1987). Neal and co-workers compared the capacities of hepatic cytosolic GST isolated from some animal species representing a range of susceptibilities to AFB₁ cytotoxicity to catalyse the conjugation of GSH with several model substrates and also with microsomally-activated AFB₁ to ascertain which model substrates most closely parallel AFB₁.

It was found that the order of activities in the various liver fractions using 1-chloro-2,4-dinitrobenzene (cDNBB) as a substrate were: mouse > pre-neoplastic nodular rat > guinea pig > control rat which paralleled the capacities of the cytosols to catalyse the formation of AFB₁-GSH from microsomally activated AFB₁ and GSH.

Neal and Green (1983) used a chicken liver microsomal system to investigate the formation of the AFB₁-reduced glutathione (AFB₁-GSH) conjugate *in vitro*. Chicken microsomes were used in this study because it was found to metabolise AFB₁ in a 30 minute incubation period and the metabolism proceeded almost exclusively via the presumed epoxide as indicated by the formation of AFB₁-dihydrodiol-tris complex.

An interesting feature observed was AFB₁-DNA binding and glutathione conjugation in rats of different ages. Behroozikha and co-workers (1992) showed that immature rats are deficient in the hepatic key factors involved in biotransformation of AFB₁. Epoxidation of ³[H]AFB₁ in the presence of liver microsomes from different age groups show that new-born rats are capable of catalysing only minimal AFB₁-DNA binding. The amount of AFB₁-GSH conjugate formed is also higher when adult GST is involved in the system. They therefore concluded that immature liver is less efficient than a mature organ in dealing with a chemical carcinogen. It has been shown that GST plays an important role in carcinogenesis by conjugating reduced GSH with electrophilic compounds and thus preventing binding to cellular macromolecules. These GST's exist in multiple forms of dimeric combinations of different subunits. On the basis of similarities in structural and kinetic properties, the mammalian GST's have been classified into three groups, i.e. α, μ, π family (Gopalan *et al.*, 1992). Several GST classes are believed to be able to conjugate the AFB₁ -8,9-epoxide and GSH. Differences between mammalian species in susceptibility to AFB₁ carcinogenesis have been shown to be the result of differences in alpha GST conjugation of the epoxide suggesting that alpha GST activity may be a key factor in deterring species

susceptibility to AFB₁. Exposure of neonatal rats to xenobiotics such as phenobarbital produced long term changes in hepatic enzymes not seen in adult animals exposed to the same compounds. These rats showed altered levels of DNA adduction as adults following the *in vivo* AFB₁ exposure (Zanger *et al.*, 1992). Zanger and co-workers exposed new-born male rats to diethylstilbestriol (DES) and other xenobiotics. They found that neonatal treatment with DES resulted in a long term protection against AFB₁ adduction to DNA, the putative initiating step in carcinogenesis. The protective mechanism seemed to be increased detoxification of the AFB₁-8,9 epoxide resulting from increased cytosolic concentration of alpha class GST's and subsequent an increase in cytosolic protein concentration. Geurgerich and co-workers (1992) found that human liver cytosol has considerably less GSH-S-transferase activity towards synthetic AFB₁8,9-oxide than rat liver cytosol. Of the human enzyme classes examined, the activities of the enzyme classes varied in the order of $\mu > \alpha > \pi$.

Wong and co-workers (1992) studied the effects of geniposide (GP), an iridoid glycoside which was found to inhibit AFB₁-induced hepatotoxicity and hepatic DNA binding in rats. Isolated from the fruit of *Gardenia jasminoides*, it was shown that GP reduced AFB₁-induced DNA repair synthesis in a dose dependent manner in hepatocyte cultures and was found to elevate the metabolism of AFB₁ and decrease the formation of AFM₁. The enzyme activities of GST and GSH peroxidase in AFB₁-treated hepatocyte cultures are enhanced in the presence of GP thus reducing AFB₁induced DNA repair synthesis through an increased AFB₁ detoxification metabolism which provides one possible mechanism for the chemopreventive activity of GP.

2.3.4.8 Oxidative damage

Kuo-Huang Ling and co-workers (1967) also showed the effect of AFB₁ on liver enzymes of the rat. Their studies revealed a decrease in the activity of succinate dehydrogenase and cytochrome c oxidase in the livers of toxin treated rats. It has been suggested that oxidative damage might contribute to the cytotoxic effects of AFB₁. Oxidative damage refers to the impairment of cellular components (enzymes, membrane lipids and proteins and nucleic acids) by reactive oxygen species such as superoxide radicals(O₂⁻), H₂O₂ and hydroxyl radicals (-OH). The latter reacts with cellular components to cause lipid peroxidation and DNA base damage. Shen and co-workers (1995) investigated AFB₁ induced lipid peroxidation and cell injury in cultured rat

hepatocytes. They used malonaldehyde generation and lactate dehydrogenase release as indices of lipid peroxidation and cell injury. Exposure to AFB₁ (72 hours) resulted in significantly elevated levels of LDG being released as well as the MDA generation in cultured hepatocytes. These effects were dose dependent. MDA generation and LDH release were found to be inhibited by the addition of superoxide dismutase and Dimethylsulfoxide (DMSO, a hydroxyl radical scavenger).

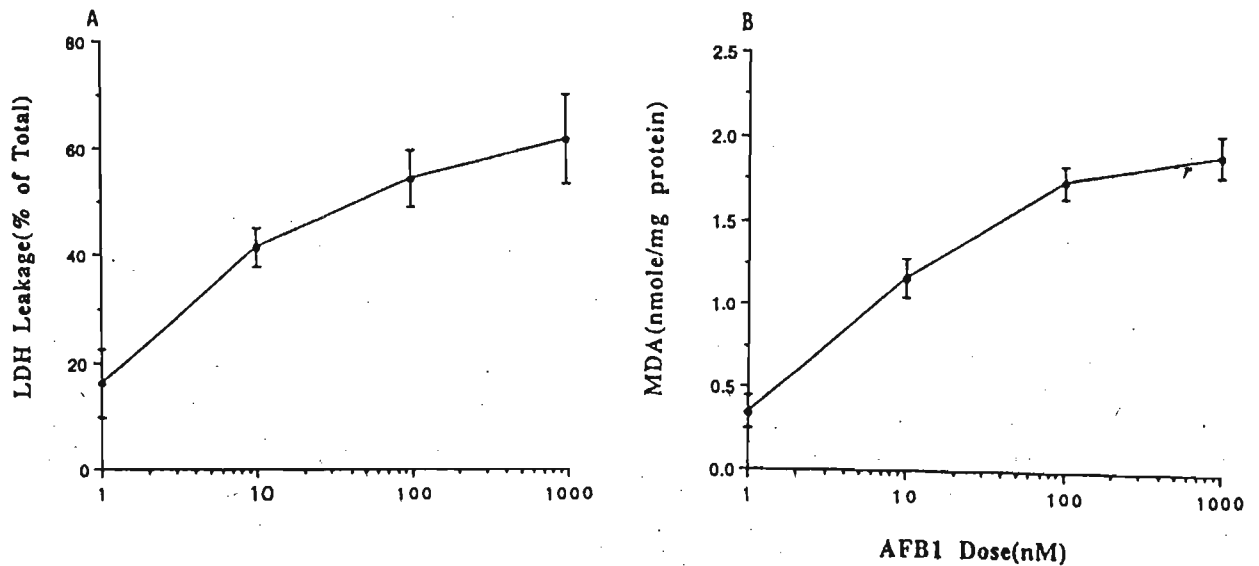


Fig 2.22 Dose -response of LDH release and MDA generation (Shen *et al.*, 1995)

2.4 Conclusion

There is at present a world-wide increase in awareness of the potential dangers posed to human health by mycotoxin contamination of food. The greatest concern today is probably the potent cancer risk posed by carcinogenic AFB₁. This chapter has attempted to review the far reaching effects of AFB₁ and Zea in the hope of demonstrating the importance of this investigation in understanding the biochemical mechanism of action.

Chapter 3

CELL CULTURE AND CYTOTOXICITY ASSAYS

3.1 INTRODUCTION

Since mycotoxins display a wide diversity of chemical structure, there are no common methods of analysis for these toxins. The most practical methods for detection are physico-chemical in nature. However, the ultimate test of toxicity of a mycotoxin is to experimentally test the effect of the suspected toxin in a living system (Smith and Moss, 1985). During the last three decades the use of cell culture systems for investigations involving acute toxicity has increased. Several cell culture systems have been developed for rapid and economical *in vitro* tests for the identification of potentially toxic compounds and to characterise the toxicity of mycotoxins. The measurement of toxicity *in vitro* is purely a cellular event and hence the toxic response may be measured by changes in cell survival or metabolism.

The need for sensitive, rapid, reliable and economical methods has led to the development of several standard assays. Such assays include the measurement of protein and DNA synthesis using an array of radiolabelling techniques. Recently, proliferation assays have become available for analysing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. In 1980, Mosmann described a Methyl Thiazol tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) reduction assay which is readily used in cytotoxicity evaluations.

3.1.1 The methyl thiazol tetrazolium(MTT) assay

The use of MTT assays for various medical, microbiological and toxicological approaches: *in vitro* assessment of chemotherapeutic agents against tumour cells; assessment of radio sensitivity and cytotoxicological evaluation of chemical compounds has proven to be very successful (Hanelt *et al.*, 1994). The principle of this reduction assay is that the tetrazolium salt is reduced to formazan (Fig 3.1) by the “succinate-tetrazolium reductase” system which belongs to the respiratory chain of the mitochondria and is only active in viable cells (Mosmann, 1980). Another theory suggests that most cellular bioreduction of MTT is associated with enzymes of the endoplasmic reticulum and involves the reduced pyridine nucleotides NADH. Succinate is a weak electron donor for mitochondrial MTT reduction (Berridge *et al.*, 1996). Therefore cellular reduction of MTT is more related to the glycolytic rate per se and thus to NADH production rather than to respiration. Berridge *et al.* (1996) showed that MTT is reduced by microsomal enzymes that require pyridine nucleotides but agree that succinate can also act as an electron donor in MTT reduction through mitochondrial succinate dehydrogenase, however this reduction is slow and contributes little to total cellular MTT reduction.

An increase in the number of viable cells results in an increase in the overall activity of enzymes in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye produced which directly correlates with the number of metabolically active cells in the culture. The formazan dye produced is quantified spectrophotometrically. The following figure illustrates the MTT reaction.

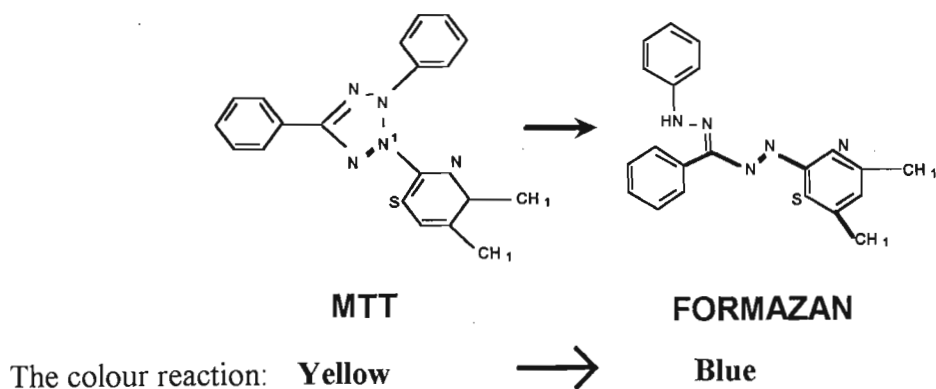


Fig 3.1 . The chemical structures of the reactant (MTT) and the product (formazan) following the exposure of the tetrazolium salt to viable cells.

This assay has been employed by Reubel et al. (1987); Hanelt et al. (1994) Shier et al (1991); Visconti et al. (1991); Dombrink et al. (1993) to mention a few who have successfully assessed the cytotoxicity of mycotoxins. To date, the results obtained have proven to be valid and reliable.

3.2. CELL CULTURE

3.2.1 The cell and its environment

Presumably the best environment for growing cells is one providing as nearly as possible the conditions they experience *in vivo*. The essential requirements for an optimum cell culture environment are temperature, pH, the substrate, gas phases and the medium containing the necessary nutrients.

a) Temperature

One of the fundamental properties of living matter is the ability to perform chemical reactions at comparatively low temperatures. The optimal temperature for cell culture depends on the body temperature of the animal from which the cells were obtained. Therefore the temperature required for optimal growth of most human cell lines

is 37°C. The temperature at which cells can be stored in is usually at -70°C. Cultures can be stored for years in a frozen state, thus ensuring a continuous stock of cells in cases of loss due to contamination.

b) Gas phase

The most important gases are oxygen and carbon dioxide. Most dispersed cells in culture prefer low oxygen tensions. The depth of the culture medium influences the rate of oxygen diffusion to the cells and is normally within the range of 2 to 5mm. HEPES(N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) (20mM) buffer is often added in the medium as an alternative to bicarbonate to stabilise the pH (7.4). HEPES is a satisfactory buffer in tissue culture media for the growth of many different cell types, mainly because it does not require an enriched atmosphere to maintain the correct pH.

c) Substrate

Most cells cultured *in vitro* have been grown as monolayers on an artificial substrate, in order to proliferate. Single-use polystyrene flasks provide a simple, reproducible environment for cultured cells.

d) Medium

The culture medium is by far the most important single factor in culturing cells. Its main function is to provide the physical conditions of pH and osmotic pressure required for survival and the complicated chemical substances required by the cells which it cannot synthesise itself. Eagles minimum essential medium (EMEM), is a commercially prepared growth medium that contains essential amino acids, vitamins and salts. One of the most important nutrients required is glutamine which is required by the cells as an energy and carbon source (Freshney, 1983). Additional proteins are required to enhance cell growth. Various types of animal sera are used to supplement culture media preparations for cell

growth. The serum contains important proteins such as albumin, globulins and fetuin which act as carriers for minerals, fatty acids and hormones and have been found to be beneficial, promoting cell attachment to the substrate. The serum used is normally from a non-human source, to exclude human antibodies which could be inhibitory to the subsequent isolation of human viruses. Foetal calf serum (FCS) is usually used as an additional protein source. A complete culture medium (CCM) contains FCS, EMEM, glutamine and antibiotics (penicillin/ streptomycin/ fungizone) and therefore sustains continuous cell growth (Reubel, 1987).

In addition to CCM, Hanks balanced salt solution (HBSS) containing inorganic salts, glucose and phenol red is routinely used to wash cultured cells in order to remove cellular debris and dead cells.

e) Trypsin

Cells in culture are routinely subcultured so that they can be propagated for the purpose of increasing cell numbers. Cells are removed from culture flasks using a variety of agents such as chelating agents, e.g., ethylenediaminetetraacetic acid (EDTA), or enzymes such as trypsin. Trypsin, an aqueous crude extract prepared from porcine pancreas is a proteolytic enzyme that aids in the digestion of connective tissue of cells and their cell membranes. Trypsin breaks down the intracellular matrix that binds cells to each other and the substrate surface allowing adjacent cells to detach from each other. The optimum conditions for trypsin activity are at a pH range of 7.6-7.8 and a temperature of 37°C.

3.2.2 The maintenance of the cell line

The major requirement that distinguishes cell culture from many other laboratory techniques is the need to maintain asepsis. Media for the cultivation of cells are highly

nutritious not only for the cells but also for foraging invaders such as bacteria and fungi. The majority of these microorganisms have a much more rapid growth rate than cells and frequently produce toxins which are lethal to them. The most important part of cell culture is to eliminate contamination. The laminar flow cabinets with sterile air blown over the work surface affords greater control of sterility.

Once the culture is seeded it needs a periodic medium change followed by passaging and subculturing to attain confluence. The required time for cell growth *in vitro* is approximately one week. The characteristic features which determines whether or not a culture could be passaged includes cell density, rate of cell division and cell morphology (Freshney, 1983).

3.3 MATERIALS AND METHODS

All cell culture consumables were purchased from Polychem S.A., unless otherwise stated.

3.3.1 Materials

a) Base medium

Eagles Minimum Essential Medium with Earle's salts, Hepes, non essential amino acids and L-glutamine were routinely used. Constituents of each of these products are shown in appendix 1. Foetal calf serum (FCS) was heat inactivated at 56°C for 30 minutes in a water bath with agitation. The FCS was filter sterilised using a 0.45 μ filter (Millipore), aliquoted into 5ml vials and stored at -20°C until required. It was necessary to aliquot FCS since it was previously found that repeated thawing of heat inactivated FCS at 37°C resulted in the presence of granules, flocculent material and appeared turbid. To enhance cell growth, 5 to 10% FCS was added into the culture medium for growing cells while 0.5 to 5% was added when the cells formed confluent layers. To eliminate contamination, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2.5 μ g/ml fungizone

were added to the culture medium. To 100ml of culture medium a 1% penicillin/streptomycin/fungizone mixture was added.

b) HBSS

The HBSS (Appendix 1) was added to wash off dead cells and cellular debris.

c) Trypsin

Lyophilised trypsin (2.5g) rehydrated with 20ml sterile distilled water was made up to 100ml with HBSS (calcium and magnesium free). The final mixture was then dispensed in 2ml aliquots in sterile Bijou bottles and stored at -20°C.

d) Mycotoxins

The mycotoxins zearalenone (Zea), Aflatoxin B₁ (AFB₁) and AFB₂ were obtained from CSIR. The epoxide derivatives of both Zea and AFB₁ were synthesised in the laboratory as outlined below.

Synthesis of the AFB₁-8,9-epoxide

Synthesis of the AFB₁-8,9-epoxide involved oxidation of AFB₁ (10mg) with dimethyl dioxirane (DMOX) (1ml) prepared from potassium peroxydisulphate as a distilled 0.05M solution in acetone (Baertschi *et al.*, 1988). AFB₁ was dissolved in acetone (2ml) and reacted with two fold excess of DMOX. The reaction was complete within thirty minutes at room temperature. Solvents and excess DMOX were removed by evaporation under a stream of N₂ leaving the epoxide which was recrystallized using methylene chloride (1.5ml). (¹H NMR spectral data not shown).

The synthesis of the Zea epoxide

Zea (10mg) was dissolved in dry analar acetone (2ml) to which was added 3 moles of dioxirane. The reaction was stirred in a reaction vial for 12 hours at room temperature. A sample (5 μ l) was spotted onto a thin layer chromatographic plate (TLC) (10 cm x10cm, aluminium backed) and developed in hexane : DCM : ethanol (15:10:15). The plate was dried under a stream of warm air and tested for the presence of the epoxide. The tlc plate showed more than one product. Using silica 7729, the components were separated using column chromatography. Comparison of the ¹HNMR spectra of zearalenone supported the epoxide formulation.

Mycotoxin stock solutions

Stock solutions of the mycotoxins were prepared by dissolving 0.5 mg of each of the mycotoxins in 30 μ l ethanol, 170 μ l DMSO and 4.8ml CCM to produce toxin concentrations of 0.1mg/ml. The stock solutions were then mixed and filter sterilised through 0.45 μ filters (Millipore). Mycotoxin concentrations were prepared ranging from 1.56 μ g/ml to 25 μ g/ml by serially diluting the stock solutions with CCM. The controls were prepared in the same way without the addition of mycotoxins.

e) Cell lines

All cell lines were purchased from Highveld Biologicals.

It has been recognised that several cell lines should be used in parallel to test the cytotoxicity of mycotoxins since toxin activating systems and effects may differ with the type of cells utilised. Therefore in this study AFB₁, AFB₁-8,9-epoxide and AFB₂ have been evaluated using both HepG2 (liver carcinoma) and A549 (lung carcinoma) cell lines while Zea and its epoxide were assessed using HepG2 and Hela (cervical carcinoma) cell lines. The selection of the biological system must reflect the purpose of the study. For the study of organ-specific toxic mechanisms the similarity of the system to the *in vivo* target

organ is essential. Since the liver is involved in the detoxification processes HepG2 cells were chosen to assess the cytotoxicity of the selected mycotoxins, especially AFB₁.

f) Preparation of the tetrazolium salt (MTT)

The MTT (Boehringer Manneheim) (5mg) was dissolved in phosphate buffered saline (1ml) to give a concentration of 5mg/ml and filtered through a 0.45 μ filter (Millipore) to remove insoluble residues of the yellow powder. The sterile stock solution was stored in the dark at 4°C.

3.3.2 Methods

a) Cell maintenance (Appendix 2)

Culture flasks (25cm³) that contained cells with a 20% confluency needed a routine medium (supplemented with 5% FCS) change. The cells were passaged by discarding the medium with depleted nutrients. Flasks were then rinsed in HBSS (5ml) (37°C) to remove cellular debris and further washed twice in HBSS. Cell detachment was detected microscopically or with the naked eye as detached cells gave the medium a cloudy appearance. Complete culture medium (5ml) was then added and the flasks were capped and returned to the incubator (37°C) (Labotec).

The medium from culture flasks of cells with 100% confluency was discarded and the cells were washed twice with HBSS described above. A 1ml trypsin solution (0.25%) was added to the confluent flasks and trypsin digestion was allowed to proceed for approximately 1 to 5 minutes until microscopic observation showed the cells to have rounded off slightly but not to have become dislodged from the surface. The trypsin was then removed with a sterile pasteur pipette. To the flask was added 1.5ml CCM containing 10% FCS and the cells were dispersed in the solution by tapping the flask hard against the hand. The resultant suspension was repeatedly pipetted with a sterile pipette

to separate clumps of cells. To a 0.5 ml cell suspension was added 4ml of medium for 1:3 split. Flasks were then stoppered and incubated at 37°C without further disturbance to allow cells to attach to the substrate.

Culture flasks containing confluent cells (not immediately required for cytotoxic testing) were stored by freezing the cells. The cells were first trypsinised and then suspended in freezing medium (EMEM + 10% Dimethylsulfoxide + 10% FCS). The cell suspension (1ml) was dispensed in 2 ml vials and stored at -70°C.

When cells were required for cytotoxicity assays, the frozen cells were rapidly thawed by centrifugation (10000g) at 25°C. The supernatant was discarded and the pellet was resuspended in fresh CCM (1ml). The pelleted cells were dispersed in the culture medium by gentle shaking and finally transferred to a culture flask for incubation (37°C). It is important to note that the procedures described thus far were all under strict aseptic conditions.

b) The cytotoxicity assay

A culture flask (25cm³) of confluent HepG2 cells were trypsinised and resuspended in CCM (5ml) to give a cell number of 4×10^4 cells/ml, using a haemocytometer (Freshney, 1983). This cell suspension was dispensed (100µl) into each well of a 96 well microtiter plate giving a final cell count of (4000 cells/well). For each mycotoxin concentration (25µg/ml to 1.56µg/ml) there were five replicates of a 100µl each. The control cells consisted of 100µl cell suspension and a serial dilution of DMSO and ethanol (five replicates of 100µl each). Plates were incubated for 48 hours at 37°C.

The MTT reduction assay

After the cells were incubated (48hours) with the mycotoxins, the wells were aspirated to remove growth medium and 100µl of CCM together with 10µl of MTT

(5mg/ml) was immediately added to each well. The microtiter plate was then incubated (37°C) for a further four hours. Thereafter the supernatants were aspirated and 100µl of DMSO (100%) was added to each well and incubated for another hour (37°C). Finally the optical density of each well was measured spectrophotometrically using a Bio-rad microplate reader at a wavelength of 595nm with a reference wavelength of 630nm.

c) Phase contrast microscopy

In order to investigate morphological changes under the phase contrast microscope, AFB₁ (25µg/ml) was added to a confluent layer of HepG2 cells in a 25cm³ flask. The flasks were incubated (37°C) for 24 hours. Cells were microscopically examined to observe the cytotoxic effects of AFB₁. Cells were observed through a Zeiss inverted microscope and photographed with a Zeiss photomicroscope (using green filters).

3.3.3 Statistical analysis

The results from the cytotoxicity tests were analysed using a Statsgraphic Plus programme. Mean absorbances were expressed as % cleavage activity in comparison to cell controls (100%):

$$\% \text{ Cell viability} = \frac{\text{mean absorbance of toxin treated cells}}{\text{mean absorbance of control cells}} \times 100$$

The multiple box and whisker plots show the distribution of the range of data for each mycotoxin. It is useful for comparing parallel batches of data. The (x) denotes the means and the (-) denotes the median. Bar graphs representative of dose response curves showing absorbances as a measure of cleavage activities have also been plotted in an attempt to illustrate cell viability.

3.4. RESULTS

3.4.1 HepG2 cell growth and characteristics

The HepG2 cells grew rapidly and after two days in culture the cells appeared scattered on the substrate. The cells were monolayered, polygonal, non-refractile, agranular and were tightly adherent to one another. After a week in culture cells appeared tightly packed together (Fig 3.2). Due to substrate limitations, the cells appeared more rounded as they tried to anchor in the intercellular matrix. Many cells displayed prominent nuclei which were observed by phase contrast microscopy (x1600). At a lower magnification (x1000) a confluent monolayer of cells was clearly observed, together with small areas containing cellular debris (Fig 3.3).

3.4.2 HepG2 cells treated with AFB₁

After 24 hours incubation of confluent HepG2 cells with AFB₁ (25µg/ml) a distinct change in cell morphology was observed. Cell death was characterised by cell rounding, a detachment from the substrate and a marked inhibition of cellular growth (Fig 3.4). The culture medium also appeared turbid due to the high content of cellular material floating around. In addition cells appeared granulated.



Fig 3.2 A phase contrast micrograph of HepG2 cells growing in a 25cm³ culture flask after a week in culture. The cells appeared bi-nucleated (x1600).

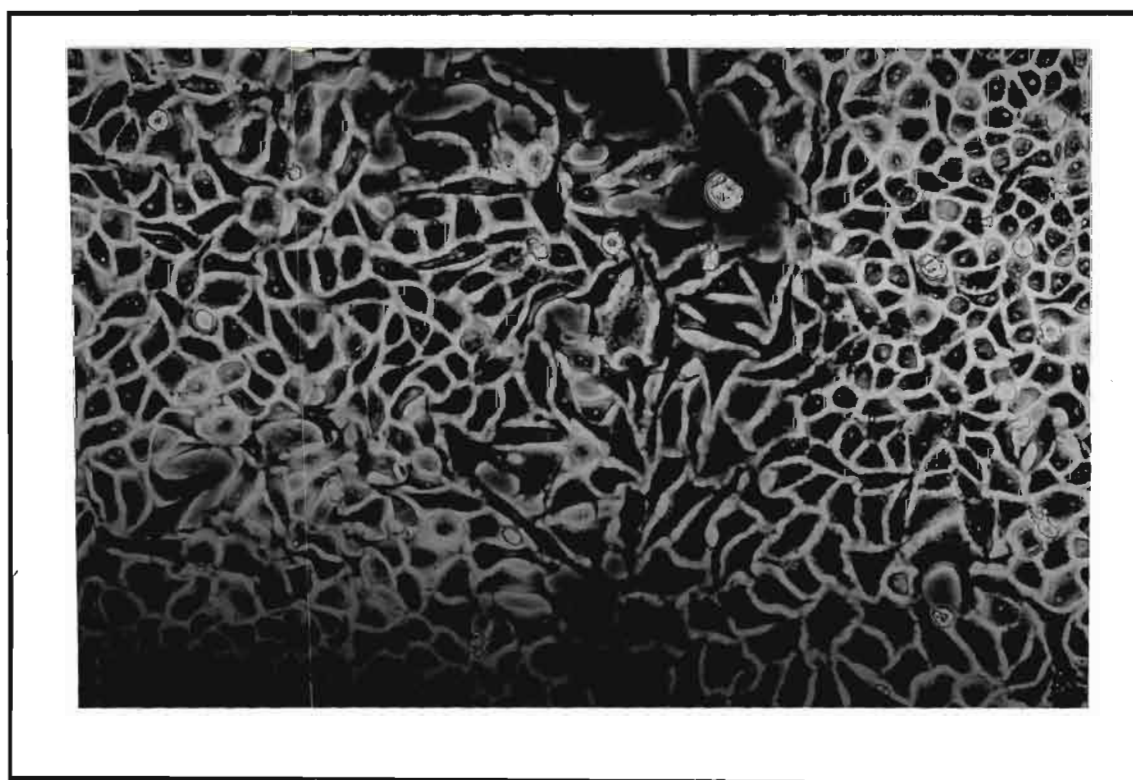


Fig 3.3 A phase contrast micrograph of a confluent monolayer of HepG2 cells , after a week in culture attached to the substrate (S), with some cellular debris (x1000).

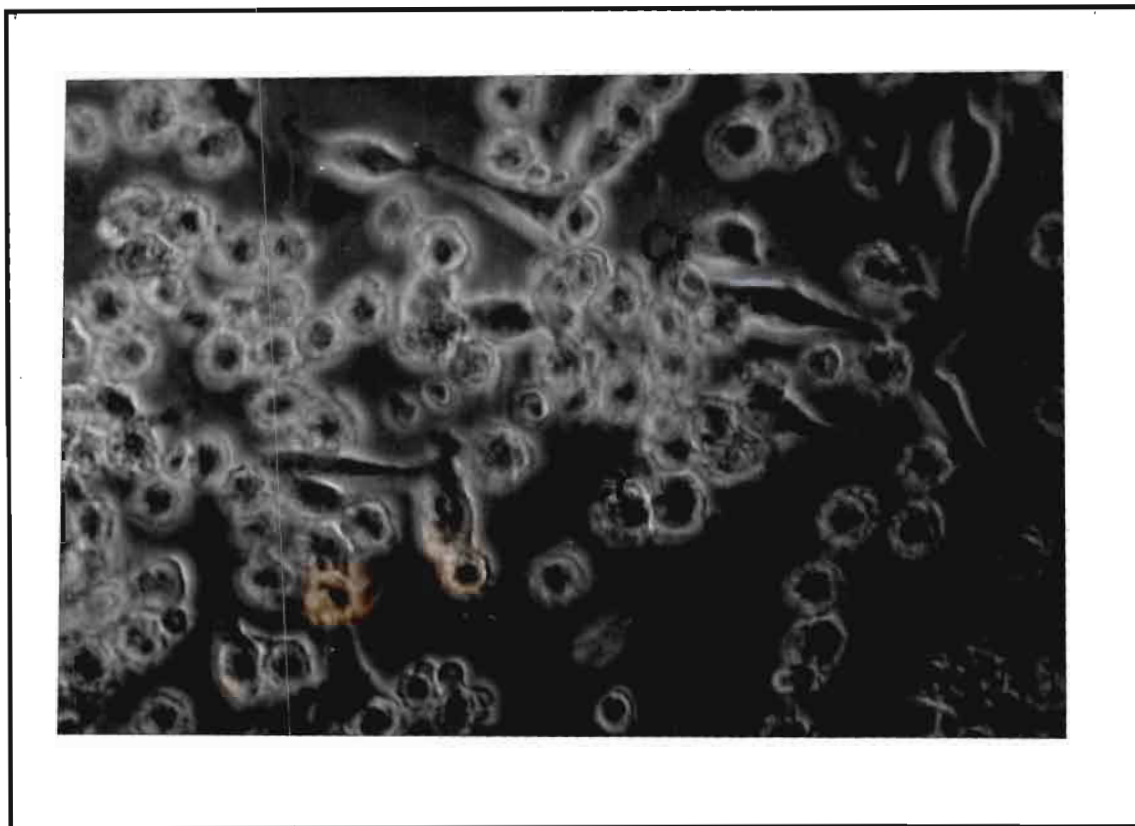


Fig 3.4 AFB₁ (25µg/ml) treated cells following a 24 hour exposure period, showing characteristic features of cell death such as cell rounding (Cr) (x1600).

3.4.3 The colour reaction of the HepG2 cells

Following treatment with the respective mycotoxins the colour of the culture medium remained pink indicating viable cells (Fig 3.5a). However, after the 48 hour incubation period of the cells with mycotoxins the cells reacted with the added tetrazolium salt (MTT) to form purple crystals. DMSO solubilizes the crystals and results in the production of a purple dye (formazan) (Fig 3.5b).

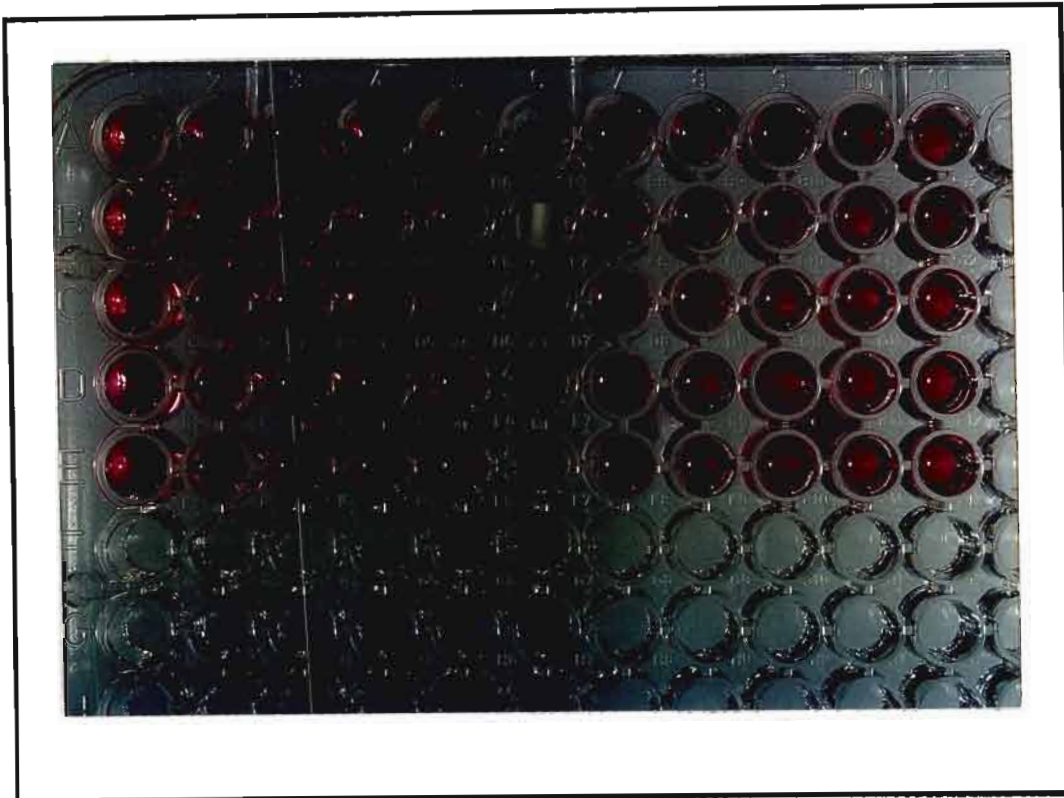


Fig 3.5a Cells exposed to a serial dilution of mycotoxins ($25\mu\text{g/ml}$ to $1.56\mu\text{g/ml}$) in a 96 well microtiter plate for 48 hours.

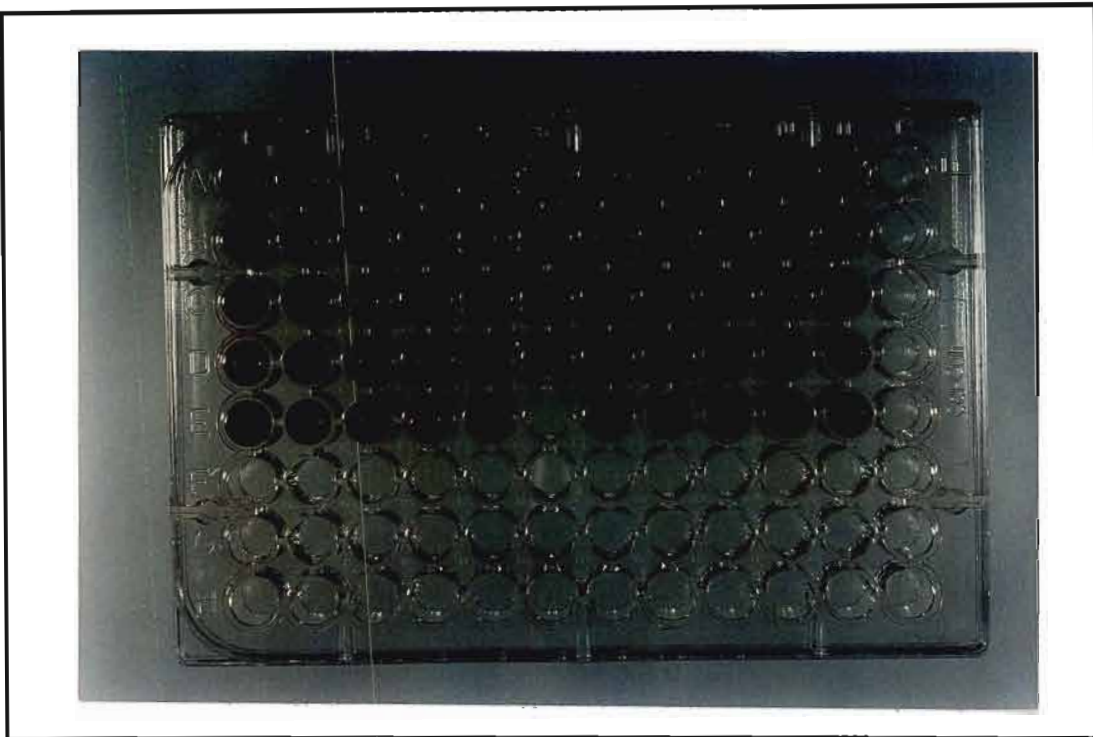


Fig 3.5b Following the exposure period to the mycotoxins cells reacted with MTT to form purple crystals which are solubilized in DMSO to form a purple dye.

3.4.4 The cytotoxic evaluation of AFB₁ and the AFB₁-8,9-epoxide using HepG2 cells

The results showed that HepG2 cells were particularly sensitive to the AFB₁-8,9-epoxide as compared to AFB₁. The multiple box and whisker plot showed a significant difference ($p = 0.007$) in the distribution of the toxicity levels of AFB₁ and the AFB₁-8,9-epoxide at a 95% confidence interval (Fig 3.6.1). The bar graph representative of the dose response curve showing cleavage activity displayed a 72% cell viability for AFB₁ at 25 μ g/ml while the epoxide treated cells showed a 61% cell viability (Fig 3.6.2). At the lower concentrations (1.56 μ g/ml) AFB₁ showed a 100% cell viability while the AFB₁-8,9-epoxide showed a 95% cell viability. The results suggests that the AFB₁-8,9-epoxide is more cytotoxic than AFB₁.

3.4.5 Effects of the AFB₁-8,9-epoxide and AFB₂ on HepG2 cells

A comparison of the cytotoxic effects of the AFB₁-8,9-epoxide and AFB₂ using the multiple box and whisker plot showed a significant difference ($p = 0.02$) at 95% confidence interval between the two toxins (Fig 3.7.1). The bar graph (Fig 3.7.2) of each of the toxins showed a distinct difference in the distribution of the toxic levels for both toxins. Upon analyses of the bar graph it was found that AFB₂ elicited minimal cytotoxic effects at all concentrations tested (100% cell viability at 25 μ g/ml) while the AFB₁-8,9-epoxide showed significant cytotoxic damage (25 μ g/ml showed only 58% cell viability).

3.4.6 The effects of AFB₁ and the AFB₁-8,9-epoxide on A549 cells

The A549 cells reacted differently to the exposure to AFB₁ and the AFB₁-8,9-epoxide. From the multiple box and whisker plot it was found that there was no significant difference between the effects of both the parent toxin and its derivative ($p=0.4$) (Fig 3.8.1). The distribution of the toxicity levels was similar for both toxin treatments (Fig 3.8.2). This result suggests that the HepG2 cells are more sensitive to AFB₁ than the A549 cells. From the dose response curve it was found that at 25 μ g/ml AFB₁ displays a 75% cell viability while the AFB₁-8,9-epoxide displays an 80% cell viability and at 1.56 μ g/ml AFB₁ displayed 94% cell viability and the epoxide 97% cell viability.

3.4.7 The cytotoxic effects of Zea and its epoxide on Hep G2 cells

Results using a multiple box and whisker plot showed that there is a significant difference ($p=0.0002$) between the toxicity levels of Zea and its epoxide with the latter being highly cytotoxic (Fig 3.9.1). The bar graph showed that at 25 μ g/ml Zea displayed a 66% cell viability while the epoxide displayed only an 18% cell viability (Fig 3.9.2).

3.4.8 The cytotoxic effects of Zea and its epoxide using Hela cells

When Hela cells were exposed to these mycotoxins there was no significant difference between the toxicity levels ($p=0.7$) (Fig 3.10.1). The absorbancy levels were similar in distribution. The bar graph showed an increased cell viability at 25 μ g/ml with Zea showing a 89% cell viability and the epoxide derivative showing a 96% cell viability. At 1.56 μ g/ml Zea showed a 64% cell viability and the epoxide derivative showed a 72% cell viability (Fig 3.10.2).

3.4.8 The cytotoxic effects of AFB₁ and Zea on HepG2 cells

It was decided to investigate the cytotoxicity of these two mycotoxins produced from two different toxigenic genera in an attempt to assess cytotoxicity levels as they co-occur as reported in epidemiological studies. The dose response curve showed 100% cell viability at all concentrations of Zea while AFB₁ produced an increased cell viability with a decrease in AFB₁ concentrations with 100% cell viabilities below 6.25µg/ml. However when both mycotoxins co-occur there is a distinct increase in cell mortality with increased toxin concentrations with the curve showing only 46.7% cell viability at 25µg/ml and 100% cell viability at 1.56µg/ml (Fig. 3.11).

From the results obtained it is clear that there were distinct differences between the cell lines in relation to their sensitivity to the mycotoxins and their derivatives. The statistical significance obtained using dose response curves and multiple box and whisker plots were effective in illustrating the varying cytotoxicity levels of the toxins. Concentrations of mycotoxins exhibiting higher absorbancy values than the controls may be attributed to enhanced cell growth.

**Multiple box-and-whisker plot of AFB1
and its epoxide derivative in HepG2 cells**

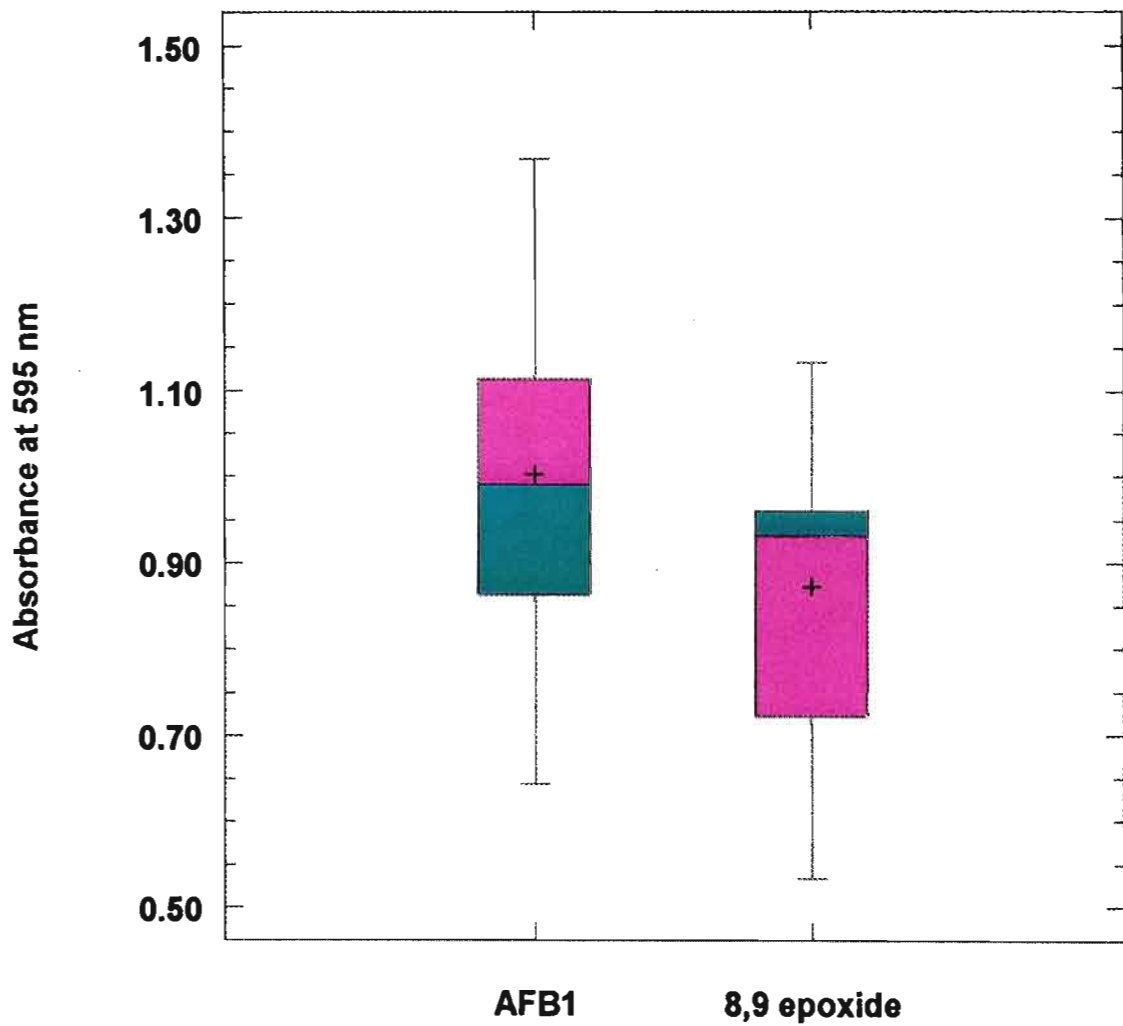


Fig 3.6.1 A multiple box and whisker plot showing the distribution of the toxicity levels of AFB₁ and AFB₁-8,9-epoxide in HepG2 cells

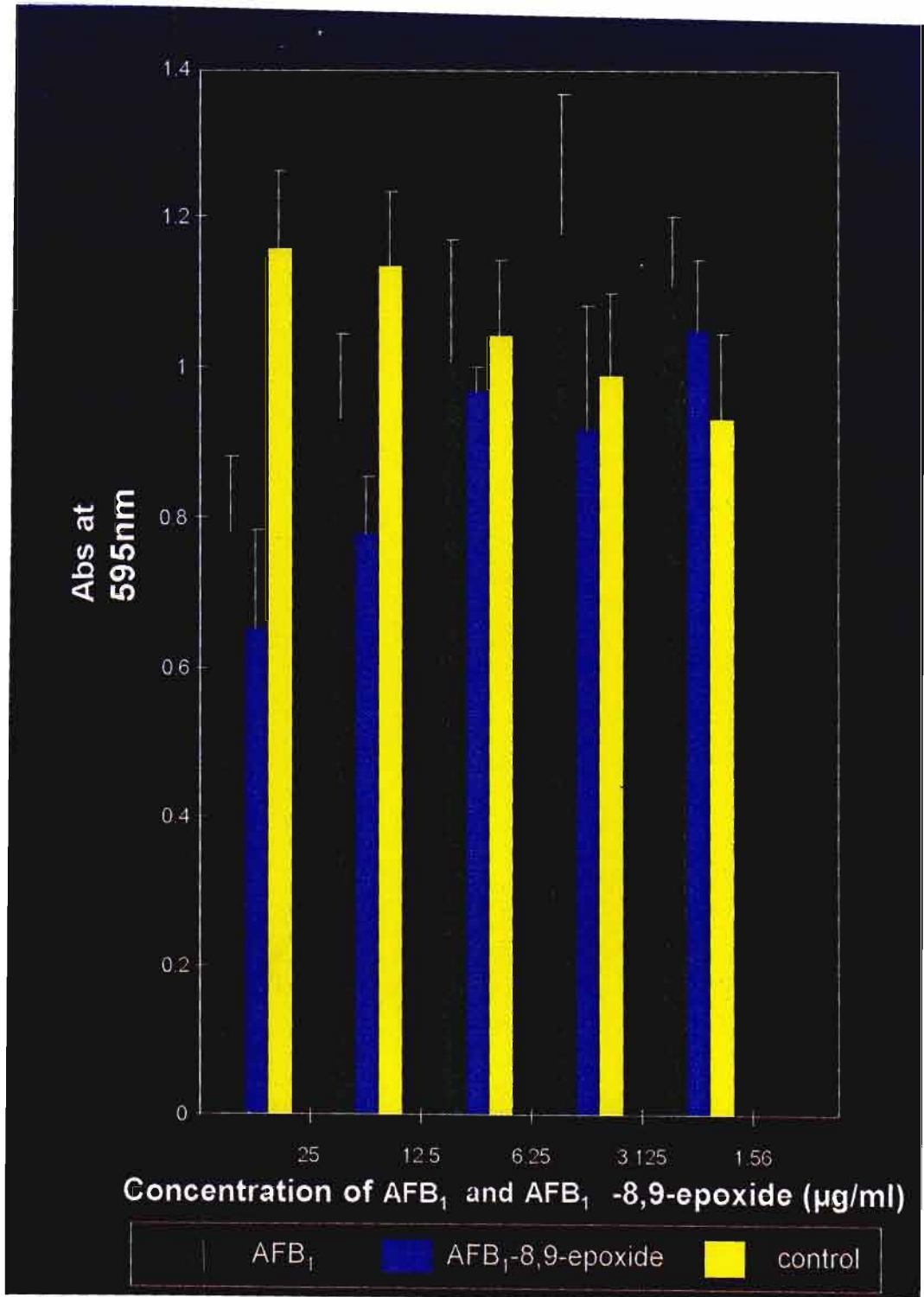


Fig 3.6.2 A bar graph showing the cleavage activity of viable HepG2 cells as a measure of absorbance following the exposure to AFB₁ and the AFB₁-8,9-epoxide at various concentrations. Error bars represent standard deviations, n=5.

Multiple box-and-whisker plot of
8,9 epoxide derivative and AFB₂ in HepG2 cells

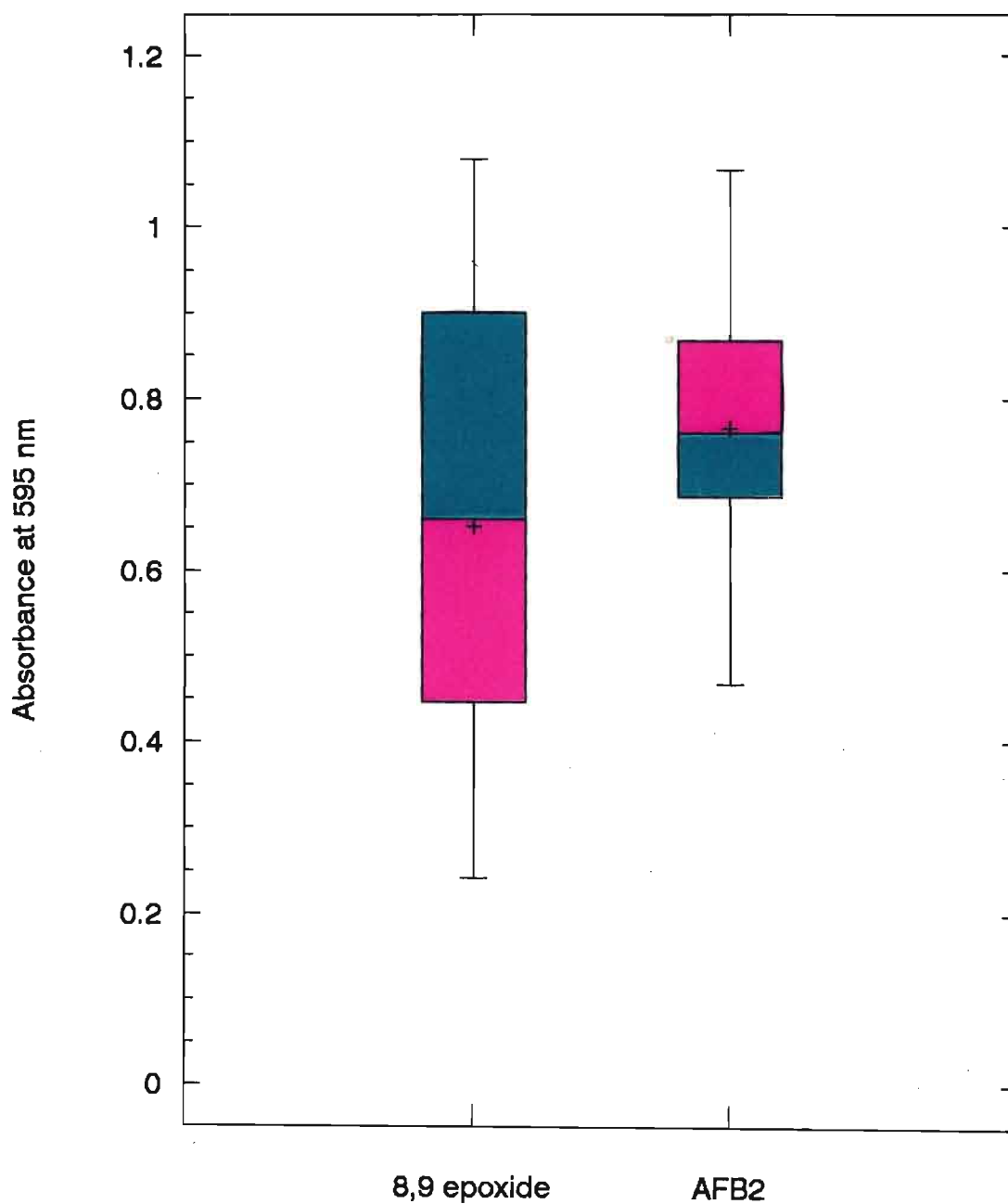


Fig 3.7.1 A multiple box and whisker plot showing the distribution of the toxicity levels of AFB₁-8,9-epoxide and AFB₂ in HepG2 cells.

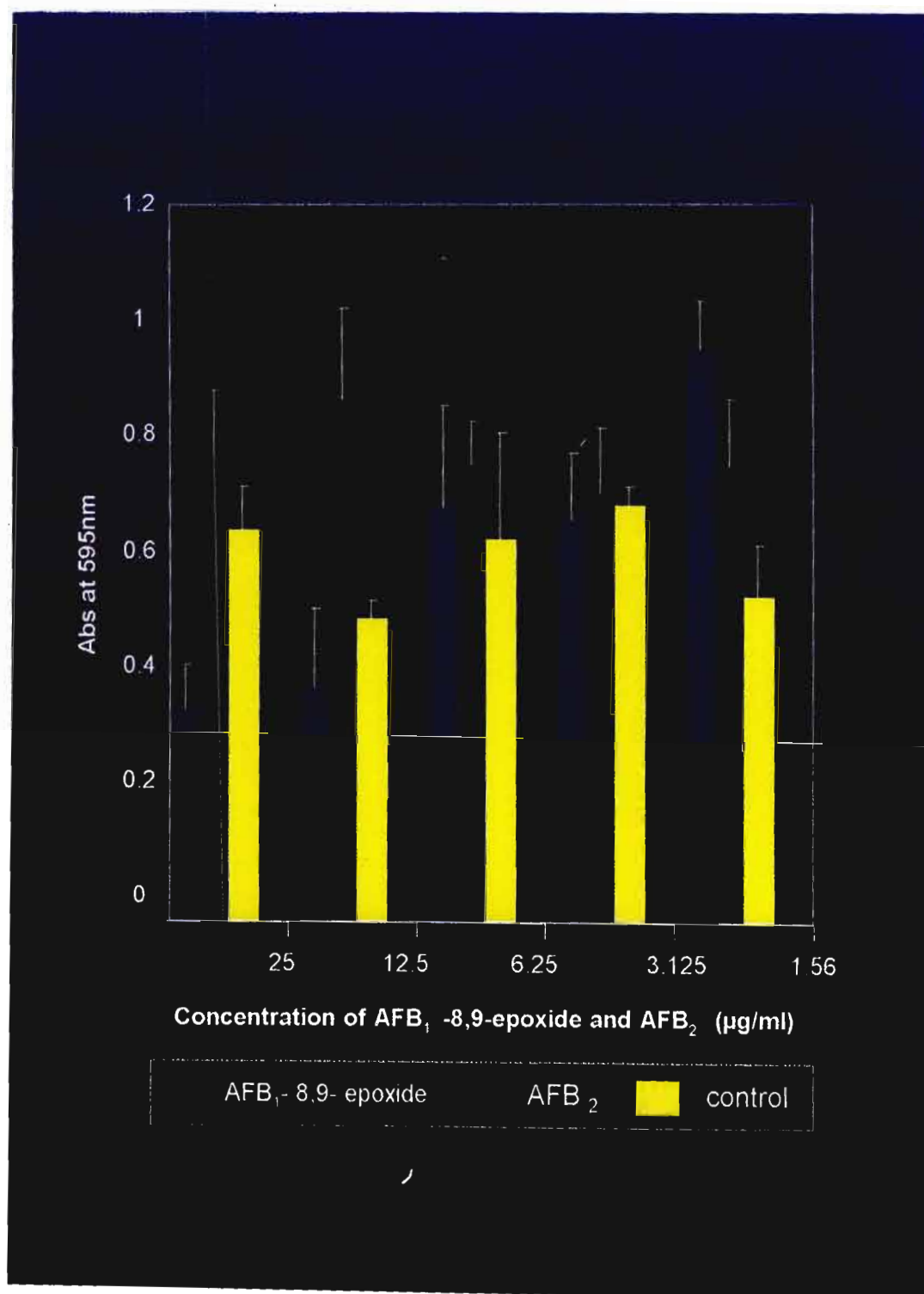


Fig 3.7.2 A bar graph showing the cleavage activity of viable HepG2 cells following exposure to AFB₁-8,9-epoxide and AFB₂. Error bars represent standard deviations, n=5.

Multiple box-and-whisker plot of AFB1 and its epoxide derivative in A549 cells

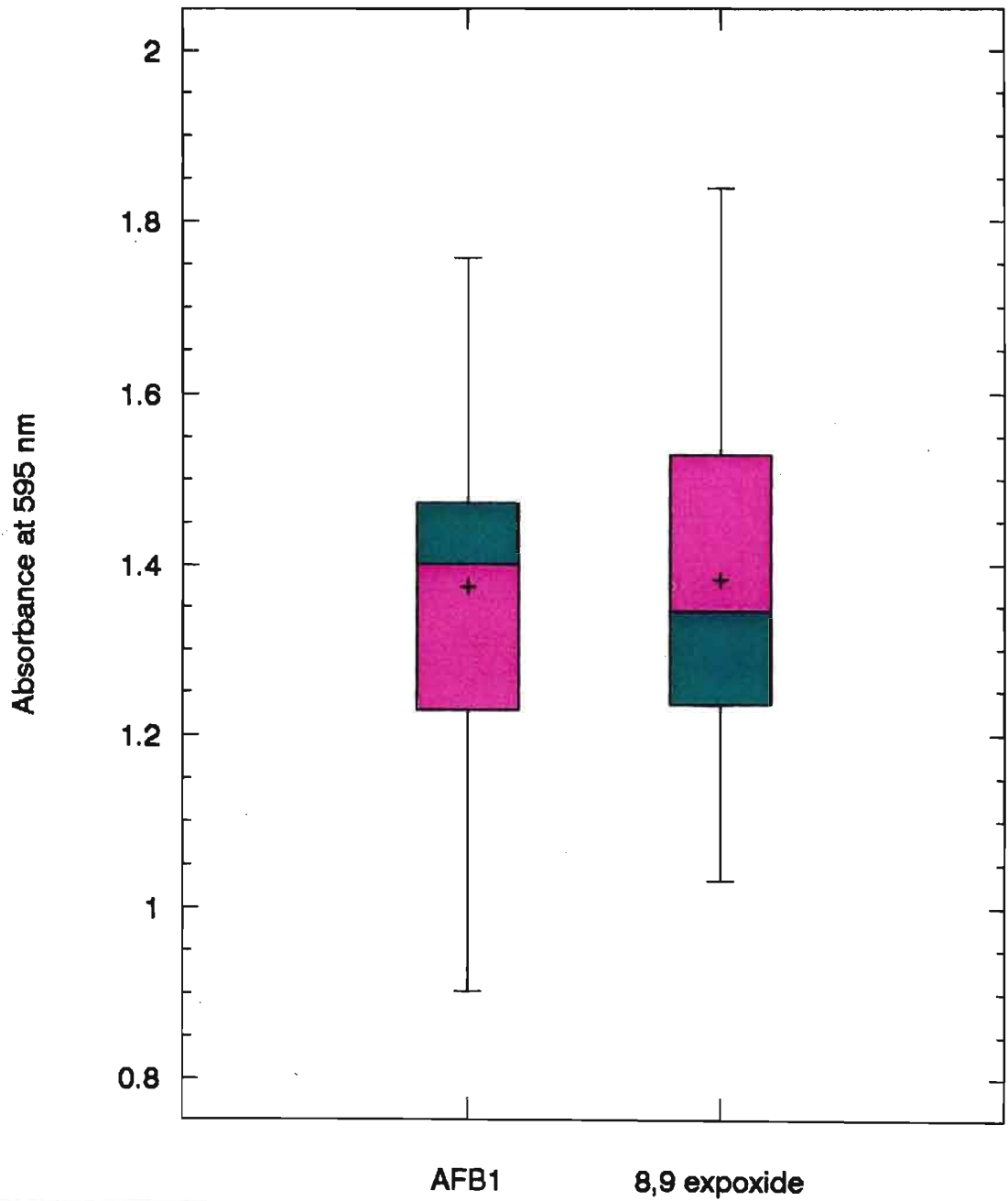


Fig 3.8.1 A multiple box and whisker plot showing the distribution of toxicity levels of AFB₁ and AFB₁-8,9-epoxide in A549 cells.

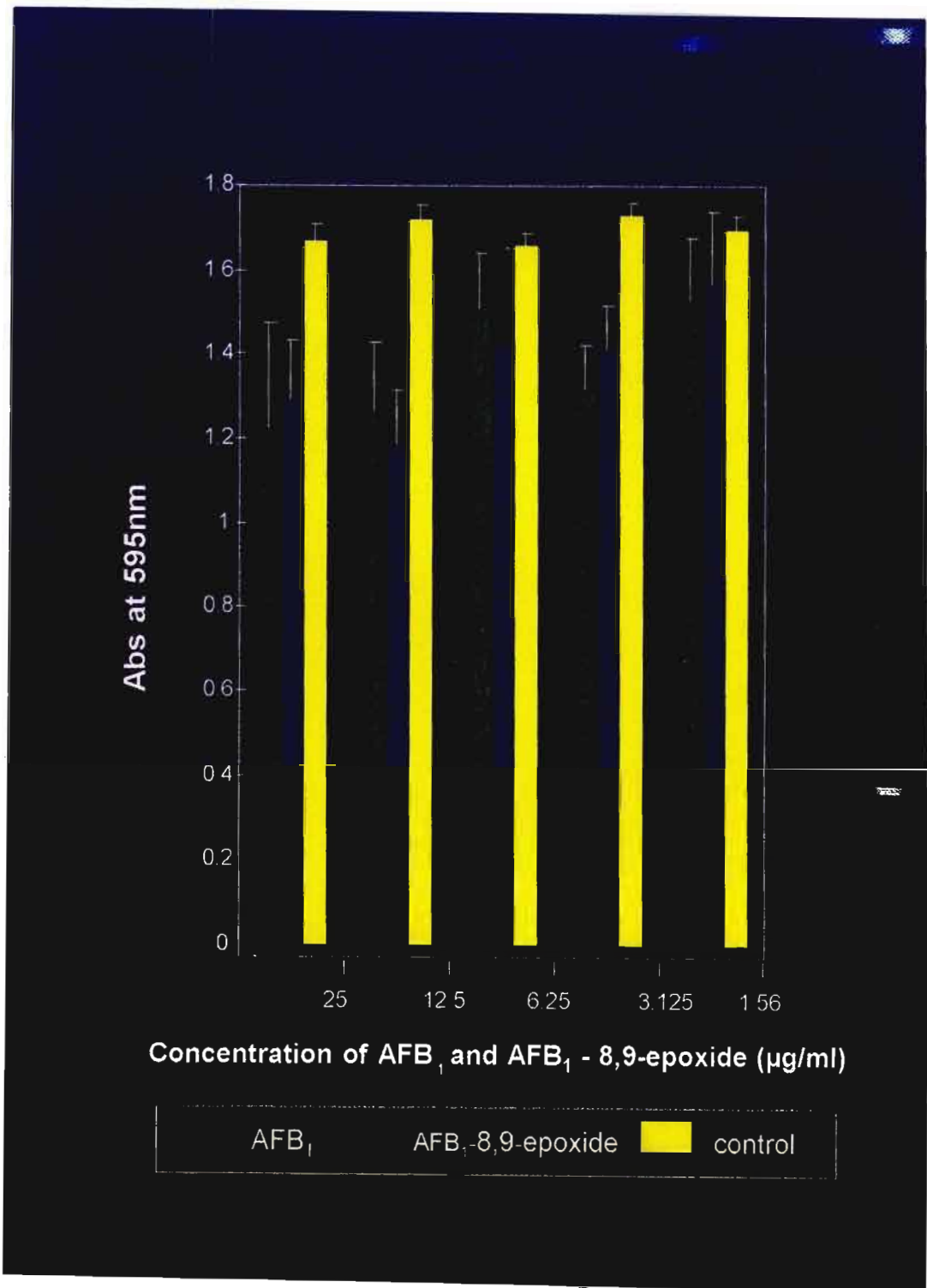


Fig 3.8.2 A bar graph showing the cleavage activity of viable A549 cells following the exposure to AFB₁ and AFB₁-8,9-epoxide at various concentrations. Error bars represent standard deviations, n=5.

Multiple box-and-whisker plot of Zea and its epoxide derivative in HepG2 cells

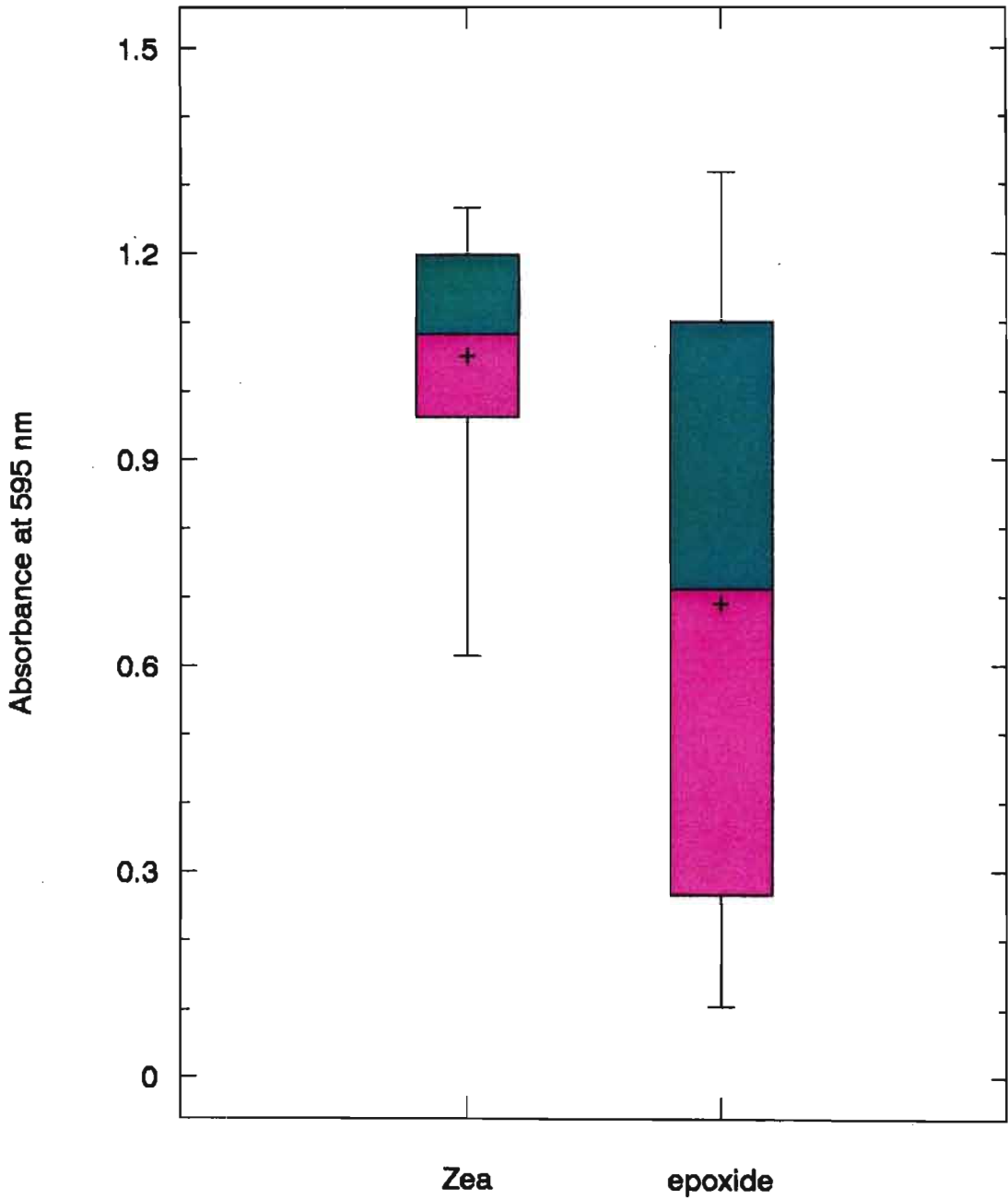


Fig 3.9.1 A multiple box and whisker plot showing the distribution of the toxicity levels of Zea and the zea-epoxide in HepG2 cells.

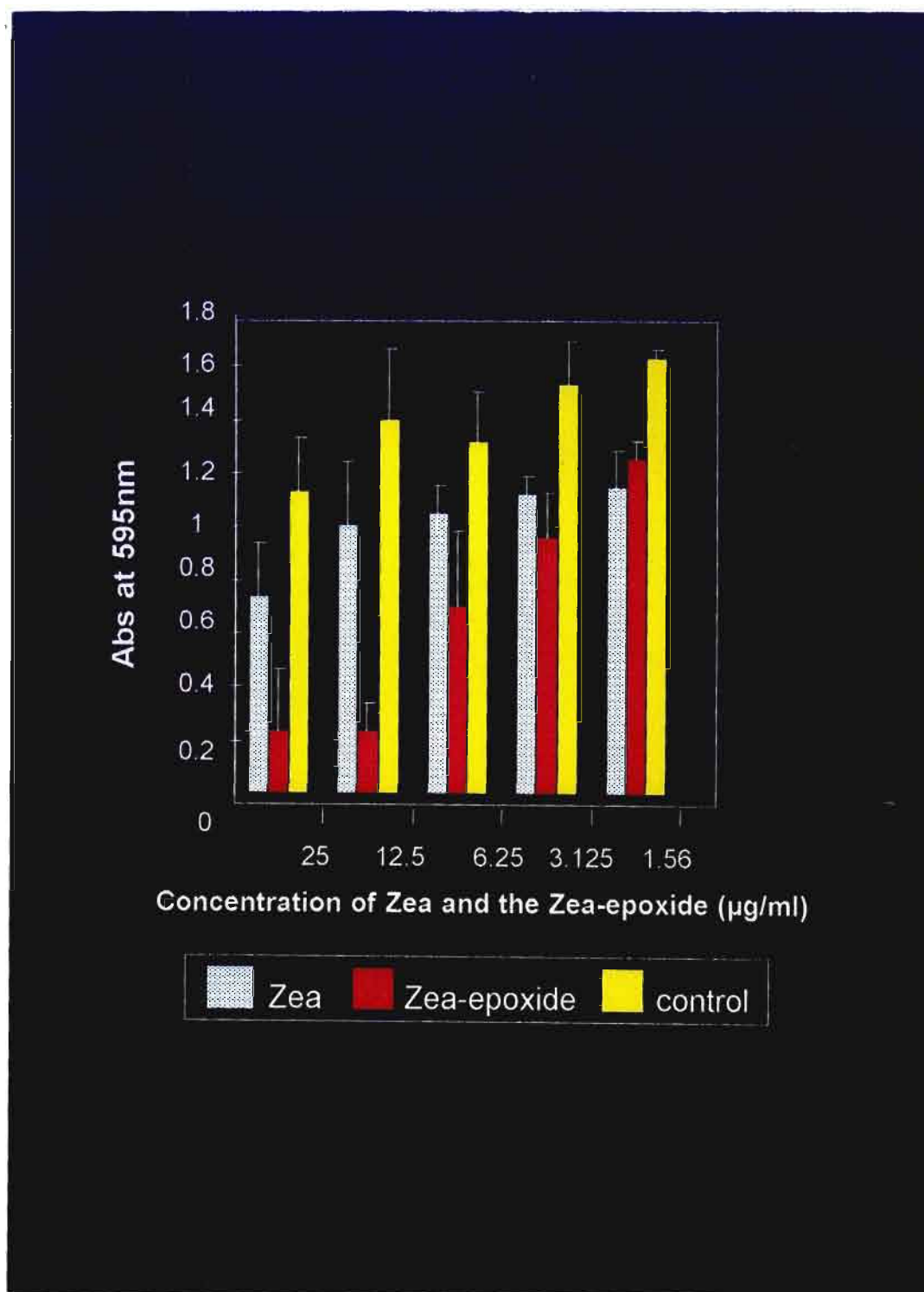


Fig 3.9.2 A bar graph showing the cleavage activity of viable HepG2 cells as a measure of absorbancy levels following exposure to Zea and the zeae-epoxide at various concentrations. Error bars represent standard deviations, n=5.

**Multiple box-and-whisker plot of Zea
and its epoxide derivative in Hela cells**

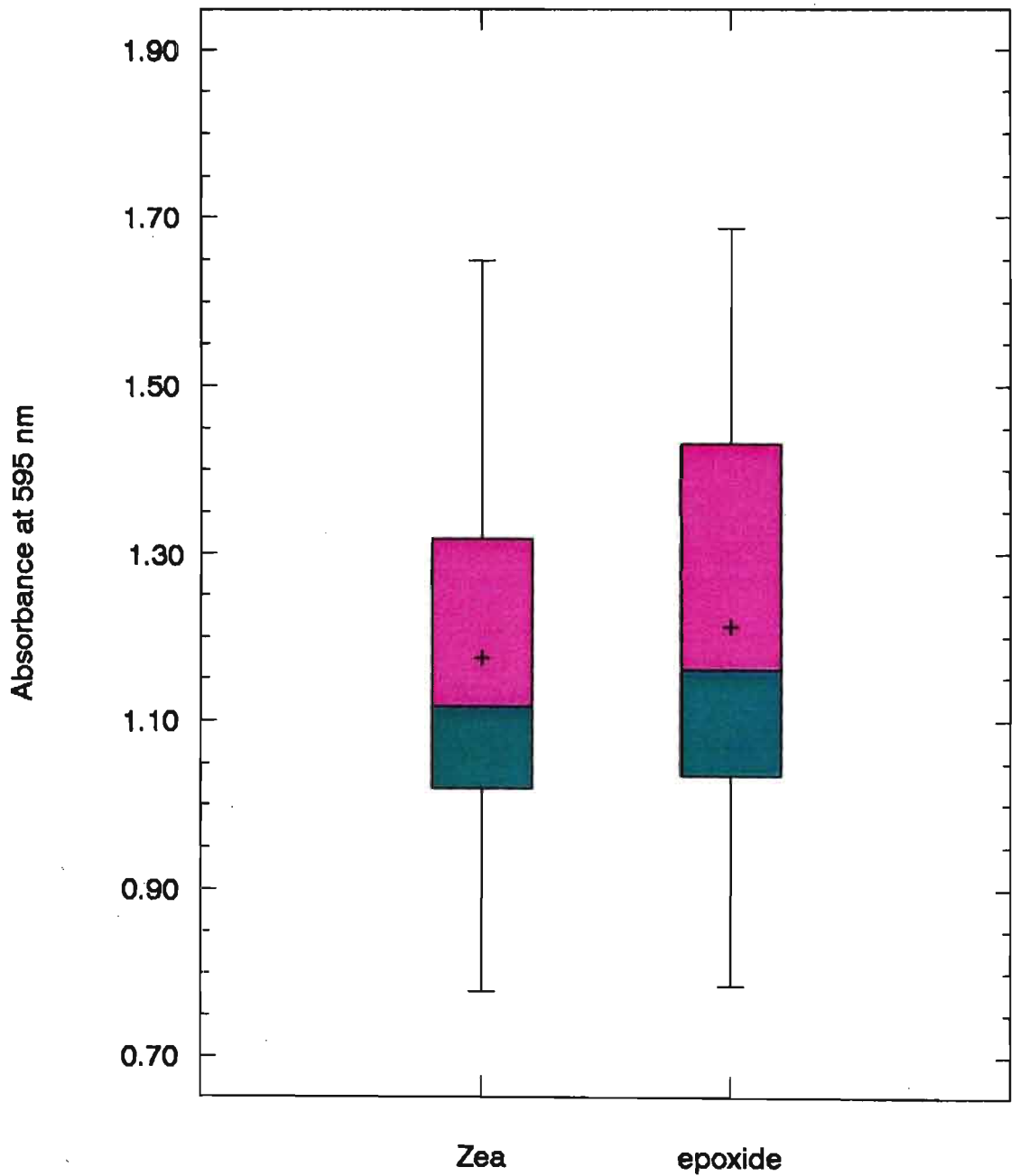


Fig 3.10.1 A multiple box and whisker plot showing the distribution of toxicity levels of Zea and zea-epoxide in Hela cells.

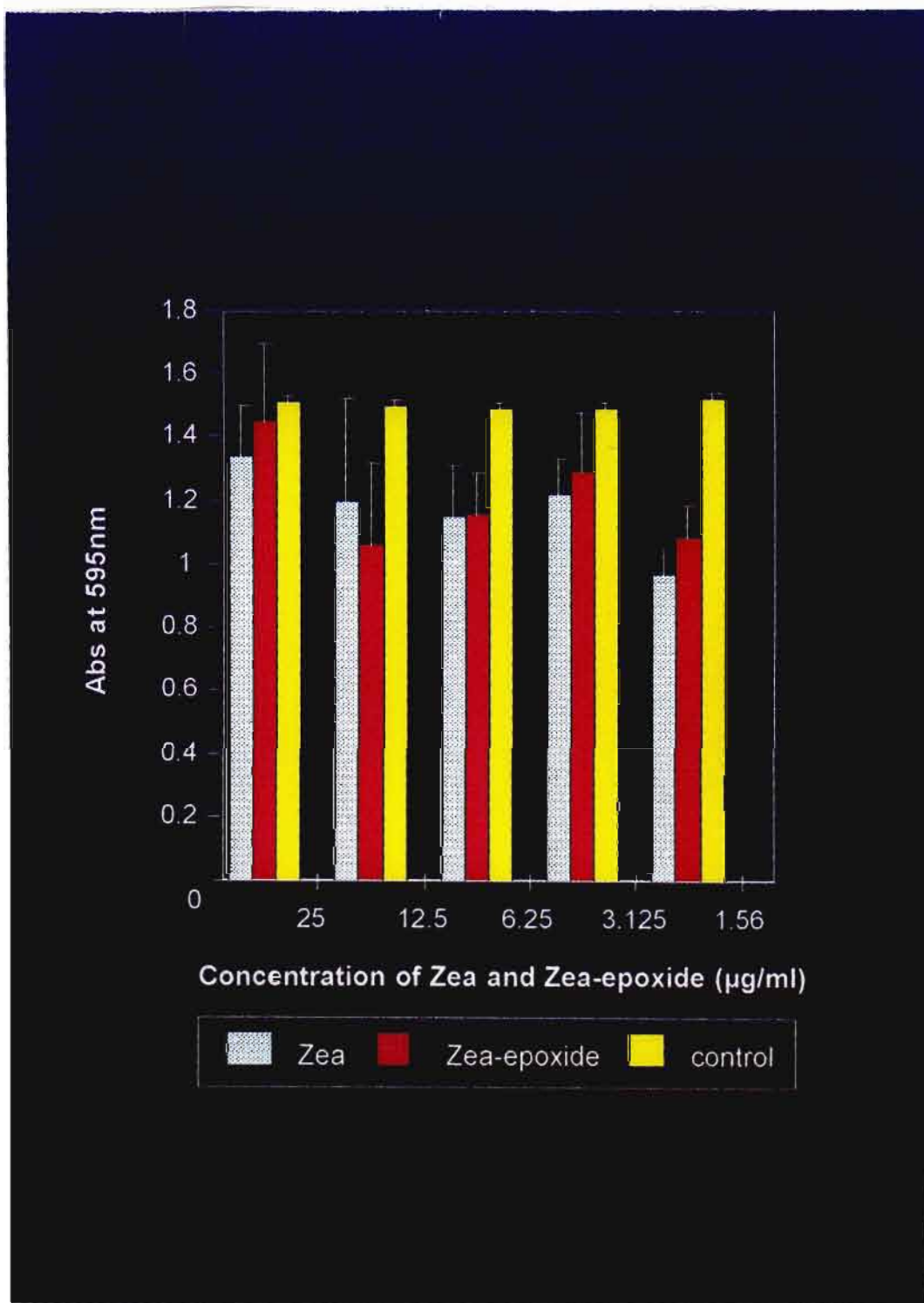


Fig 3.10.2 A bar graph showing the cleavage activity of viable hela cells as a measure of absorbancy following exposure to Zea and the zea-epoxide. Error bars represent standard deviations, n=5.

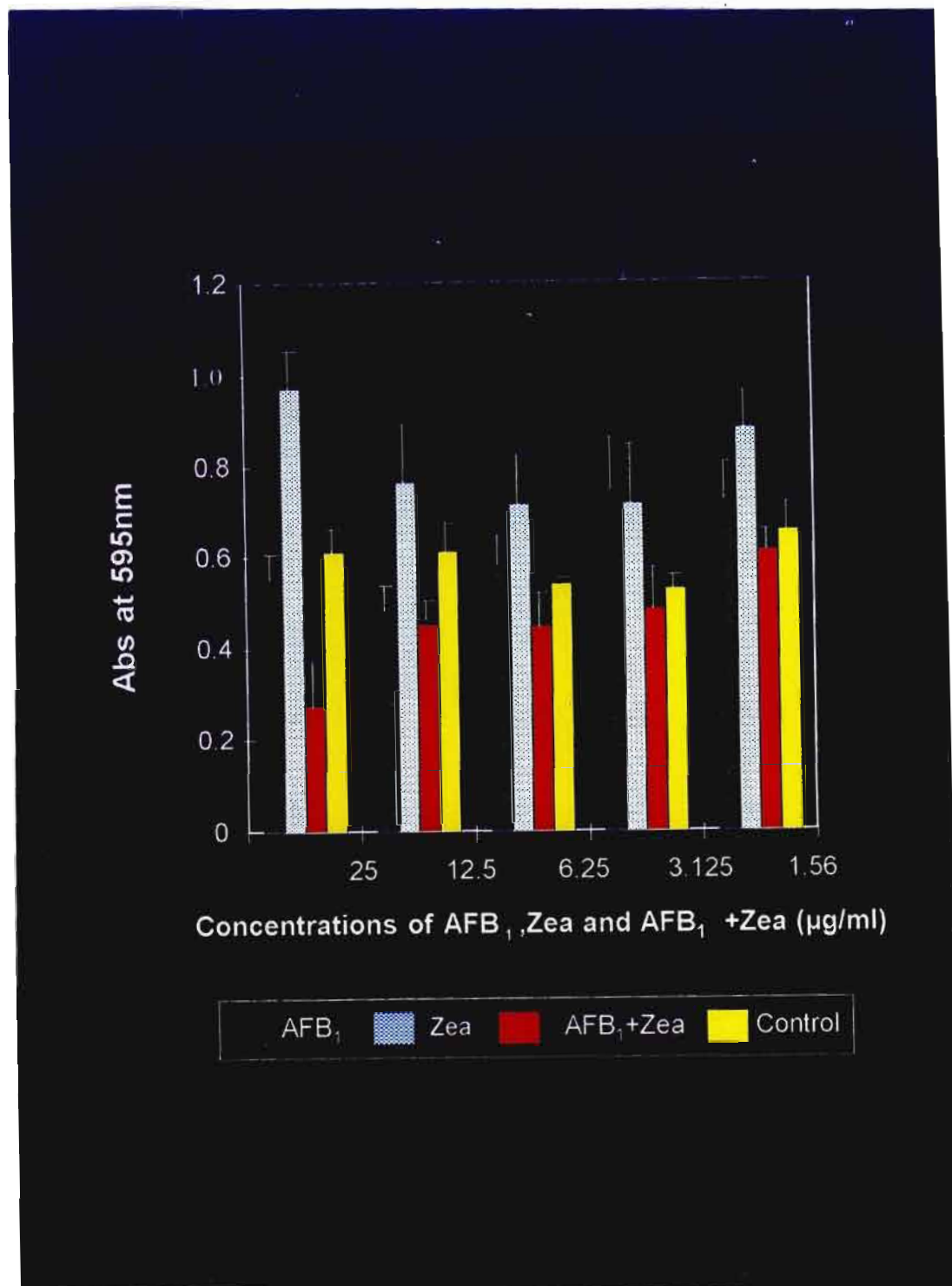


Fig 3.11 A bar graph showing the cleavage activity of viable HepG2 cells, as a measure of absorbancy following the exposure to AFB₁, Zea and as they co-occur in the environment. Error bars represent standard deviations, n=5.

3.5. DISCUSSION

In most biological systems, a proper determination of the dose is difficult. This is also true for cell culture work. In fact the dose should be measured as the concentration of the test compound at the biochemical target, but in general this is not possible because we seldom know the exact target (Syversen, 1991). As in this investigation, the dose is represented as the amount of material added to the cell culture well. The cell culture medium may modify the behaviour of the compound in several ways. The single most important component in the medium is the serum which may cause a masking of toxicity (Syversen, 1991). Therefore increased FCS concentrations (10%) was used only during freezing of cells and its maintenance. During the cytotoxicity assays only 2% FCS was used. The high FCS concentrations is ideal for freezing because it does not crystallise and surrounds the cells preventing water from entering and crystallising the cells.

Dimethylsulfoxide is a dipolar, organic solvent which is also active in biological systems as a cryoprotective agent which protects the cells during freezing by minimising the formation of ice crystals which could be damaging to the cell. It also maintains the microsomal monooxygenase activities in cultured hepatocytes which is important in the biotransformation processes in the liver cells (Villa *et al.*, 1991). In this investigation DMSO was also employed as a solubiliser of the mycotoxins during the preparation of the mycotoxin serial dilutions and the dark purple formazan crystals formed in the MTT assay.

Alteration in cell morphology is a common effect of toxic substances on cells (Toivola and Eriksosson, 1991). Liver cells are affected by a great number of compounds acting by various subcellular mechanisms. Cell detachment would suggest a loss of viability, while the inhibition of cell growth suggests that mitosis of cells is in some way hindered by the toxin. Anchorage dependency is a characteristic feature of these cells (Evans, 1991). Cell division normally requires an appropriate substrate thus its detachment from the substrate would suggest an inhibition of mitosis and subsequent cell

growth. Cell rounding could be attributed to a loss of cell turgidity and the subsequent disruption of the cytoplasm.

The twenty four exposure period proved too long a period for the cells. The toxin concentration was also very high resulting in a loss of cells. Their inability to cope with the toxic insult resulted in an inevitable loss of cell proliferation. Cells exposed to the toxins in the cytotoxicity assays also exhibited similar morphological alterations at high concentrations. Since the cytotoxicity testing was carried out in microtiter plates a number of variables were investigated such as varying concentrations and types of toxins.

For the cytotoxic evaluation, the three different cell monolayers used in this study reacted differently to the toxins. The different cytotoxic effects elicited by the mycotoxins could be due to various factors and mechanisms which are not yet understood in detail. Main factors seem to be the ability of the mycotoxin to bind to cellular receptors and or penetrate cell membranes which is dependent on the size, structural conformation of the toxin molecule and the polarity of the compound (Thompson as cited by Hanelt *et al.*, 1994). The toxic modes of action of the mycotoxins studied is beyond the scope of this particular investigation but some limited conclusions have been drawn. One of the observations made is the variation in sensitivity of the cell lines to the toxins. The three most commonly encountered explanations for tissue to tissue variability in toxin sensitivity are tissue to tissue variability; in the amount of target enzymes or other cellular function which interacts with the toxin and in the amount of metabolic enzymes which activate a non toxic precursor molecule into a metabolite with non-specific toxicity and in the amount of detoxification mechanism that protects the tissue that are less susceptible to the toxic agent (Shier *et al.*, 1991). Each of these mechanisms may be abundant in certain differentiated cell types but present at low levels in all cell types; however these cytotoxicity evaluations do not distinguish between these mechanisms.

Many have been investigating the metabolism *in vitro* of toxins by liver preparations from various experimental animals and humans to provide vital information in

order to elucidate the possible mechanisms responsible for species differences in susceptibility to toxins.

From the MTT assay important conclusions were drawn. HepG2 cells displayed an increased sensitivity to the AFB₁-8,9-epoxide as compared to AFB₁, a difference which is significant in terms of toxicity. Such a result supports the extensive documented data on the biotransformation of AFB₁ into the AFB₁-8,9-epoxide. It is well documented that the mechanism of toxicity of AFB₁ is mediated by the covalent binding of the AFB₁-8,9-epoxide, a chemically reactive metabolite, to cellular proteins and nucleic acids. It is this reaction of the electrophilic epoxide with various nucleophilic centres in cellular targets which is believed to be of critical importance in the carcinogenic process. The consequences of this "activation" reaction poses a biological hazard to the cell, exerting toxic, carcinogenic and genotoxic effects.

Of the toxic actions associated with AFB₁ exposure, the most serious are mutagenicity and carcinogenicity which have been linked to the metabolic activation of the compound. As stated earlier P₄₅₀ is the principle enzyme system involved in the biotransformation of AFB₁. Independent of the tissue under investigation, activation of AFB₁ to the AFB₁-8,9-epoxide is an absolute requirement for AFB₁ to manifest its mutagenic, carcinogenic and DNA binding actions (Massey, 1995). The results obtained for AFB₂ cytotoxicity is of particular interest when one compares it with that of the AFB₁-8,9-epoxide. It is known that the activation of AFB₂ does not readily occur in rat species with its potency reduced by more than 150 times compared to that of AFB₁ (Roebuck *et al.*, 1977). It has been shown in the duck liver that AFB₂ is desaturated to form AFB₁. The latter compound would then be available for activation through the epoxidation pathway (Roebuck *et al.*, 1977). In these results it was shown that AFB₂ had no cytotoxic effect on the HepG2 cells which is in accordance with various previous studies (Roebuck *et al.*, 1977).

Even though in most species the carcinogenic effects of AFB₁ is expressed in the liver, the extreme toxicity and carcinogenicity of AFB₁ (especially in the rat) suggests that in human exposure other organs may also be affected. AFB₁ has been detected in human lung tumour tissue. It has been suggested that the respiratory system may be a target for the carcinogenic effects of AFB₁, following inhalation (AFB₁-laden grain dusts) and evidence supports suggestions that human lung is potentially at risk through aflatoxin exposure to the diet (Massey, 1995). In South East Asia it was reported that several individuals died following an acute exposure to aflatoxin. Reports showed that AFB₁-DNA adducts were detected and measured by Hplc and immunochemically in lung tissue (Harrison and Garner, 1991).

There is also a growing body of animal data implicating AFB₁ as a potential lung carcinogen. It was shown that whole rabbit lung microsomes were as active as liver microsomes in catalysing the formation of a DNA-binding metabolite of AFB₁ (Daniels, 1990). Lung microsomes were however found to be more active than liver microsomes in the activation of AFB₁ when data were expressed in terms of microsomal P₄₅₀ content (Daniels, 1990). It has been shown in this study that the A549 cells have the ability to rapidly biotransform AFB₁ into the AFB₁-8,9-epoxide since the cytotoxic assay showed no significant difference between the toxicities of the parent toxin and its derivative. This can be supported by the fact that the lung cells contain an increased P₄₅₀ content as compared to the liver cells.

The cytotoxic effect of Zea and its derivative on HepG2 cells showed a significant difference between the toxicities but the dose response curves were similar. An increased toxin concentration was associated with an increased cell death. It is generally accepted that the liver is one of the target organs for oestrogen (Nagasue *et al.*, 1986). As already stated Zea mimics oestrogen. The discovery of oestrogen receptors in the cytosol of hepatocytes has stimulated research attempting to define a possible role for steroid hormones, particularly oestrogens in the pathogenesis of hepatic disorders. Eagon *et al.* (1991) have suggested that certain liver diseases may be unusually oestrogen responsive.

Adenoma and hepatoma are disorders which have been associated with the use of steroidal agents such as oestrogen. It has further been postulated that a decreased content of cytosolic oestrogen receptors is linked to increased hepatocyte proliferation (Francavilla et al. 1984). As seen in the dose response curve the high doses of Zea and its epoxide administered to HepG2 cells caused a regression of hepatoma cell proliferation while lower doses enhanced cell proliferation. It has been shown that small doses of oestrone and 17β estradiol promotes cell proliferation while higher doses have the opposite effects (Mishkin *et al.*, 1983). Neuer et al (1994) showed that Zea stimulated the production of prostacyclin (has vasodilatory and antiaggregatory properties) in endothelial cells cultures from human umbilical cords production at low concentrations (10^{-7} M) and inhibited it at higher concentrations (10^{-5} M).

However in HeLa cells both Zea and its derivative greatly enhance cell proliferation at high doses. The observed signs of hyperoestrogenism in reproductive studies have prompted a number of investigators to examine the interaction of the resorcylic acid lactones at the oestrogen target tissue level in order to determine how closely these compounds in fact mimic the actions of the endogenous oestrogens at these sites (Goodman *et al.*, 1987). From the results obtained it appears that both Zea and the epoxide elicit similar cell proliferative effects which suggests that both compounds initiate a sequence of events that follow oestrogenic stimulation. The results obtained are in accordance with various documented evidence that shows an increase in uterine weight and protein content. They have suggested that Zea and its metabolites form a receptor complex which translocates to the nucleus in a manner analogous to the estradiol receptor complex and enhances cellular proliferation as observed in this result.

There has been increased recognition that several species of toxigenic fungi can contaminate crops through the production system. Research has further led to the conclusion that toxigenic fungi typically make metabolites which would suggest that food containing a particular mycotoxin such as aflatoxin can and usually does contain other mycotoxins. Widiastuti et al. (1988)(a) showed the presence of aflatoxins and Zea in

samples of Indonesian corn analysed. Another interesting study showed the presence of cyclopiazonic acid in combination with aflatoxins, Zea and ochratoxin A in Indonesian corn (Widiastuti, 1988) (b). The possibility of synergistic toxicity is very real. It has been well recognised that naturally contaminated grain can be more toxic than the known, pure mycotoxins (Miller, 1993). The co-occurrence of Zea and aflatoxins has been reported in Brazil and Indonesia. It was therefore necessary to evaluate the toxicities of these mycotoxins as they co-occur in nature. From the results obtained it is clear that the effects of pure Zea are antagonised by the presence of pure AFB₁ at the higher concentrations and is enhanced at lower concentrations.

3.6. CONCLUSION

The MTT assay proved to be a reliable and reproducible technique in assessing the cytotoxicity of these mycotoxins and their derivatives. As documented previously AFB₁ requires metabolic activation via epoxidation to exert its biological activities and express its toxic properties. The use of different cell lines was effective in illustrating the specific sites of action of these mycotoxins as was clearly shown by the varying degrees of toxicities expressed by Zea and AFB₁ in the different cell lines used in this investigation.

Chapter 4

A COMPARISON OF THE ULTRASTRUCTURAL EFFECTS OF AFB₁ AND THE AFB₁-8,9-EPOXIDE ON HepG2 CELLS

4.1 INTRODUCTION

Microscopy has proven to be an invaluable tool during this “mycotoxin gold rush era” in understanding the mechanisms of action of some mycotoxins such as AFB₁. Various researchers have explored the use of microscopy in both human (cell lines) and animal models (biopsies) exposed to AFB₁. To date, many have concluded that morphological and biochemical lesions caused by the acute administration of AFB₁ to animals appears almost exclusively in liver and persist for variable lengths of time, depending upon the dose levels and other parameters (Pong and Wogan, 1970). In this particular investigation microscopy has been utilised to determine the sequence and progression of morphological changes observed in mycotoxin treated human hepatoma cells.

The liver has been studied as a model system for many aspects of both normal and abnormal morphology, biochemistry and cell biology. These studies have reflected both the abundant availability of tissue and wide range of hepatic structure and functions. Data on the interactions of carcinogens with macromolecules in mammalian systems has been obtained from the liver using varying doses of the carcinogen which result in acute injury rather than neoplasms. Extensive work on the effects of AFB₁ on rat liver ultrastructure has been documented since it was discovered that the liver is almost exclusively the organ of attack (Scaife, 1971). However, cells from other tissue can be affected but may require higher concentrations or longer exposure periods to achieve the same results. Work done on the effects of AFB₁ at a light microscopic level in Hela and human lung cells showed that AFB₁ elicits many toxic responses (Legator, 1966). The earliest observed effects of the toxicant was the suppression of DNA synthesis and the formation of giant cells which suggests that specific organelles are being targeted by the toxin

(Legator, 1966). This seems likely as the effects of many toxic agents on cells which are mediated via damage to one or more of these specialised subcellular compartments. Specific organelle systems may become damaged by the toxic agent when the organelle begins to play a primary role in the metabolism of a particular toxicant, when a toxicant is stored intracellularly, or as a result of an inherent sensitivity of some essential biochemical pathway in the organelle to perturbation (Fowler *et al.*, 1989). As organelle compartments play crucial roles in facilitating metabolic processes, their alteration due to the effects of a toxicant may hinder cell growth and decrease cellular viability (Fowler *et al.*, 1989).

Many investigators in the field of mycotoxicology have used light and transmission electron microscopy (TEM) to assess the effects of toxins on rat hepatoma cells (Svoboda, and Higginson, 1968). Their work revealed invaluable information about the toxic properties of AFB₁, particularly its effects on the nucleus. The TEM results obtained showed that AFB₁ interferes with DNA synthesis in hepatoma cells of rats fed AFB₁-contaminated feed by altering the structure of the nucleolus (Svoboda and Higginson, 1968). It was therefore pertinent to examine the cellular alterations in human hepatoma cells exposed to AFB₁, AFB₁-8,9-epoxide and AFB₂ using TEM and light microscopy in an attempt to correlate these alterations with biochemical events associated with cell death.

To confirm the presence of antigens in altered organelles many researchers have used antibodies to detect cellular antigens thus expanding our knowledge of both internal structure and the classification of cells (Goers, 1993). In immunocytochemical staining of cells, antibodies bind to surface or intracellular antigens and are then detected by one of many labels such as enzymes; isotopes or colloidal gold. Cells suitably fixed to preserve morphology can be visualised by either electron or light microscopy to localise antigens. Since the introduction of immunocytochemistry (ICC) into biological research, a tremendous amount of research with often spectacular data have appeared (Leeuwen, 1982 as cited by Aggenbach, 1986). These ICC staining methods take advantage of the extraordinary specificity of immune reactions to identify antigens in tissue (Snyman, 1988). In the present study, ICC has been employed in the identification and localisation

of AFB₁ its AFB₁ 8,9-epoxide derivative and AFB₂ using a polyclonal AFB₁ antibody. The advantage of using polyclonal antibodies for ICC is that they contain a population of antibodies which can recognise many epitopes on the antigen (Snyman, 1988). Tissue sections are exposed to the primary antibody (anti-AFB₁) and is then visualised by a labelled secondary antibody (goat anti-rabbit IgG gold probe). This gold probe is a gold sphere of 5 to 10nm. In addition, confocal laser scanning microscopy (CLSM) was used to complement ICC, since there are pitfalls to the use of ICC. Such pitfalls include the loss of label following post embedding techniques due to the masking of the antigen during tissue processing and embedding (Snyman, 1988).

Confocal laser scanning microscopy is a new optical microscopic technique, which offers significant advantages over conventional microscopy. Light which is emitted from regions other than the focal plane, is cut off by introducing a diaphragm in the beam path (Fink-Puches *et al.*, 1995). The result is an optical "slice" which shows more detail because the blurring from out of focus haze disappears. Researchers have begun to use CLSM in conjunction with immunofluorescence and fluorescent *in situ* hybridisation in an attempt to trace biological compartments within cells and tissue (Messerli and Perriard, 1995). Another advantage of CLSM is the computerised rendering of digital images which permits image processing such as contrast stretching, pseudo-colour presentations and morphological analyses, using software packages (Ockleford, 1995). It allows one to examine surface morphology by viewing the phase contrast image and visualising the fluorochrome labelled antigen. Superimposition of the labelled area on the phase contrast image enables one to localise the florescent label (toxin) within the cell. The confocal technique has been employed in this study to detect the presence of AFB₁ based on its fluorescent properties at a wavelength of 450μ (Wogan, 1966). The CLSM offers the best approach to non destructive high resolution examination of features of living cells.

The cell surface also provides valuable information in understanding the condition of the cell following exposure to a toxic treatment. The scanning electron microscope surpasses other instruments for viewing cell surface morphology, provides a 3D image of the surface and magnifies images up to 20,000x (Wolosewick and Parker, 1979). The illuminating system of the SEM is similar to that of the conventional TEM, however the

electron beam is focused by one or more condenser lenses into a narrower pencil of the electron beam, which scans the surface of the specimen. As the beam strikes the surface of the specimen high energy backscattered and low energy secondary electrons are given off. The signal is picked up, amplified and displayed on the cathode ray tube. In this way a picture is built on the screen which corresponds point by point to the surface contours of the area of the specimen which is being scattered by the beam. A record of the image is made by photographing the image on the screen with a camera (Carr and Toner, 1982).

The following chapter aims to correlate the results obtained using the various microscopic techniques mentioned above in an attempt to determine the mechanisms of action of the selected mycotoxins and its effects on cellular metabolism.

4.2 MATERIALS AND METHODS

a) Light microscopy

HepG2 cells were grown (37°C for 48 hours) on sterile Chance glass coverslips (Boehringer Mannheim) until confluent (Robinson, 1982). Cells on one coverslip were treated with AFB₁ (12µg/ml) for 24 hours while a second coverslip of cells were treated with the AFB₁-8,9-epoxide (12µg/ml) for 24 hours. Adherent treated and untreated cells were rinsed in pre-warmed (37°C) HBSS, fixed in 1.5% gluteraldehyde for ten minutes, rinsed in HBSS for two minutes and then rinsed thoroughly in distilled water before staining with Mayer's haemotoxylin and eosin (5%) (Drury and Wallington, 1980).

b) Transmission electron microscopy (TEM)

HepG2 cells were grown in culture flasks (25cm³) until confluent. Flasks were then exposed to AFB₁, AFB₁-8,9-epoxide and AFB₂ at 12µg/ml for 24 hours. Following this exposure period both untreated and treated cells were fixed, dehydrated and

embedded *in situ*. Blocks from the flasks were sectioned with the cells sandwiched between the resin and the plastic (Robinson and Gregory, 1977). Sections were cut on the Reichert Ultracut microtome. Thick sections (1 μ m) were stained with 1% toluidene blue (Drury and Wallington, 1980) in a 50:50 distilled water:acetone solution containing 10% borax. Ultrathin sections mounted on 200 mesh copper grids were stained with uranyl acetate followed by Reynold's (1963) lead citrate. Sections were viewed with a Joel 10B transmission electron microscope (60kV) in the Physiology Department of the University of Natal and a 100S Joel Electron microscope in the Electron microscope unit at the University of Natal. The processing schedule of the various steps in TEM are summarised in the following table.

Table 9 The processing schedule for TEM

Step	Solution	Time
1.	Fixation Cells are fixed in 1% gluteraldehyde in Hanks Balanced salt solution (HBSS)	1 hour (25°C)
2.	Buffer rinse Rinse in HBSS	
3.	Post fixation Post fix in 1% osmium tetroxide	1 hour (4°C)
4.	Buffer rinse Rinse in HBSS (x2)	10 min
5.	Dehydration- 70% ethanol	20 min
6.	Dehydration- 90% ethanol	20 min
7.	Dehydration- 100% ethanol	30 min
8.	Dehydration- 100% ethanol	30 min
9.	Ethanol:Araldite 50:50	30 min
10.	Araldite	1 hour (60°C)
11.	Araldite	48 hours (60°C)

c) Immunocytochemistry

For ICC, a post-embedding technique was employed (Snyman, 1988), where the immunolabelling was carried out on sections of AFB₁, AFB₁-8,9-epoxide and AFB₂ treated cells picked up on nickel grids. The primary antibody used was a rabbit polyclonal anti-AFB₁ (1:100) (Sigma) and the secondary antibody used was a goat-anti-rabbit IgG gold conjugate (10nm). Grids were treated with hydrogen peroxide (5%) and normal goat serum to block non-specific binding sites. The primary antibody used was a polyclonal (rabbit anti-AFB₁) (1:100), while the secondary antibody was goat anti rabbit IgG conjugated to a 5 to 10nm gold probe. Sections were viewed using a JEOL-JEM 100S transmission electron microscope.

Method controls

The method specificity may be defined as the absence of staining caused by mechanisms other than the immunological interactions between primary antibodies and the antigen to be localised (Leeuwen, 1986). It involves the omission of either the primary antiserum or the secondary layer antibody or the immunogold step for immunolabelling. In this study the method control excluded the primary antibody. These method controls serve to exclude the non immunological interactions between the secondary antibody and the detection system.

Table 10 The staining procedure using Immuno-Gold on resin sections for TEM

	Procedure	Time
1.	Etch non-osmicated sections on nickle grids by placing on a droplet of 5% hydrogen peroxide	3 min
2.	Place grids on a drop of distilled water then jet wash with 10ml distilled water and dry	
3.	Submerge grids at room temperature in normal goat serum -diluted 1:20	30 min
4.	Drain on fibre free paper	
5.	Droplet of primary antiserum and one method control in PBS or normal goat serum	3 hours
6.	Droplet of 50mM Tris pH 7.2 Then jet wash with 20ml /grid	
7.	Droplet of 50mM Tris containing 0.2% bovine serum albumin (BSA) pH 7.2. Then jet wash in 5ml/grid	
8.	Droplet of 50mM Tris containing 1% BSA pH 8.2	5 min
9.	Droplet of IgG-gold diluted 1:15 in Tris/BSA pH8.2	1 hour
10.	Droplet and jet wash as before using: 10ml/grid 50mM Tris containing 0.2% BSA pH 7.2 5ml/grid 50mM Tris pH7.2 5ml/grid distilled water	
11.	Counterstain with 2% uranyl acetate Jet wash with distilled water Counterstain with Reynolds lead citrate Jet wash with distilled water	

d) Confocal microscopy

HepG2 cells were grown on glass coverslips (as described previously) until confluency was attained. The confluent cells, were then incubated with AFB₁ 12µg/ml for varying time intervals (4 hours; 8 hours; 12 hours; 24hours). After incubation, the cells were washed in HBSS(twice), fixed in 1% gluteraldehyde solution (5ml) (15 minutes), rinsed with HBSS (5ml), placed on glass slides and sealed with glycerol (2ml). Finally, the cells were viewed on a True Confocal Scanner-Leica TCS 4D microscope (AFB₁ fluoresces under ultra violet light at 325nm).

e) Scanning electron microscopy(SEM)

Cells grown to confluency on pre-treated coverslips (as described previously) were prepared for SEM. Cells were rinsed in HBSS (5ml), fixed in 1% gluteraldehyde (5ml) in HBSS for 30 minutes at room temperature, rinsed, dehydrated through ascending grades of alcohol (5ml) and critical point dried using a specially designed holder (Evers *et al.*, 1983) in a Hitachi HCP I dryer. Cultured mammalian cells are amongst the most difficult specimens to prepare for SEM. Being bound only by a cell membrane, not having the mechanical support afforded by cells in bulk tissue and often possessing long, fragile cytoplasmic processes renders this type of specimen extremely susceptible to various external disturbances (Evers *et al.*, 1983). It is fortunate that a special holder designed to carry cell cultures through critical point drying was made available during this study to minimise accidental surface tension effects during processing and reduce debris (Evers *et al.*, 1983). Five two minute purges were used over a period of one hour and the critical temperature of 45°C for five minutes. Venting after drying was completed after 10 minutes and the overall time in the dryer was at least 75 minutes. Dried coverslips were attached on stubs using silver paint, cells coated with 60:40 gold:palladium from a distance of 10cm in a gel JEE 4 vacuum evaporator.

Table 11 Processing for SEM

Step	Processes	Time
1.	Fixative- 1% gluteraldehyde	20 min
2.	Buffer rinse - HBSS (25°C)	5 min
3.	Post fixation - Osmium tetroxide(0.5%)	20 min
4.	Rinse- distilled water	
5.	Dehydration- 30% ethanol	10 min
6.	Dehydration- 50% ethanol	10 min
7.	Dehydration- 70% ethanol	10 min
8.	Dehydration- 90% ethanol	10 min
9.	Dehydration- 100% ethanol	10 min
10.	Critical point drying	+/- 90 min
11.	Sputter coating- 60:40 Palladium: silver	10 min

The coverslips were viewed using a S 520 SEM with the following settings:

- Accelerating voltages of 10 to 25 kV
- Beam current of 20nA°
- Tilt varied from 0 to 60°C
- 200µm aperture.

4.3 RESULTS

a) Light microscopy

In the untreated HepG2 cells, nucleoli were characteristically large and contained single or multiple prominent nucleoli of irregular shape (Fig 4.1). Cells treated with AFB₁ and the 8,9 epoxide (12µg/ml) showed similar morphological alterations. These included microvesicular steatosis (Fig 4.2), the presence of multinucleated cells (Fig 4.3), microsegregation of nucleoli (Fig 4.4), cytoplasmic blebbing (Fig 4.5), increased cytoplasmic vacuolation (Fig 4.6) and increased numbers of mitotic figures and abnormal mitoses (Fig 4.7).

b) Transmission electron Microscopy

Cultured liver cells (HepG2) treated with the AFB₁ and AFB₁-8,9-epoxide showed marked cellular alterations (Table 12). The TEM results showed cytoplasmic and nuclear alterations in AFB₁ and AFB₁-8,9-epoxide treated cells while untreated cells showed numerous, ovoid mitochondria (Fig. 4.8) and organelle structures (Fig 4.9) which are characteristic features of hepatoma cells (Trump, 1965). In addition the organelles most effected by both AFB₁ and the AFB₁-8,9 epoxide treatments were the mitochondria, endoplasmic reticulum (ER) and nucleolus.

The AFB₁ treated cells showed fewer mitochondria with occasional elongated and swollen forms (Fig. 4.10). AFB₁-8,9-epoxide treated cells, however, showed fewer mitochondria with many appearing larger and distorted (Fig 4.11) with a loss of membrane integrity and subsequent clearing of the mitochondrial matrix (Fig. 4.12). Another mitochondrial alteration observed in AFB₁-8,9-epoxide treated cells was the presence of cristae arranged in a parallel, whorled or oblique orientation (Fig 4.13). AFB₂ treated cells showed no change in mitochondrial ultrastructure with the mitochondria remaining structurally intact (Fig 4.14).

Swollen ER were occasionally observed, often in close proximity to the mitochondria in AFB₁ (Fig 4.15) and AFB₁-8,9-epoxide treated cells. However, the increase in swelling of ER was more pronounced in epoxide treated cells and in addition was filled with proteinacious material (Fig 4.16). Swelling of ER was also associated with a loss of ribosomes. Areas of cytoplasmic lysis was another feature frequently observed in treated cells but were more pronounced in AFB₁ (Fig 4.17) treated cells as compared to AFB₁-8,9-epoxide treated cells (Fig 4.18).

Numerous secondary lysosomes and myelin figures were present in AFB₁ treated cells (Fig 4.19). No myelin figures were observed in AFB₁-8,9-epoxide treated cells as compared to AFB₁ treated cells but large secondary lysosomes were present (Fig 4.20) which may be indicative of more rapid degenerating organelles. In both controls (Fig 4.21) and AFB₁ treated cells (Fig 4.22) cytoplasmic blebbing was displayed by several cells but blebs appeared more swollen (Fig 4.23) and grossly altered in the AFB₁-8,9-epoxide treated cells (Fig 4.24). Areas of cytoplasmic lysis were often present in the blebs and were pronounced in AFB₁-8,9-epoxide treated cells. The presence of swollen golgi was only observed in epoxide treated cells (Fig 4.25).

Nuclear alterations in AFB₁ treated cells included the presence of large prominent nucleoli (Fig 4.26) nucleolar macrosegregation and chromatin clumping at the periphery of the nucleus (Fig 4.27). The fibrillar and granular components of the nucleolus appeared as distinct light and dark zones (Fig 4.28). The untreated cells showed a distinct nucleolus (Fig 4.29) with the fibrous and granular components being indistinguishable. Epoxide treated cells showed an abundance of euchromatin and a single large nucleolus fibrillar and granular components being distinguishable (Fig 4.30).

In contrast to AFB₁ and AFB₁-8,9 epoxide treated cells, AFB₂ showed no cellular alterations. The nucleolus was centrally situated and structurally intact.(Fig 4.31).

Table 12 A comparison of ultrastructural alterations of AFB₁ and AFB₁-8,9-epoxide treated cells

AFB₁	AFB₁-8,9- epoxide
Nucleus	
Hetero and euchromatin	Abundant euchromatin
Large nucleoli - more than one microsegregation	Single large nucleolus
Many karyosomes	Occasional karyosomes
Irregular and invaginating nuclear membranes in a few cells.	Nuclear membrane is regular
Mitochondria	
Numerous	Few
Slight alteration in the orientation in the cristae	Swollen, larger and distorted. Cristae appeared parallel, whorled and oblique. Cristae were inconspicuous in many.
Occasional elongated forms	Occasional elongated forms.
A fair number of myelin figures	No myelin figures observed but numerous vacuoles present.
Mitochondrial membrane integrity maintained	Loss of membrane integrity with clearing of mitochondrial matrix
Numerous areas of cytoplasmic lysis with smaller and larger pockets.	Not as pronounced
Few swollen cisternae of rough endoplasmic reticulum	Many swollen cisternae of endoplasmic reticulum . Some filled with proteinacious material
Numerous secondary lysosomes	Numerous secondary lysosomes
Some cytoplasmic processes showed areas of lysis .	Numerous cytoplasmic processes with areas of lysis
Normal golgi -several golgi bodies	Swollen

c) Immunocytochemistry

Table 13 Intensity of label in organelles treated with the various toxin treatments

Altered organelles	AFB ₁ treated	AFB ₁ -8,9- epoxide	AFB ₂
Nucleolus	+	++	-
Mitochondria	+	++	-
Endoplasmic reticulum	++	+	-
Cytoplasmic blebs	+	++	-
Cytoplasm	++	++	-
Adjacent to the cell	++	++	-

- absence of label

+ presence of a few gold probes

++ presence of several probes

The ICC confirmed the presence of gold labelled anti-AFB₁ in both AFB₁ and AFB₁-8,9 epoxide treated cells. However label was not found in AFB₂ treated cells. Cells exposed to AFB₁ and the AFB₁-8,9-epoxide showed the presence of label in close proximity to the mitochondria and within the swollen ER (Fig 4.32). Label was often found in the cytoplasmic blebs and in close proximity to them (Fig 4.33). Label was present at the periphery and in the centre of the nucleus (Fig 4.34) in AFB₁ and AFB₁-8,9-epoxide treated cells (Fig 4.35). Cultured liver cells treated with AFB₂ showed no label using the polyclonal anti-AFB₁. In AFB₁ treated cells label was found in areas of cytoplasmic lysis (Fig 4.36), along the cell membrane (Fig 4.37) and in vesicles (Fig 4.38).

d) Confocal Microscopy

Confocal microscopy results showed that in the four hour treated cells AFB₁ was localised in the nucleolus (Fig 4.39); in the 8 hour treatment the toxin spread throughout the nucleus (Fig 4.40); in the 12 hour treated cells the toxin spread throughout the nucleus (Fig 4.41); and the 24 hour treated cells showed toxin distributed throughout the cytoplasm (Fig 4.42). During the 24 hour exposure period many cells were dead showing a high fluorescence of toxin.

e) Scanning electron microscopy

Untreated cells were attached to the substrate (Fig 4.43) with the surfaces of the cells being covered by long filamentous cytoplasmic processes [(Fig 4.44), (Fig 4.45), Fig 4.46)]. Both AFB₁ and AFB₁-8,9-epoxide treated cells displayed high cell mortality rates and alterations of cytoplasmic processes. Cell rounding, indicative of cell death was frequent in treated cells (Fig 4.47). Cytoplasmic processes in treated cells appeared shorter and rounded (Fig 4.48).

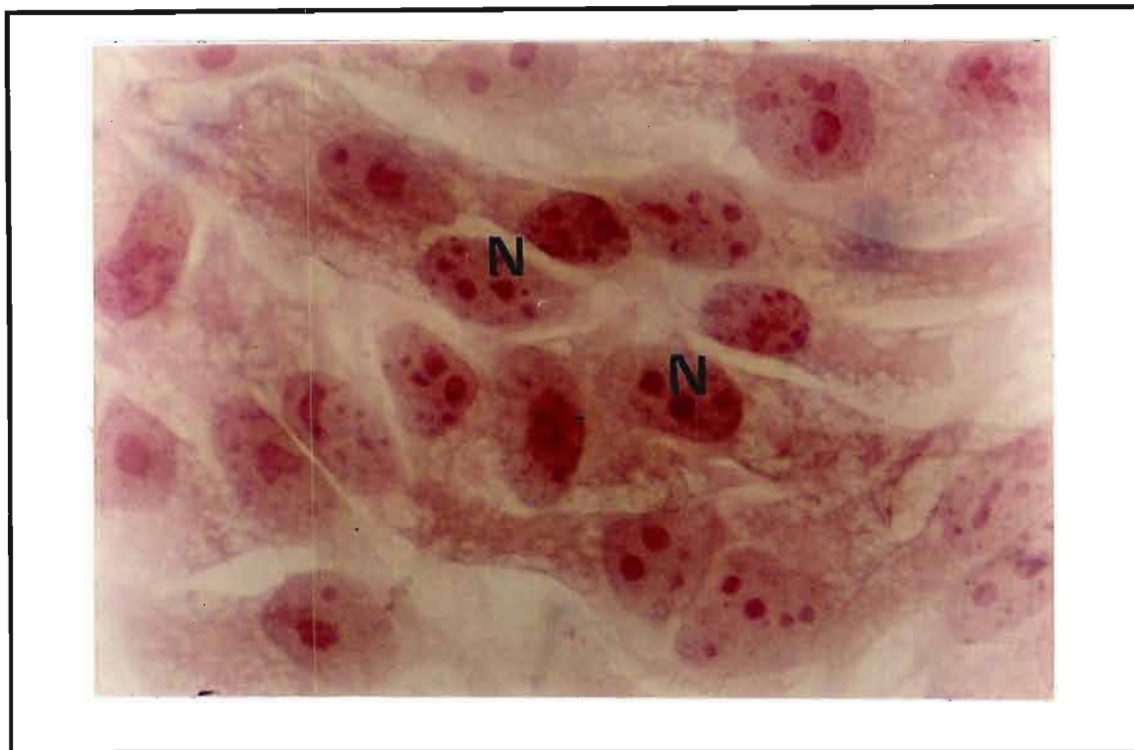


Fig 4.1 A light micrograph showing untreated HepG2 cells with large and prominent nucleoli (N) (x4000).

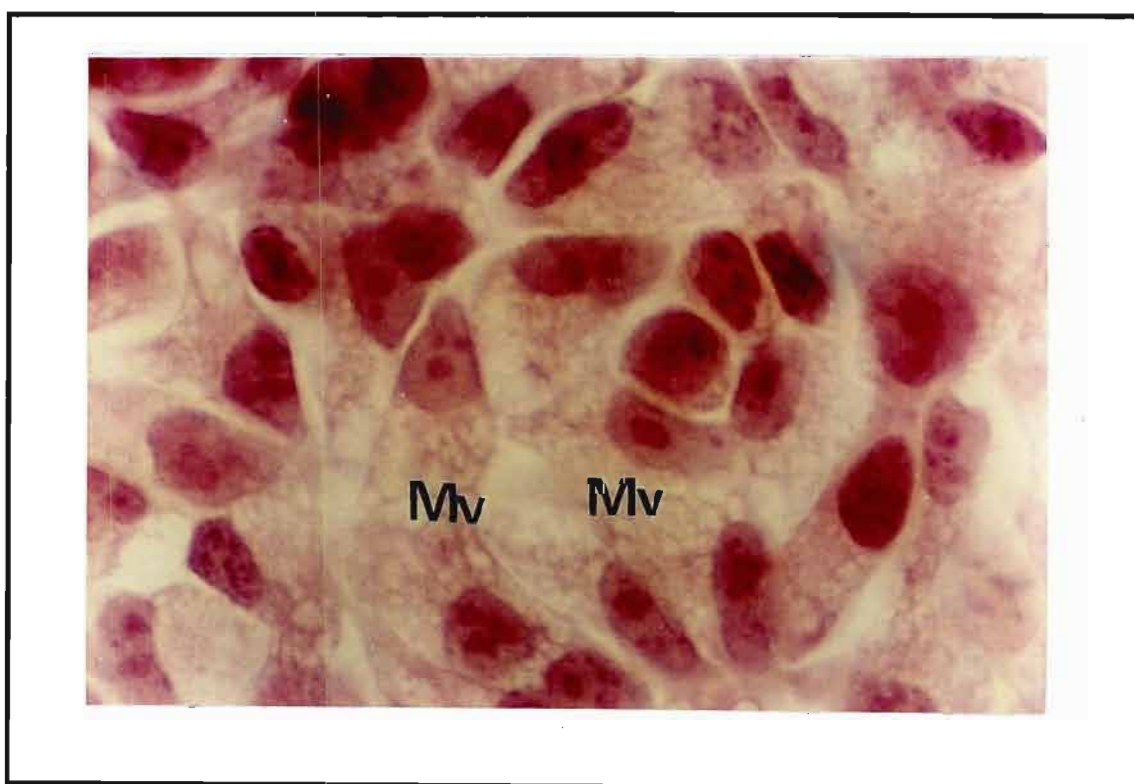


Fig 4.2 HepG2 cells treated with AFB₁ showing microvesicular steatosis (Mv) (x4000).

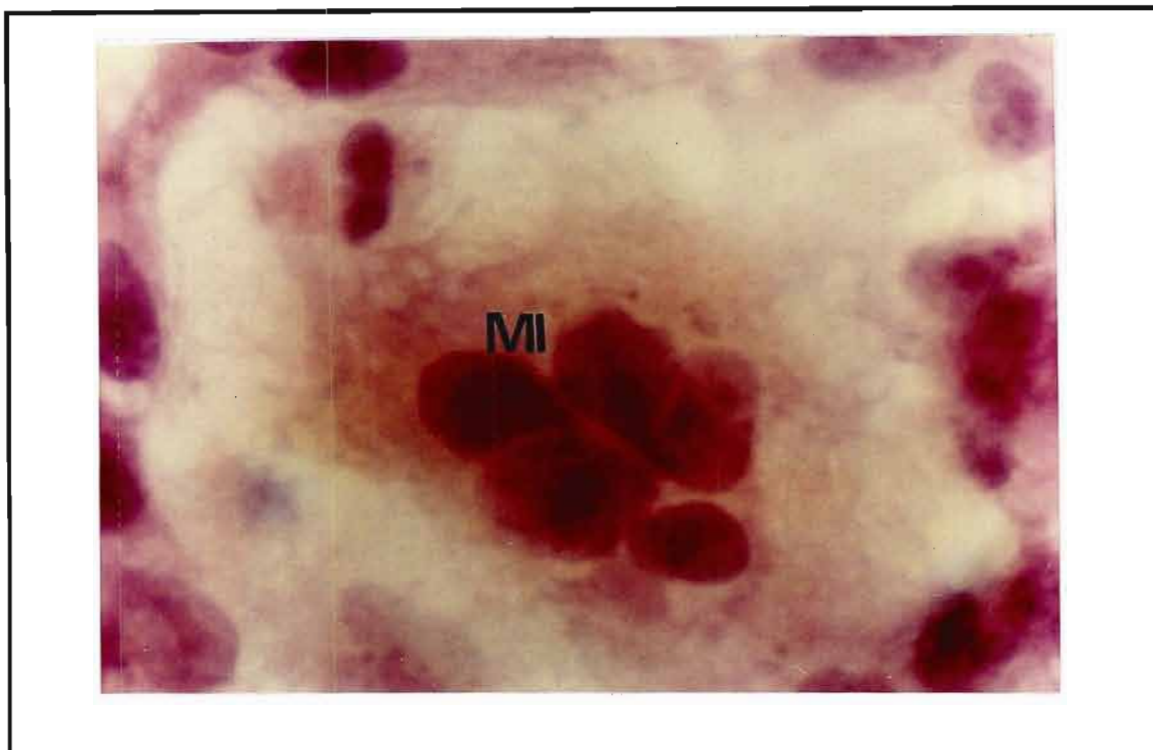


Fig 4.3 AFB₁ treated cells showing the presence of multinucleation (MI) (x4000).



Fig 4.4 AFB₁ treated cells showing the microsegregation (Ms) of nucleoli (x4000).

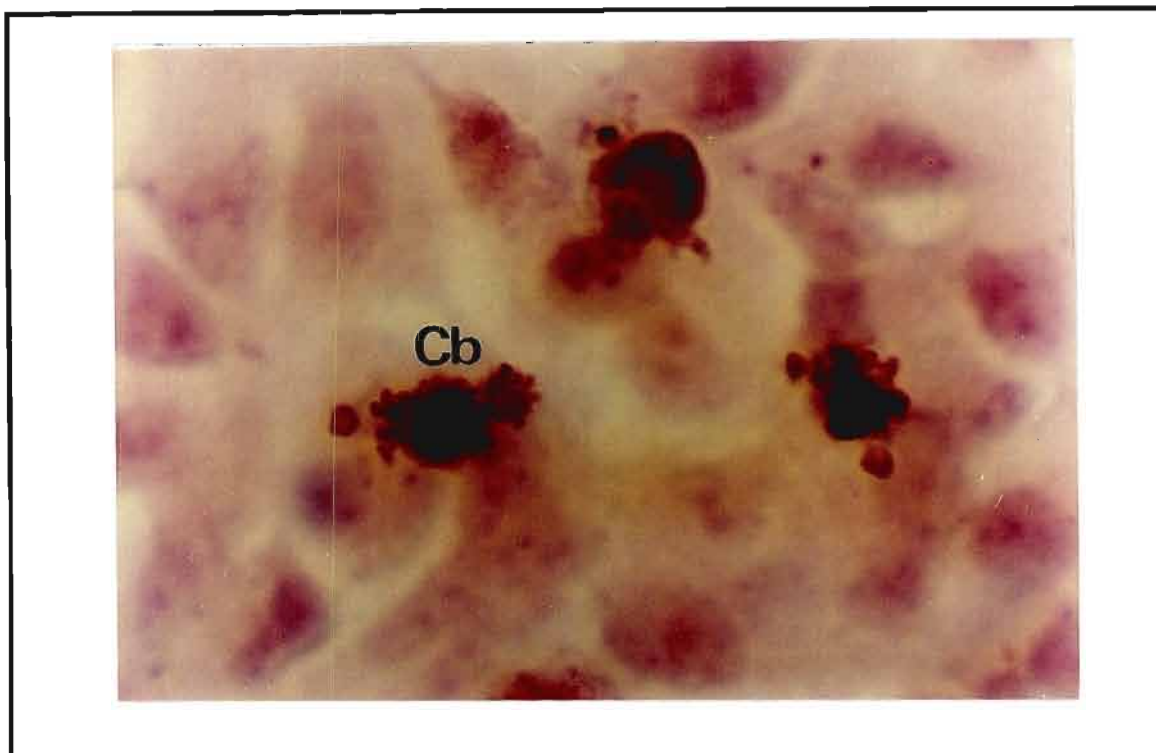


Fig 4.5 AFB₁ treated cells showing cytoplasmic blebs (Cb) (x4500).

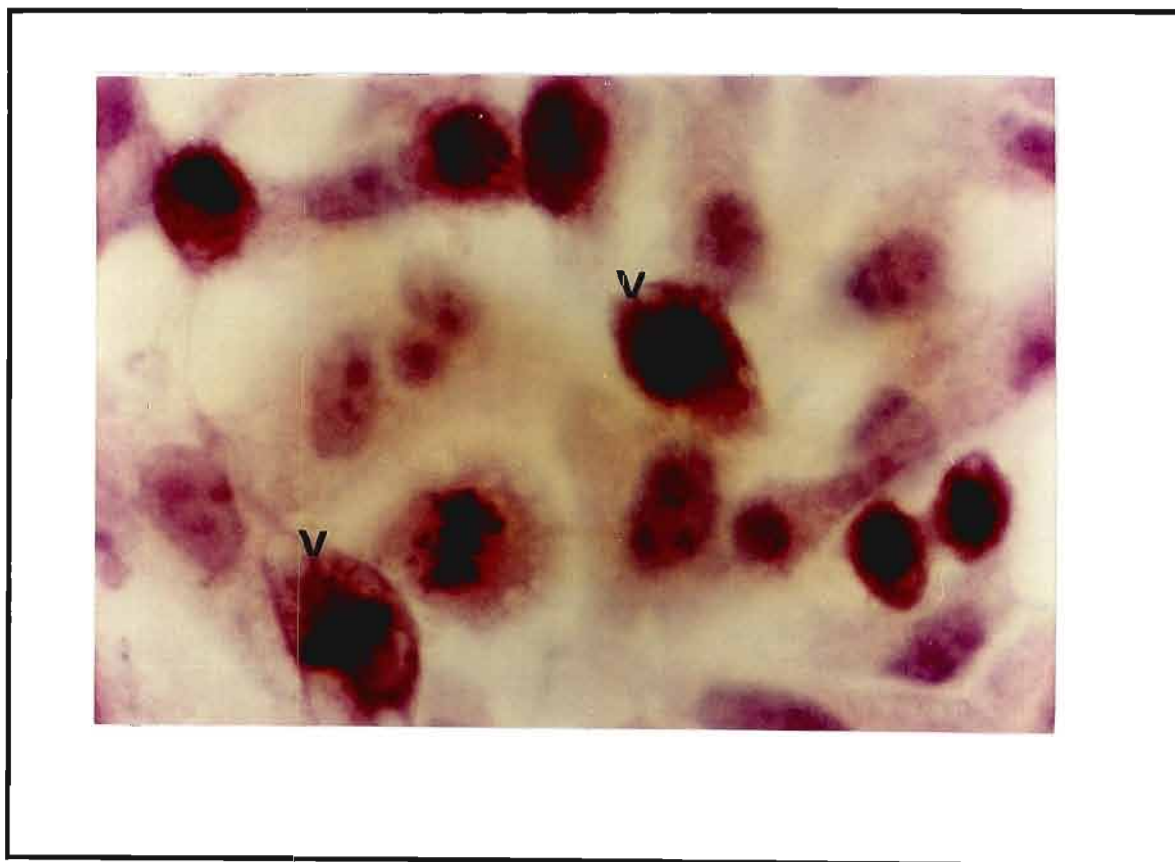


Fig 4.6 AFB₁ treated cells showing increased cytoplasmic vacuolation (v) (x4000).

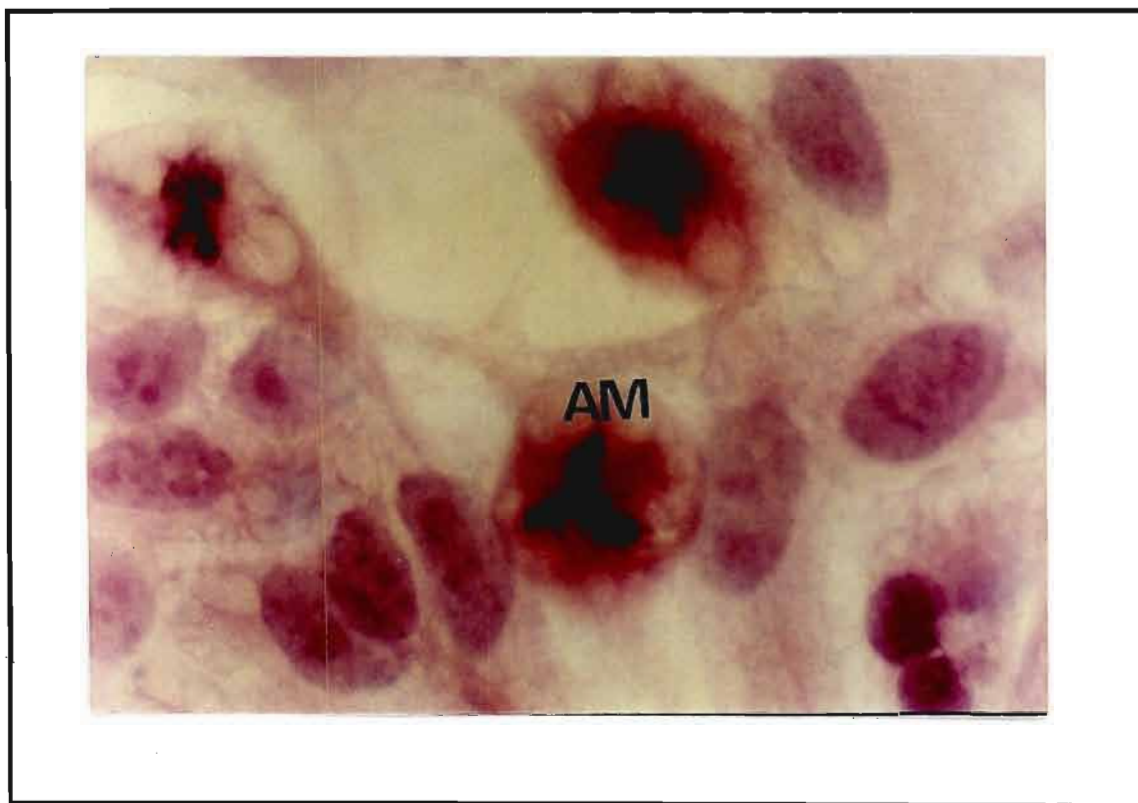


Fig 4.7 AFB₁ treated cells showing abnormal mitoses (AM) (x4000).

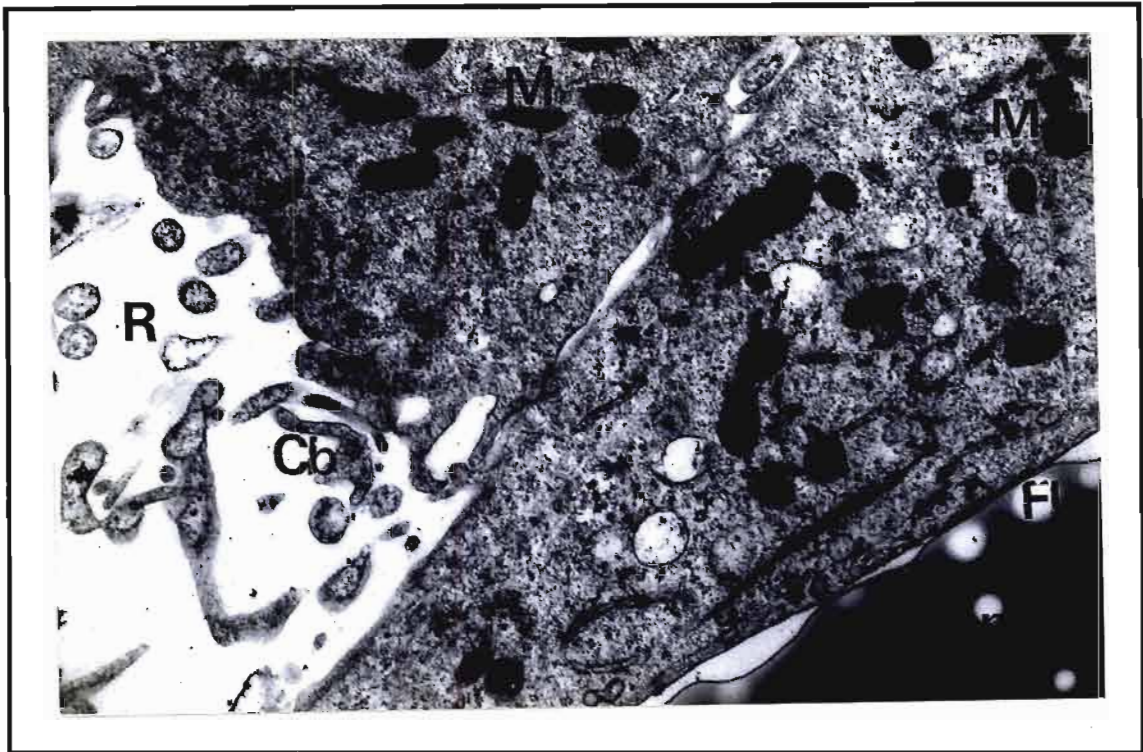


Fig 4.8 Electron micrograph of untreated HepG2 cells showing numerous ovoid mitochondria (M) which are characteristic of hepatoma cells and a few cytoplasmic blebs (Cb). The cells are sandwiched between the flask (F) and the resin (R).

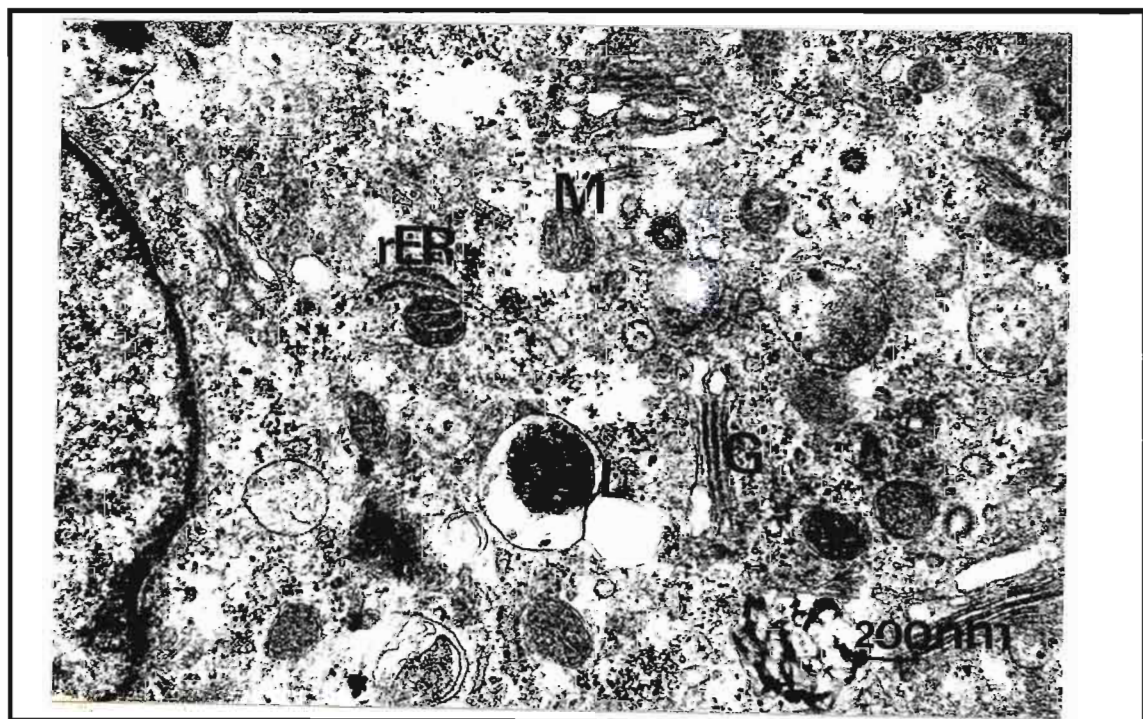


Fig 4.9 Electron micrograph of an untreated HepG2 cell showing intact organelles such as ovoid mitochondria (M), rough endoplasmic reticulum (rER), golgi (G), and secondary lysosomes (L)

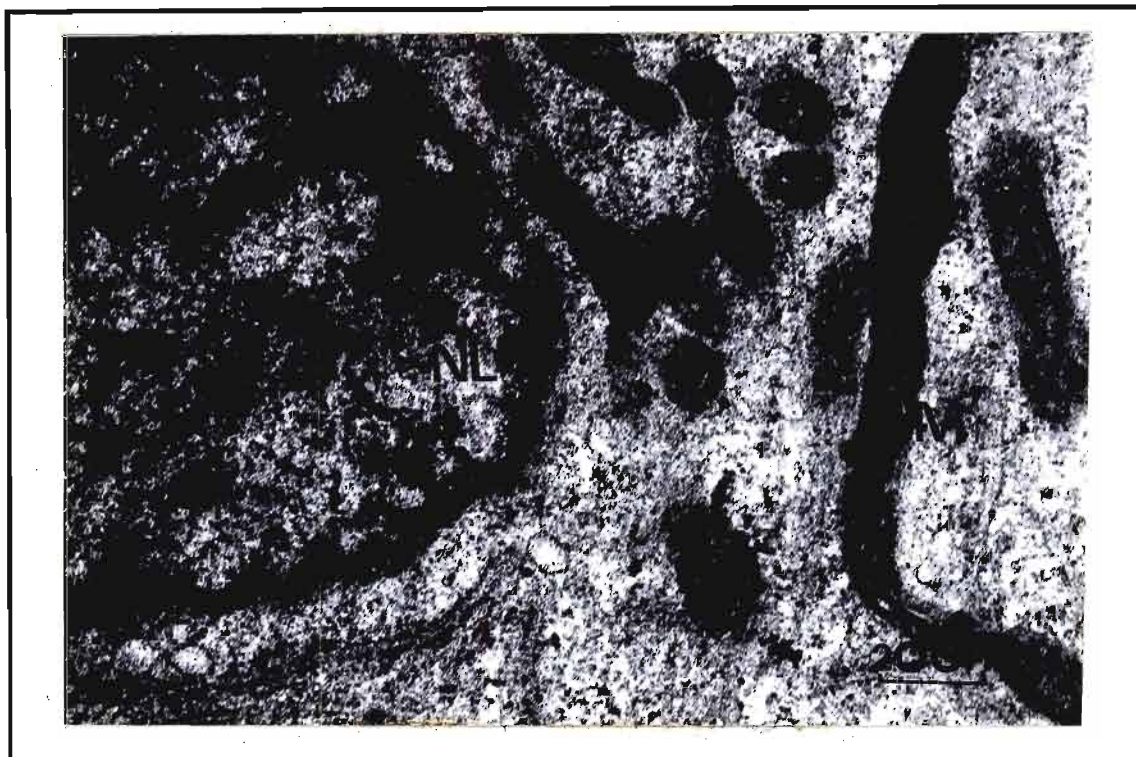


Fig 4.10 Electron micrograph of a AFB₁ treated cell showing fewer mitochondria than controls with occasional elongated forms (M) close to the nucleus (NL).

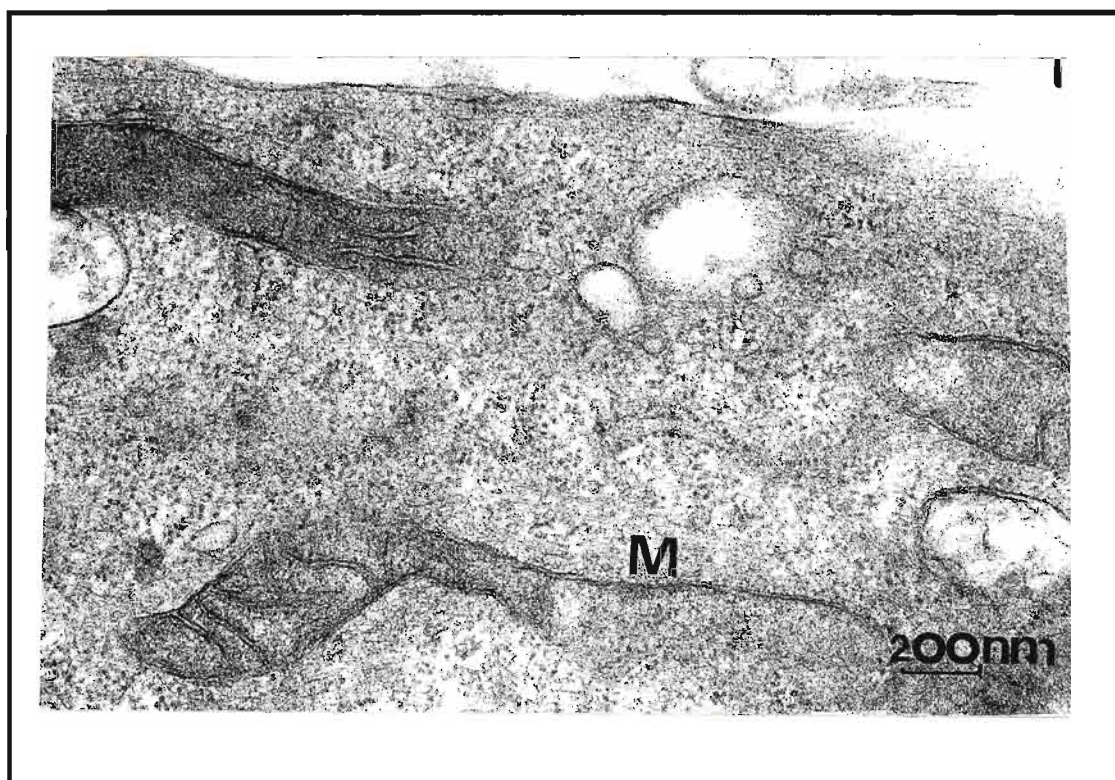


Fig 4.11 Electron micrograph of a AFB₁ -8,9-epoxide treated HepG2 cells showing larger, distorted mitochondria with a loss of membrane integrity(M).

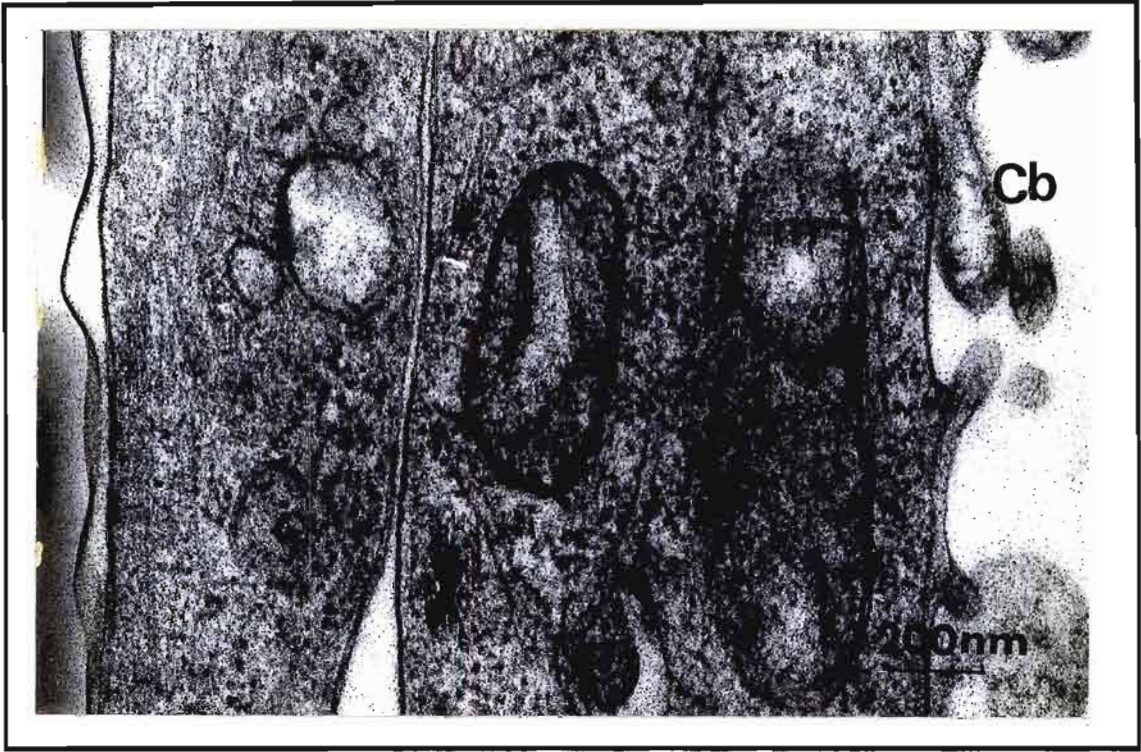


Fig 4.12 Electron micrograph of a AFB₁-8,9-epoxide treated cell showing clearing of the mitochondrial matrix (m) and the presence of cytoplasmic blebs (Cb).

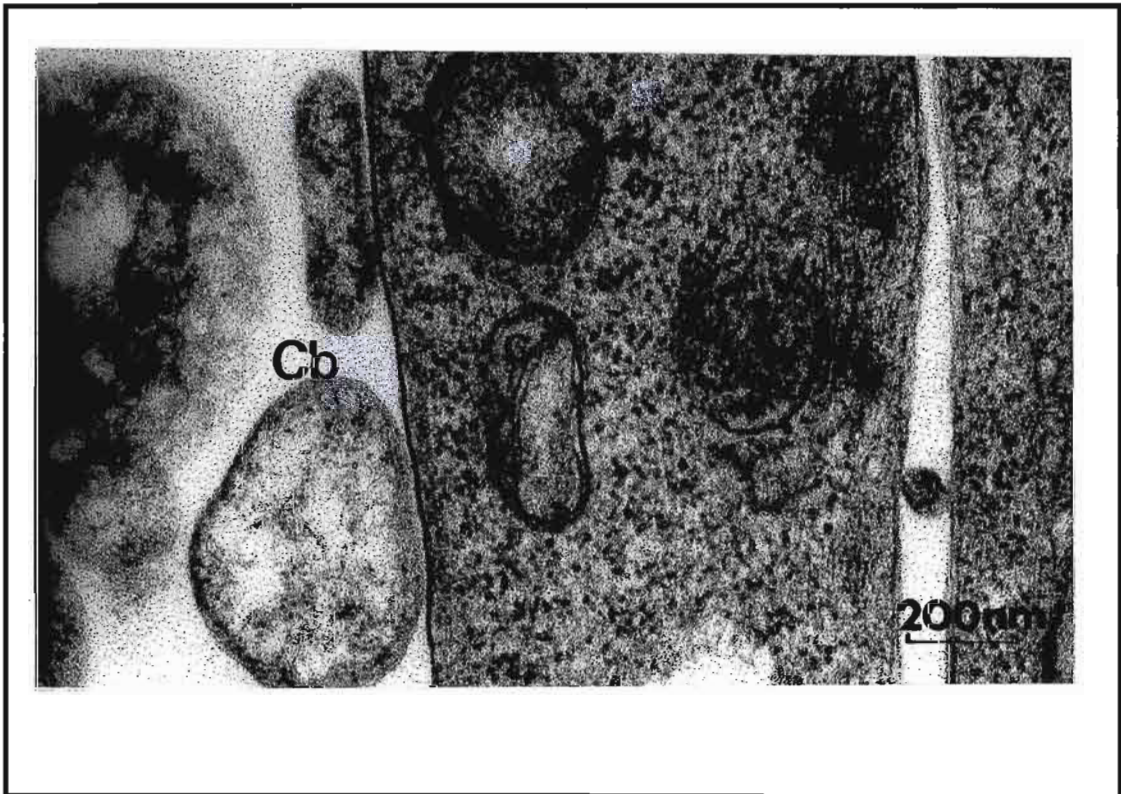


Fig 4.13 Electron micrograph of an AFB₁-8,9-epoxide treated cell showing disorientation of cristae (c) and cytoplasmic blebs (Cb).

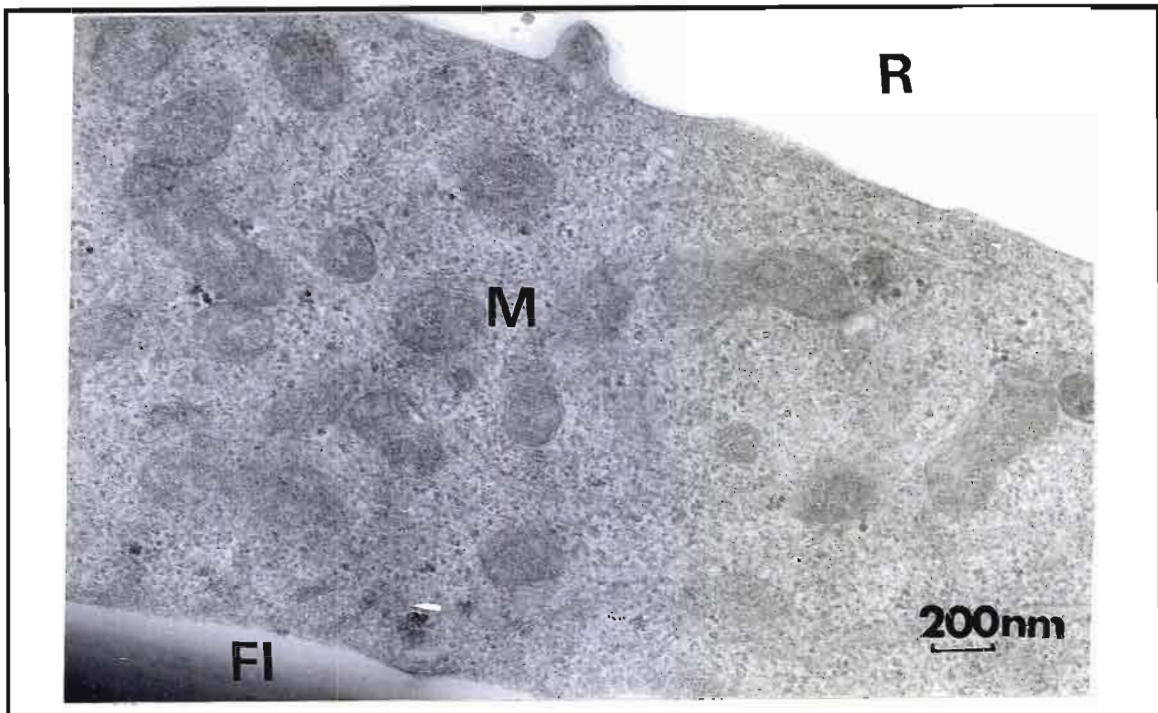


Fig 4.14 Electron micrograph of a AFB₂ treated cell showing the presence of intact mitochondria (M) Flask (FI), resin (R) .



Fig 4.15 Electron micrograph of an AFB₁ treated cell showing the presence of slightly swollen rER in close proximity to the mitochondria (M) . Areas of cytoplasmic lysis are visible together with secondary lysosomes (L)

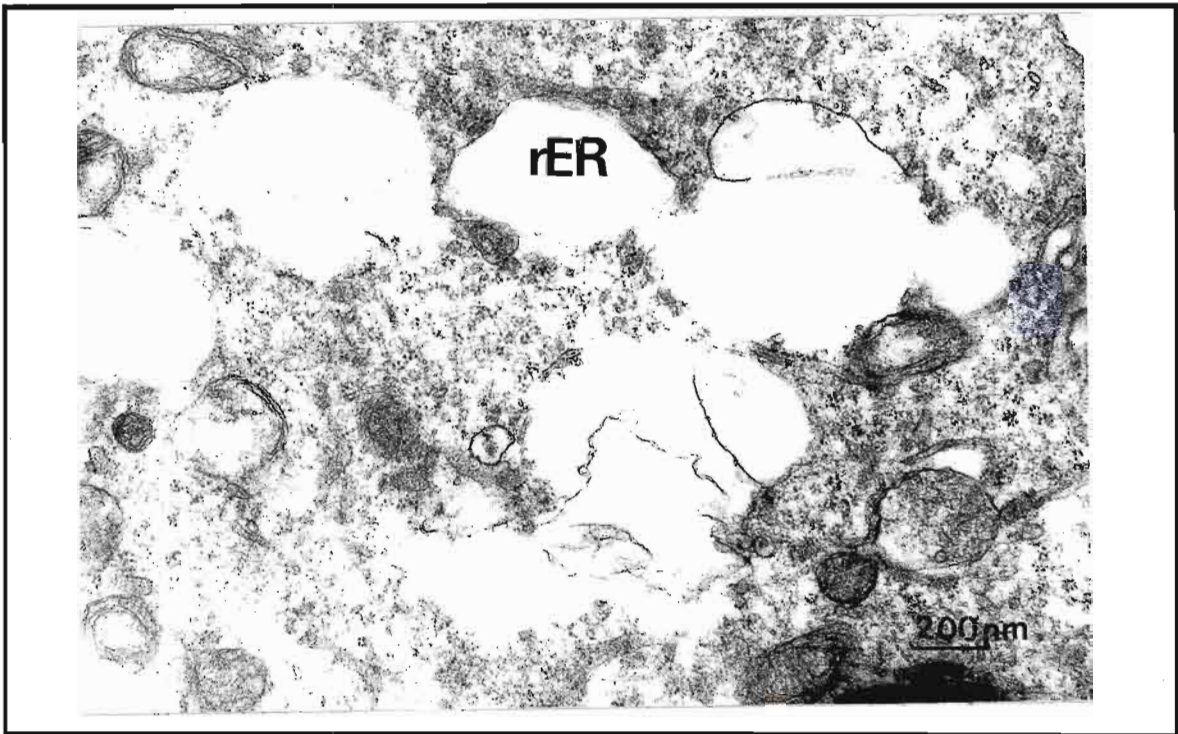


Fig 4.16 Electron micrograph of an AFB₁-8,9-epoxide treated cell showing grossly swollen endoplasmic reticulum (ER).

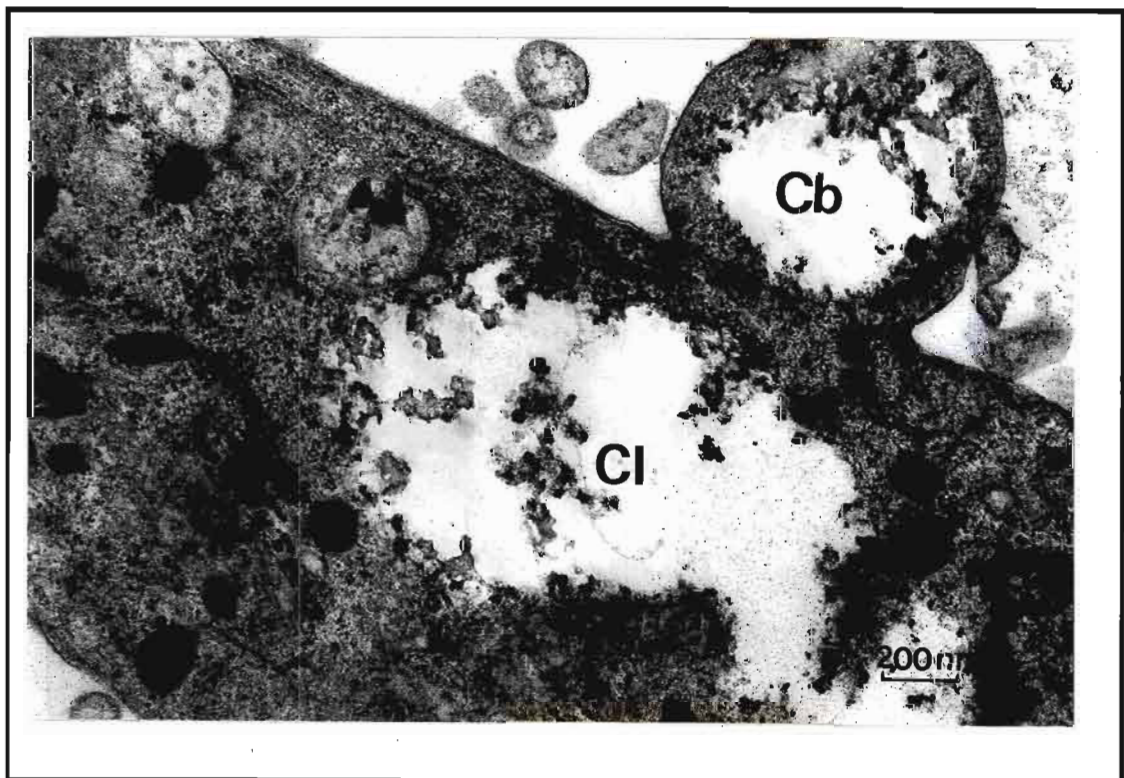


Fig 4.17 Electron micrograph showing areas of cytoplasmic lysis (Cl) observed in AFB₁ treated cells together with focal areas of lysis in cytoplasmic blebs (Cb).

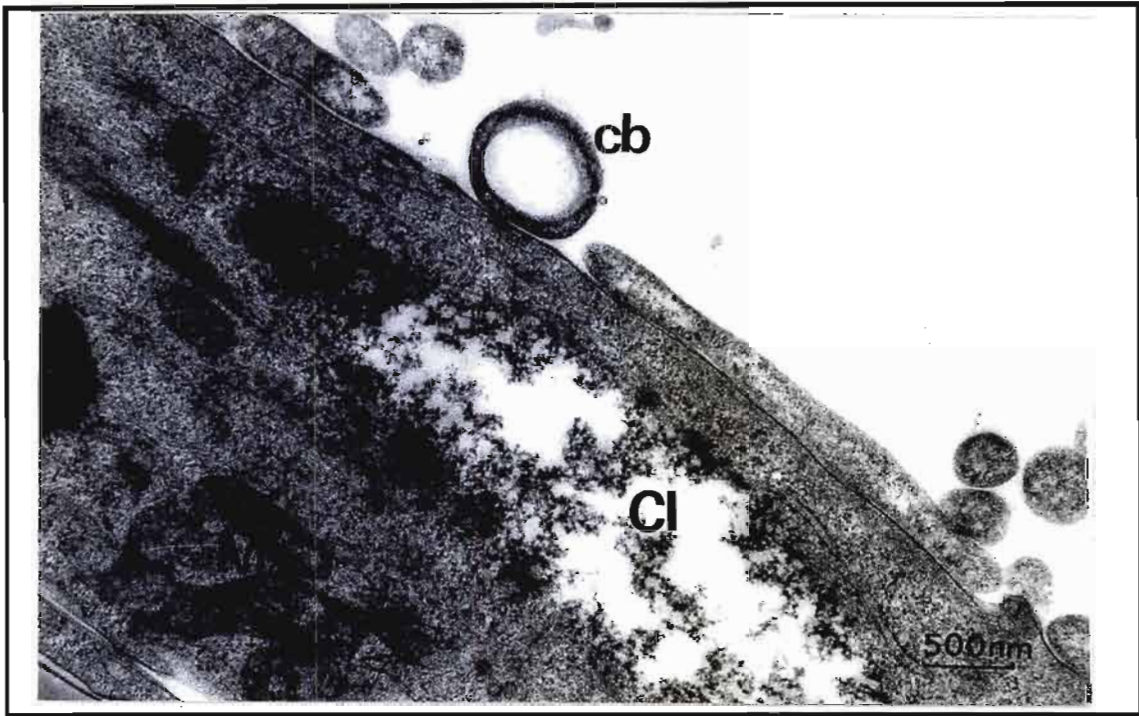


Fig 4.18 Electron micrograph showing areas of cytoplasmic lysis(Cl) observed in AFB₁-8,9-epoxide treated cells and within the cytoplasmic blebs (cb). Mitochondria (M).

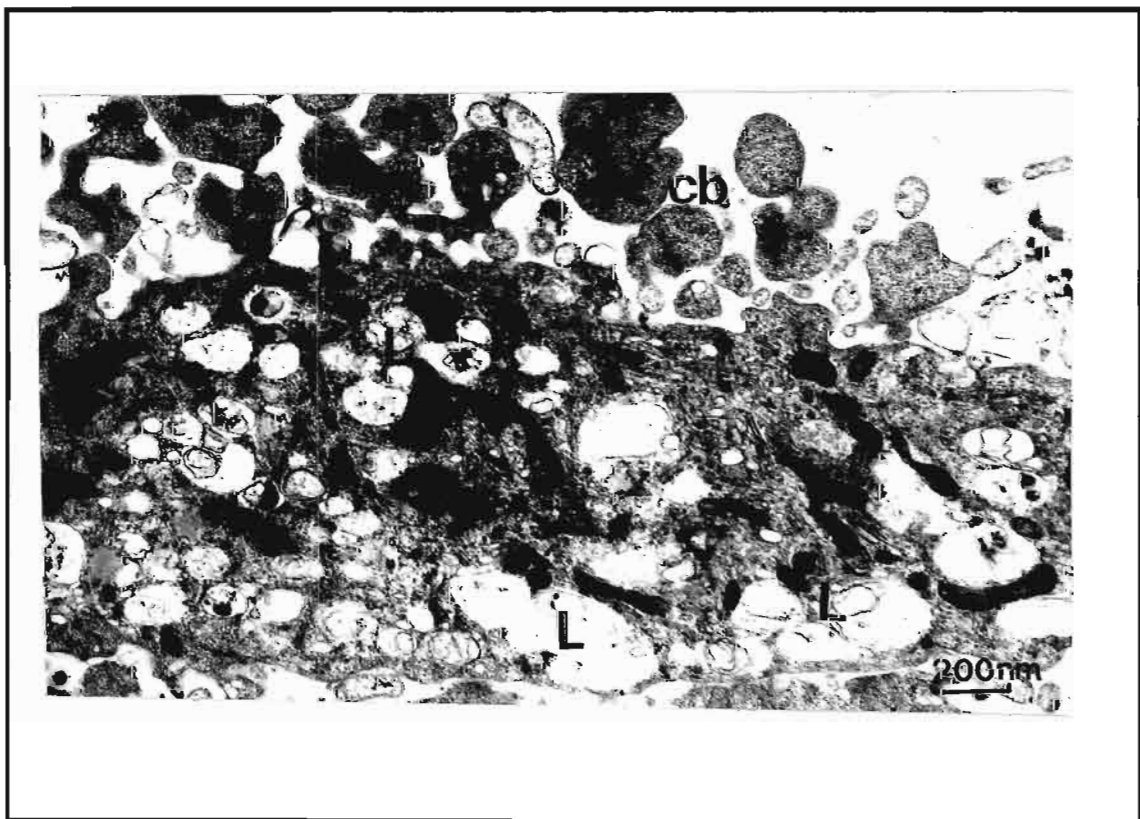


Fig 4.19 Electron micrograph showing the presence of numerous secondary lysosomes (L) and cytoplasmic blebs (cb) in AFB₁ treated cells.

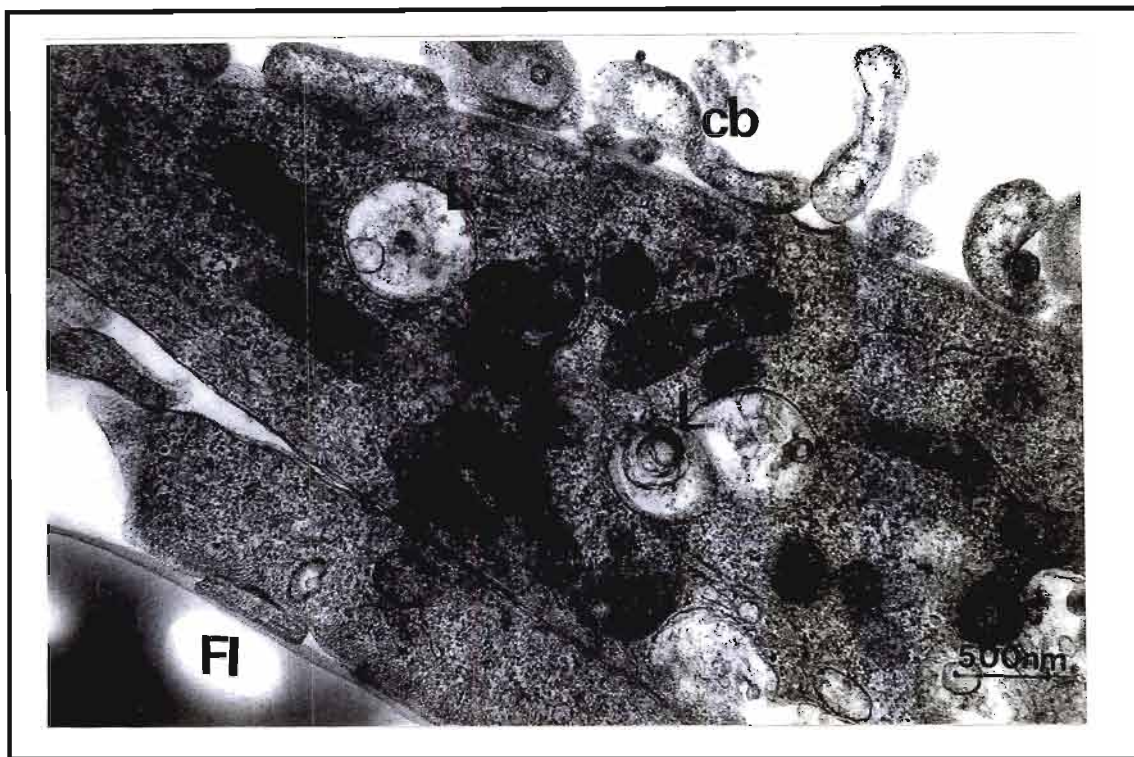


Fig 4.20 Electron micrograph showing a few secondary lysosomes (L) filled with proteinaceous material, probably degenerate organelles in AFB₁-8,9-epoxide treated cells. Cytoplasmic blebs (cb), Flask (FI).

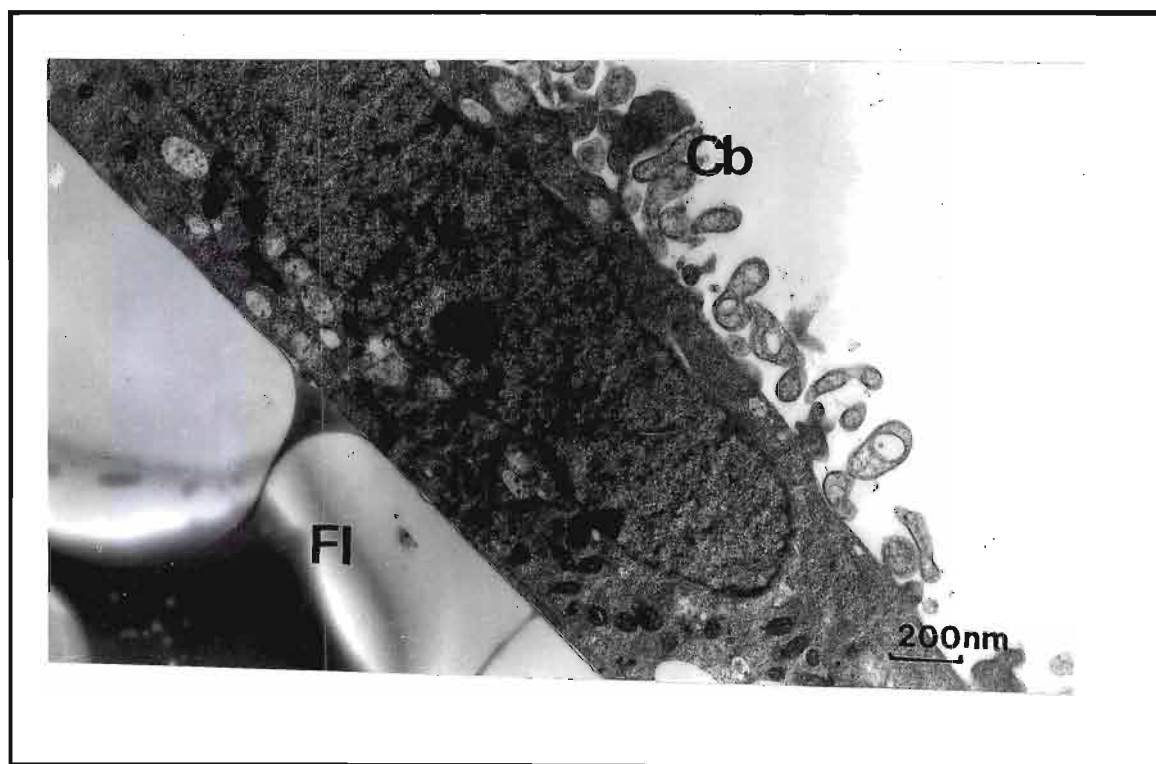


Fig 4.21 Electron micrograph of an untreated HepG2 cell showing the presence of a prominent nucleus (NL), numerous ovoid mitochondria (M) and cytoplasmic blebs (cb). Flask (FI).

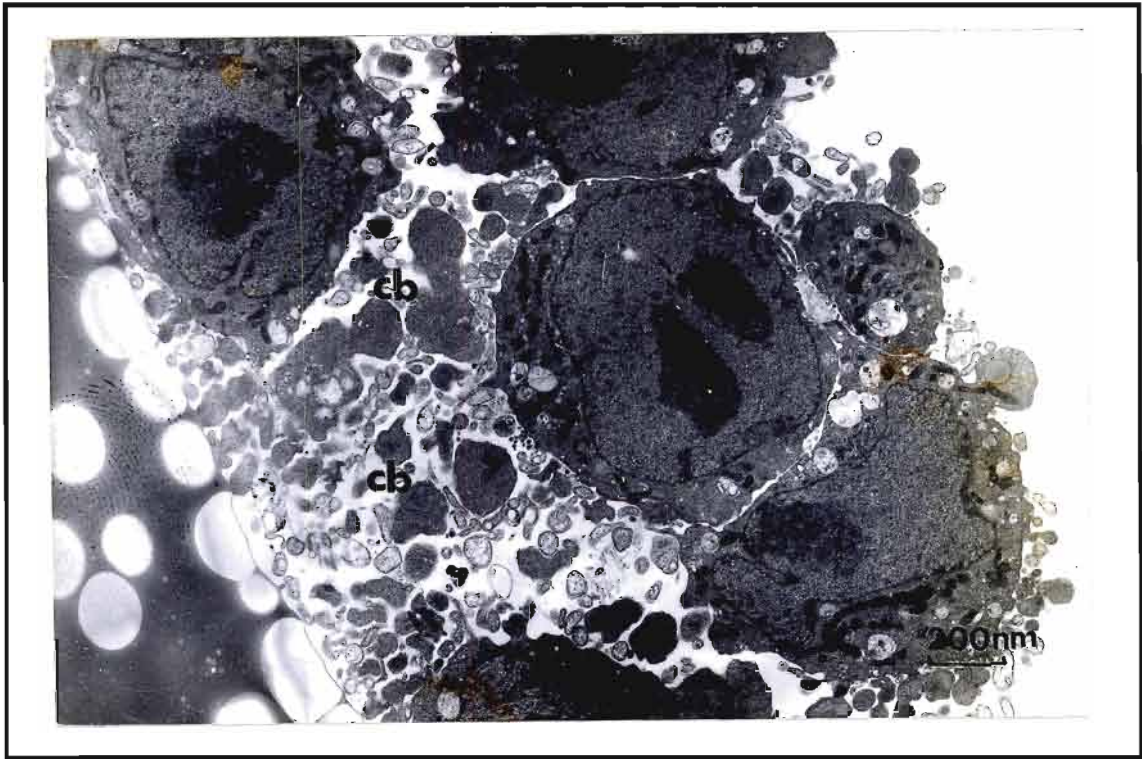


Fig 4.22 Electron micrograph showing cytoplasmic blebbing (cb) in AFB₁ treated cells.

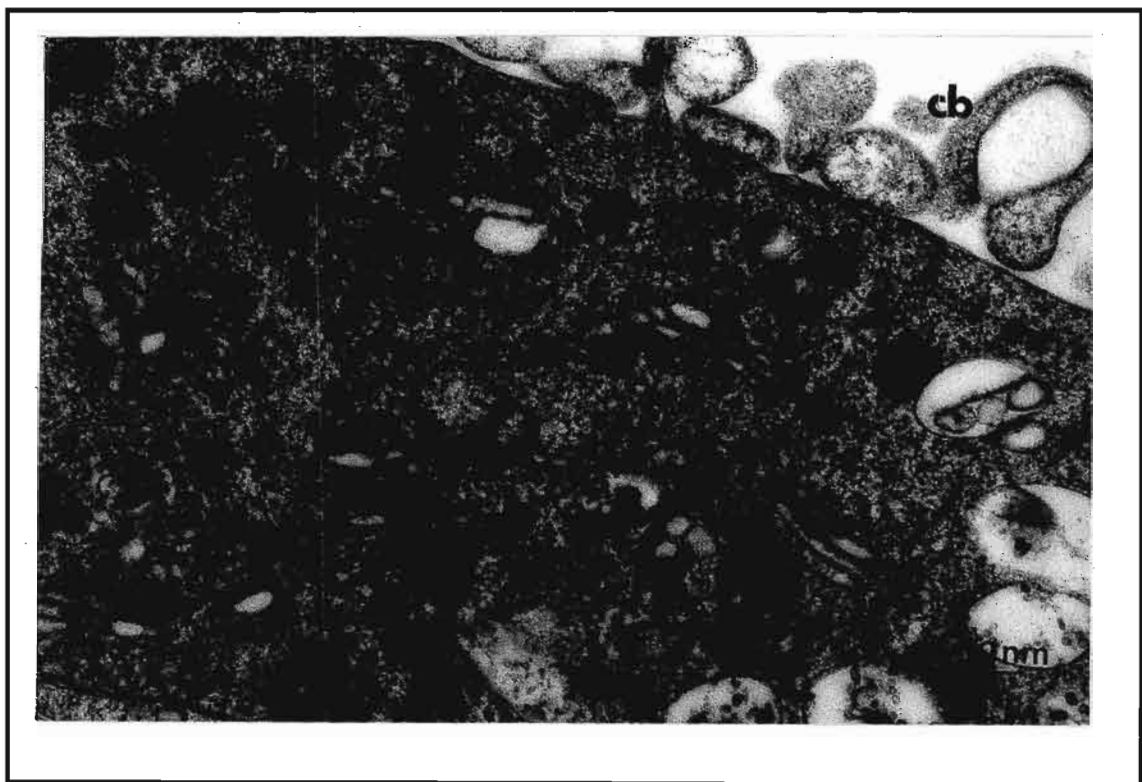


Fig 4.23 Electron micrograph of cytoplasmic blebs (cb) and slightly swollen golgi (G) in AFB₁-8,9-epoxide treated cells

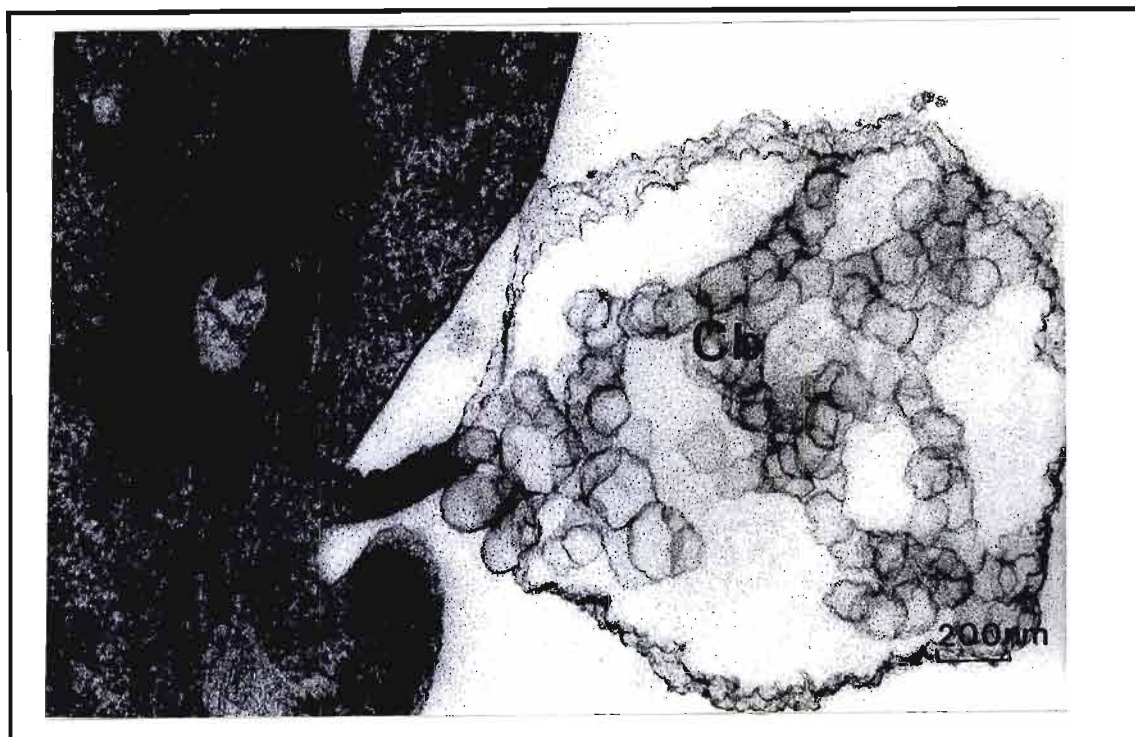


Fig 4.24 Electron micrograph of grossly altered cytoplasmic blebs (Cb) in AFB₁-8,9-epoxide treated cells.



Fig 4.25 Electron micrograph showing the presence of grossly swollen golgi observed in AFB₁-8,9-epoxide treated cells and slightly swollen rER.

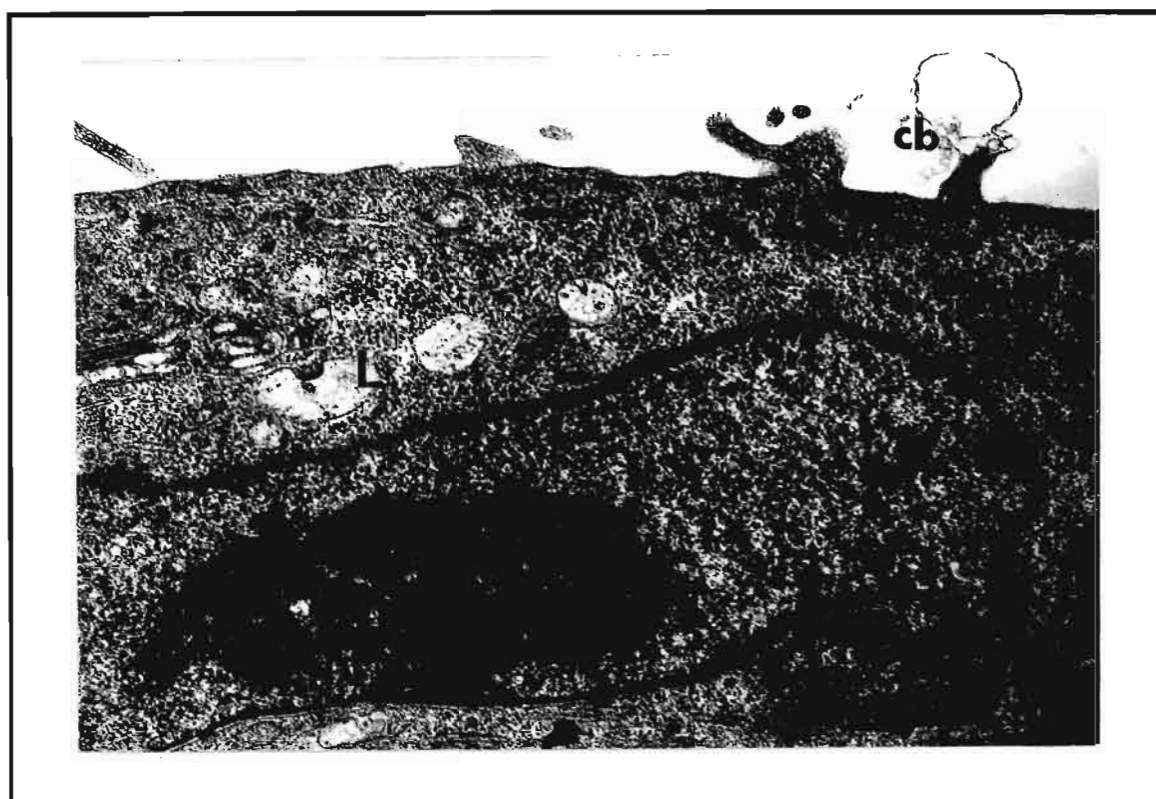


Fig 4.26 Electron micrograph of a AFB_1 -treated cell showing the presence of a large nucleolus (N), a few secondary lysosomes (L) and cytoplasmic blebs (cb).

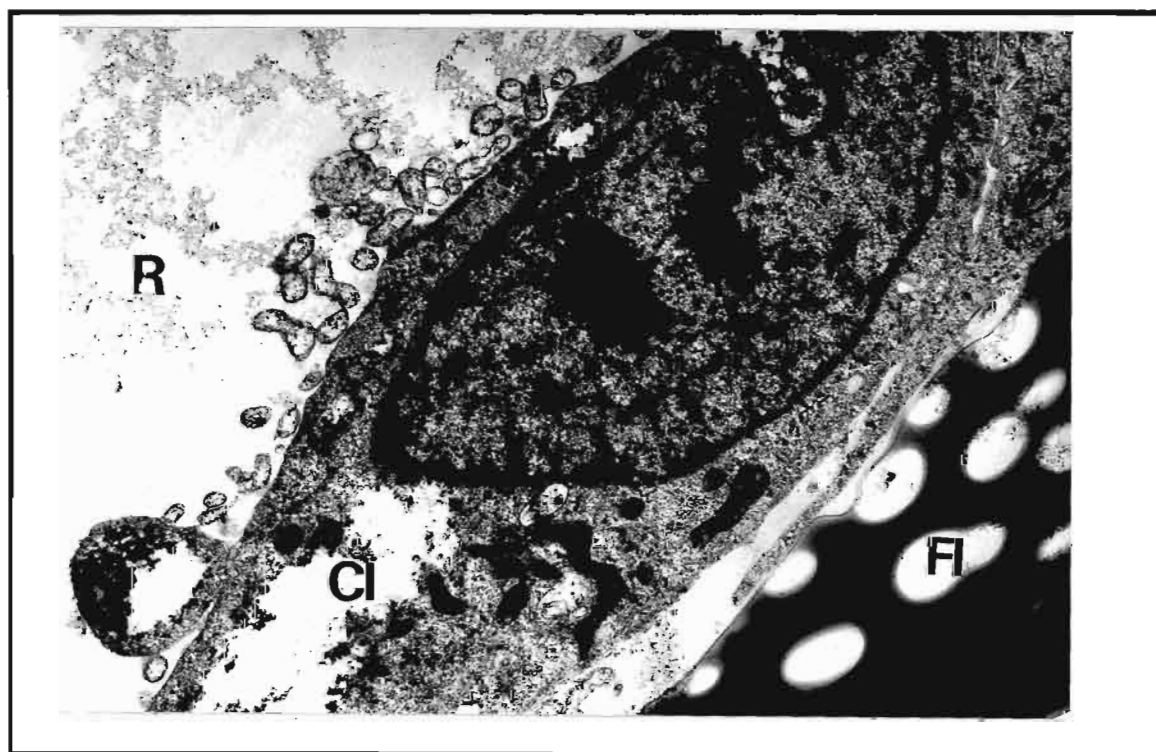


Fig 4.27 Electron micrograph of a AFB_1 treated cell showing microsegregation of the nucleoli (N), chromatin clumping (cc) along the periphery of the nucleus, and areas of cytoplasmic lysis (Cl). Flask (Fl), resin (R).

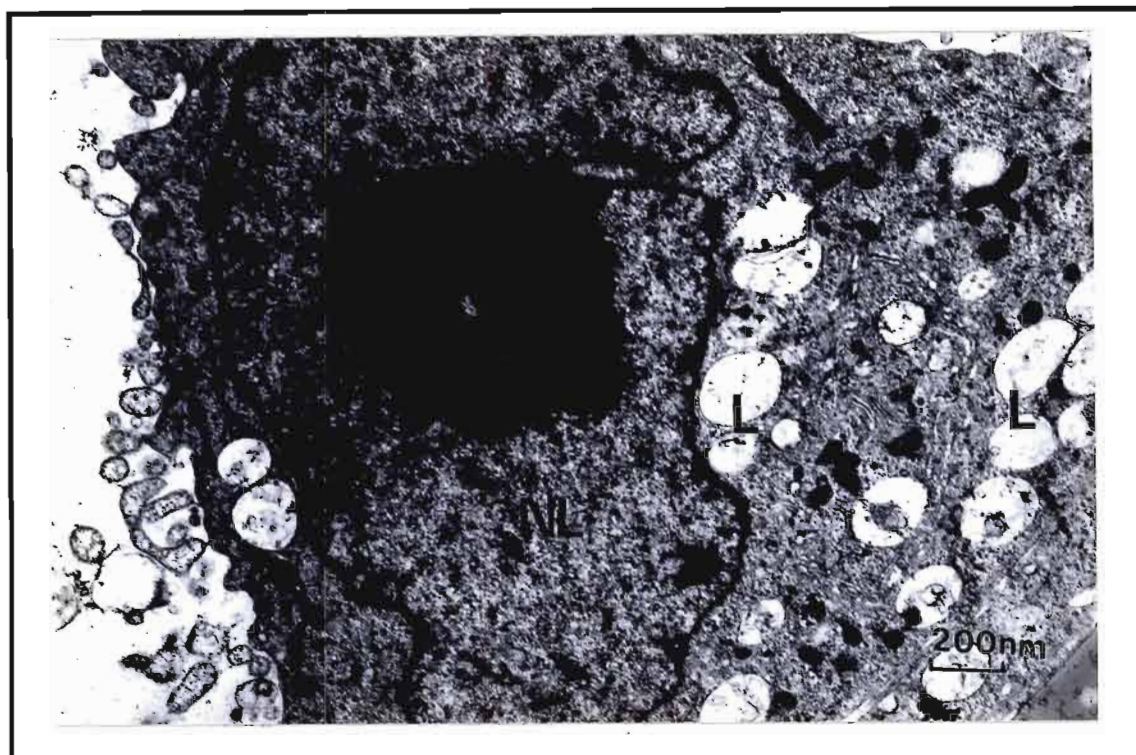


Fig 4.28 Electron micrograph showing the presence of distinct fibrillar (f) and granular (g) components of the nucleolus of AFB₁ treated cells which is indicative of an inhibition of DNA dependent RNA synthesis. Nucleus (NL), secondary lysosomes (L).

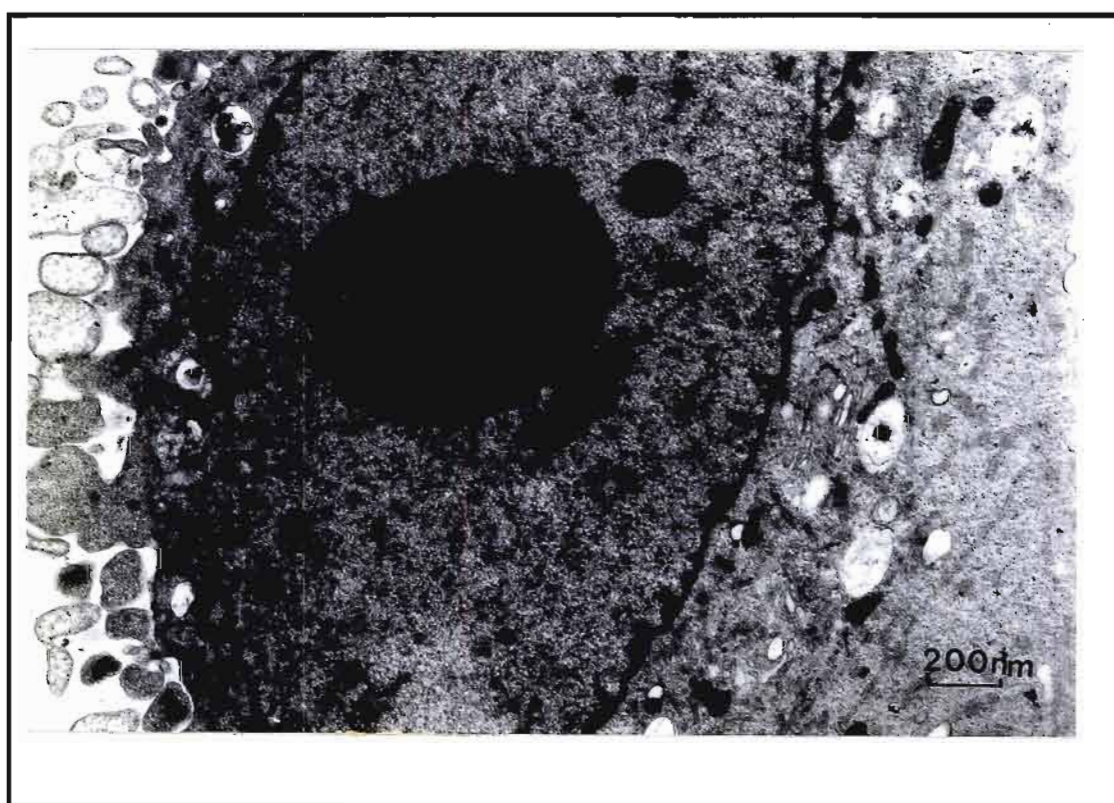


Fig 4.29 Electron micrograph showing the presence of a distinct nucleolus (N) in untreated HepG2 cells with no differentiation.

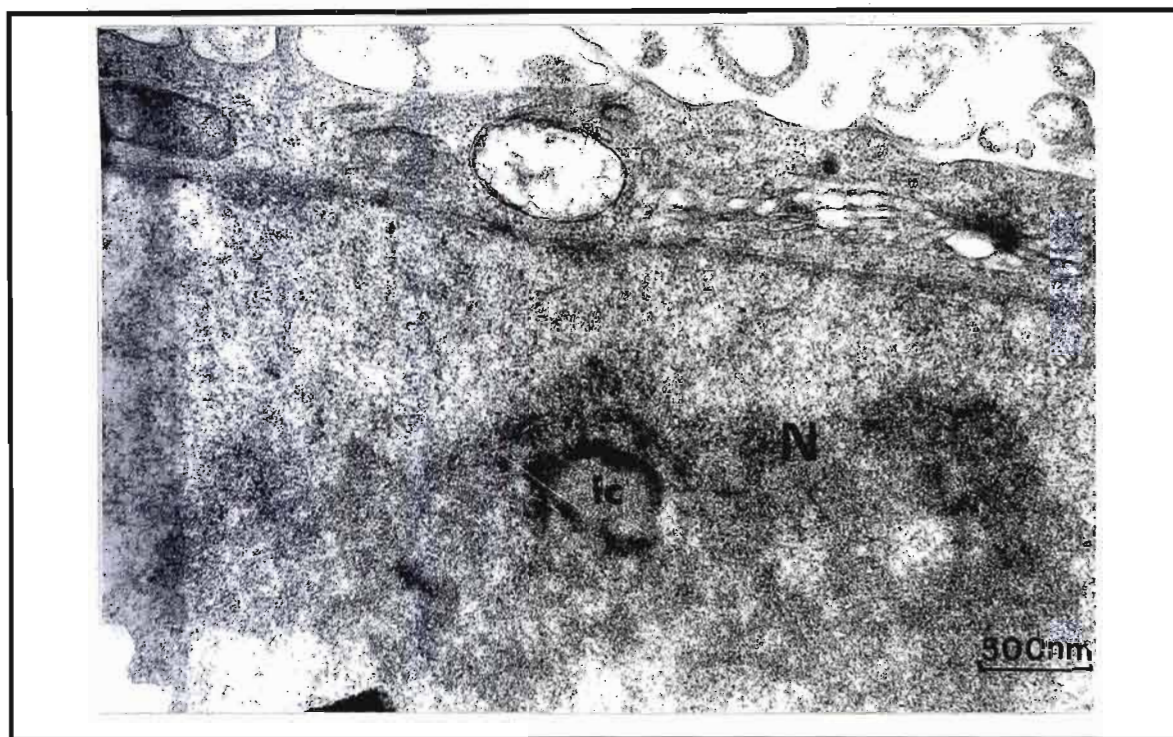


Fig 4.30 Electron micrograph showing the presence of a large nucleolus (N) and a few fibrous centers (fc) in AFB₁-8,9-epoxide treated cells.

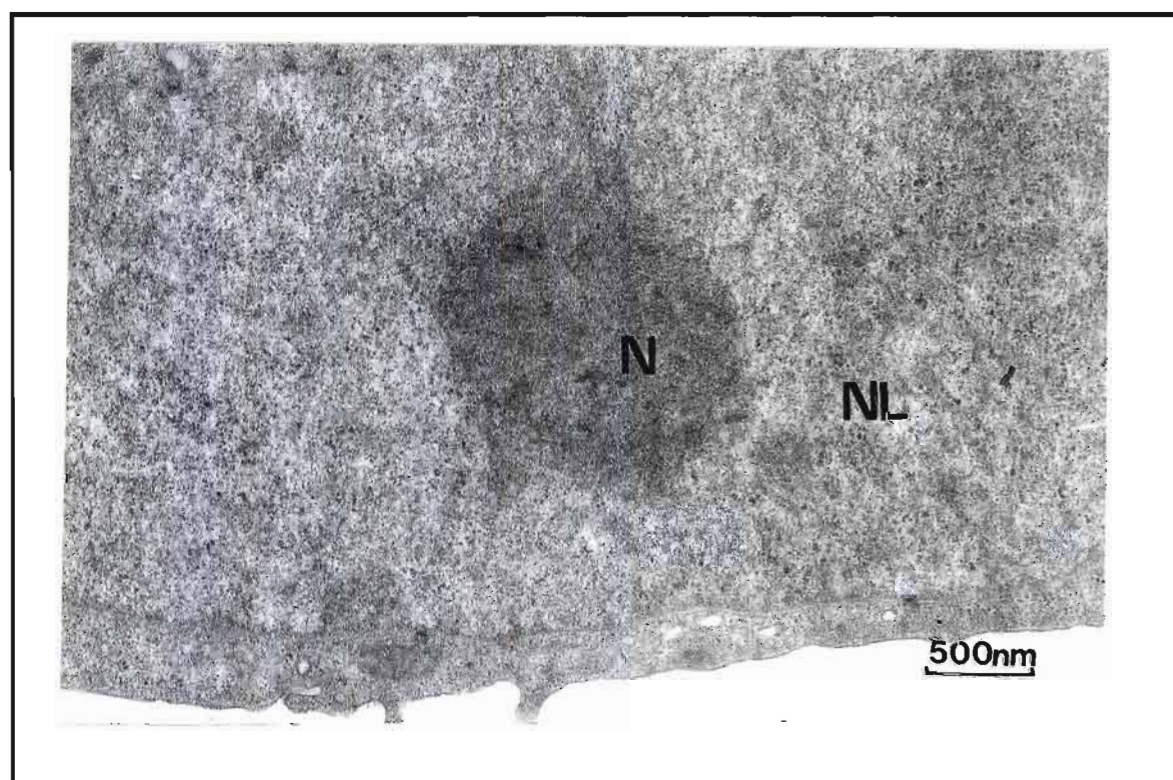


Fig 4.31 Electron micrograph of an AFB₂ treated cell showing the presence of normal looking nuclei (NL) with a distinct nucleolus (N).

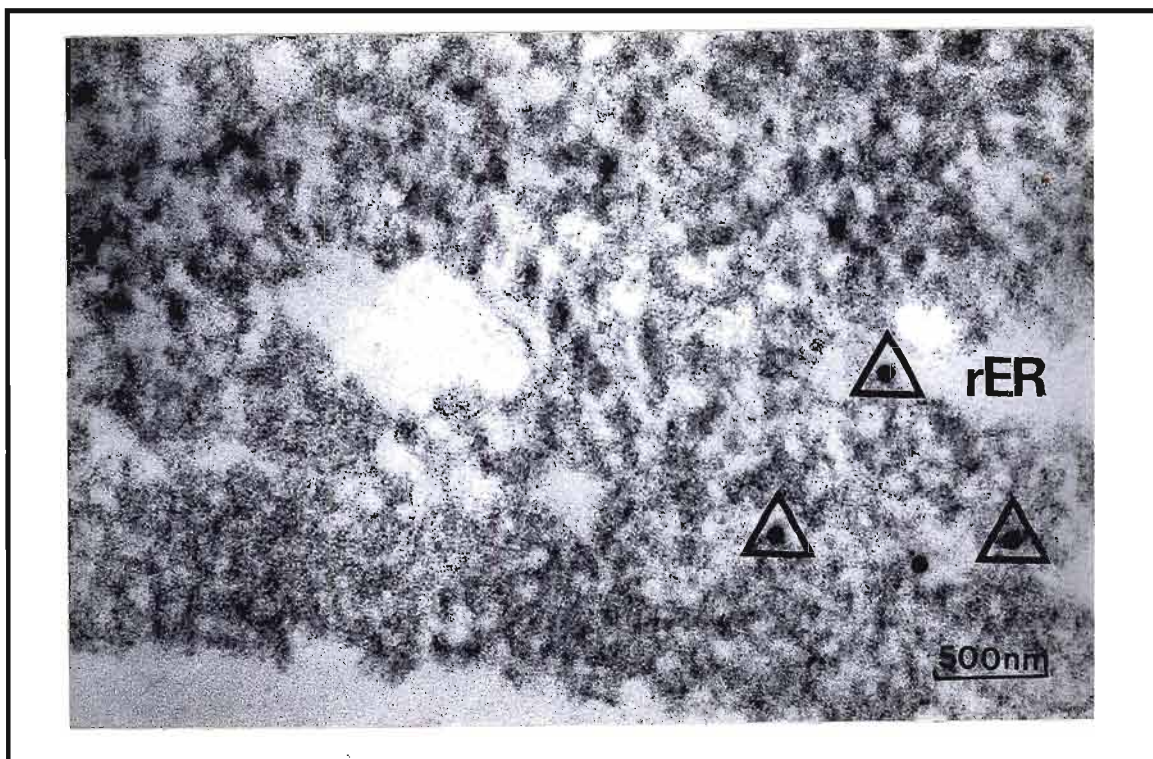


Fig 4.32 An immunocytochemical (ICC) electron micrograph showing the presence of polyclonal gold labelled anti-AFB₁ in the swollen rER and in close proximity to it in a AFB₁-8,9-epoxide treated cell .Label is encircled.



Fig 4.33 ICC electron micrograph showing the presence of 10nm gold probes within the cytoplasmic bleb (cb) and in the nucleoplasm (Np) of a AFB₁-8,9-epoxide treated cell.

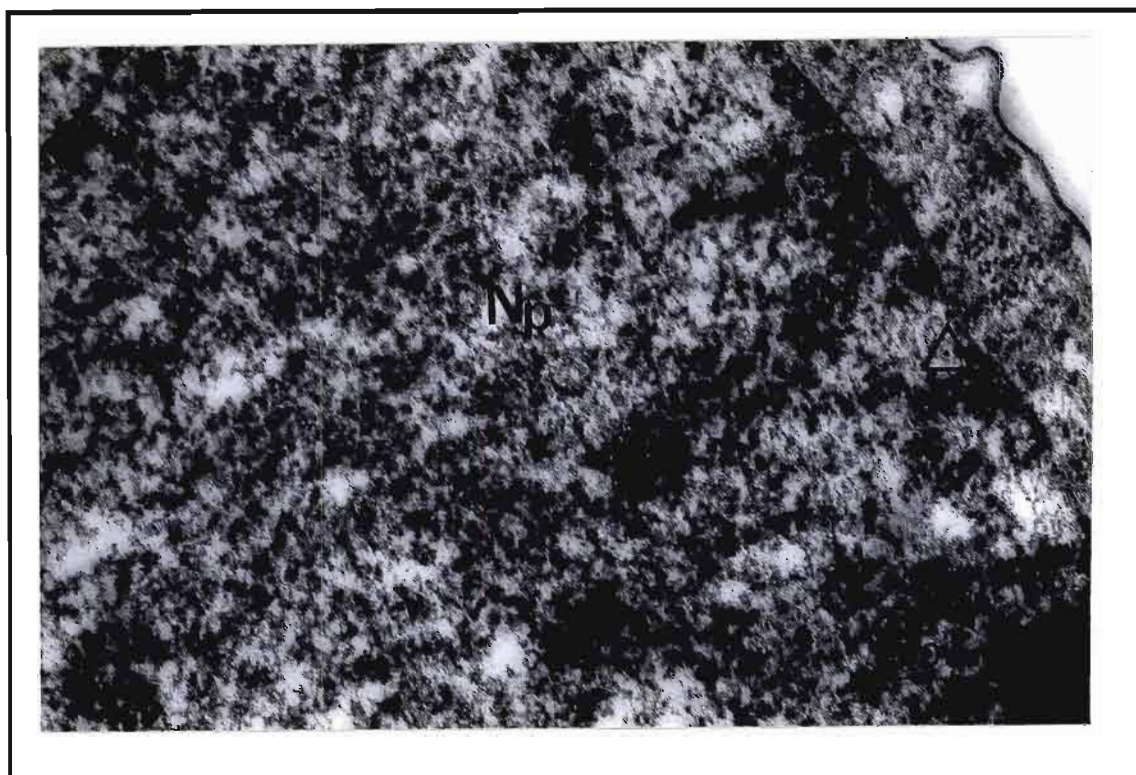


Fig 4.34 ICC electron micrograph showing the presence of label in the nucleoplasm (Np) in an AFB₁ treated cell.

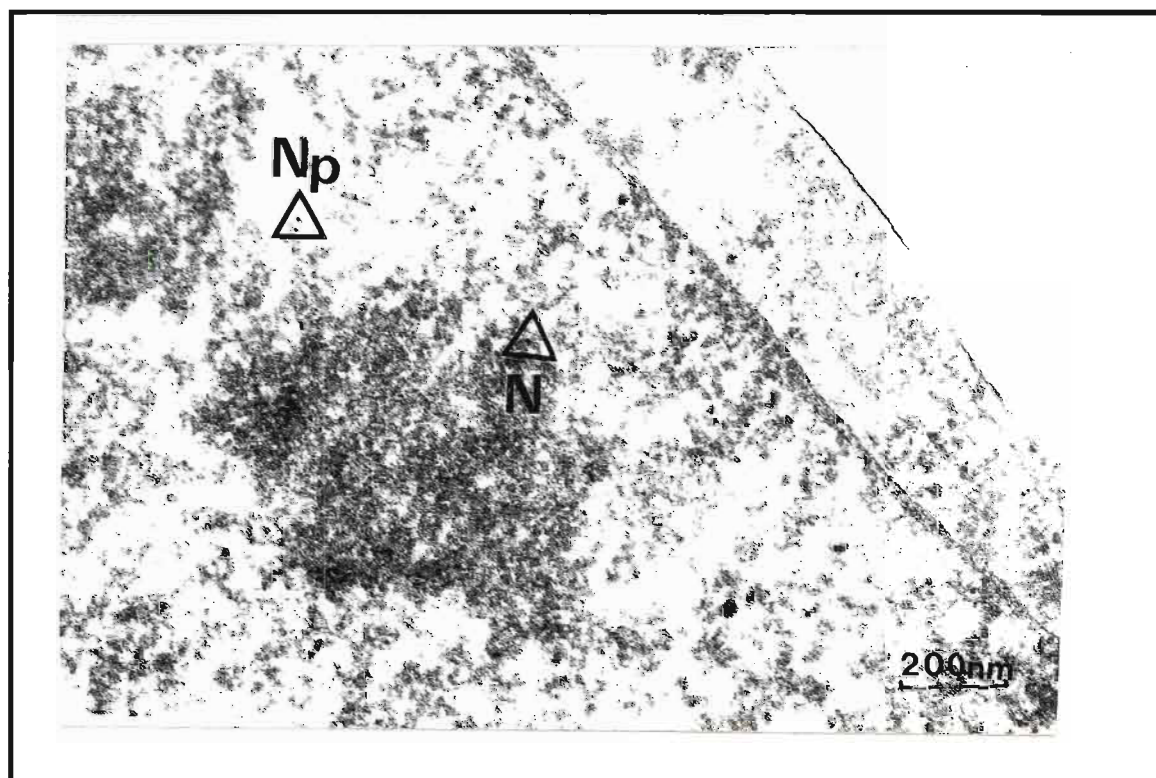


Fig 4.35 ICC electron micrograph showing the presence of label in the nucleoplasm (Np) and within the nucleolus (N) in AFB₁-8.9-epoxide treated cells.

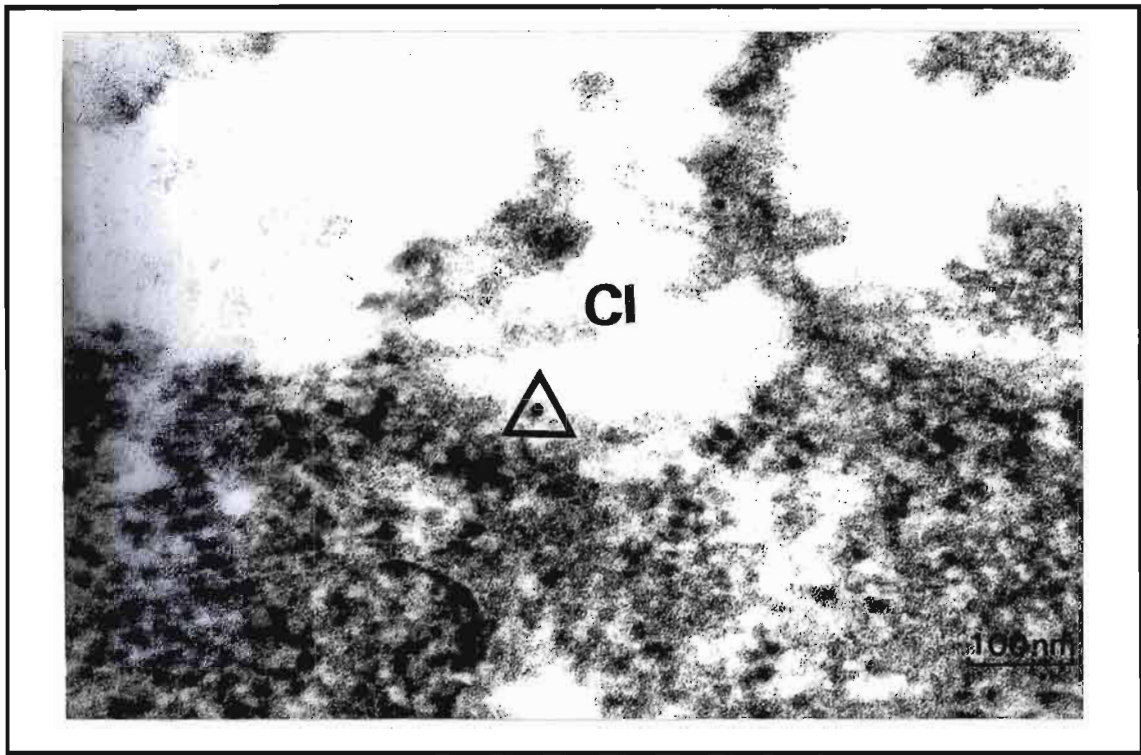


Fig 4.36 ICC electron micrograph showing the presence of label in areas of cytoplasmic lysis (CI) of AFB₁ treated cells.

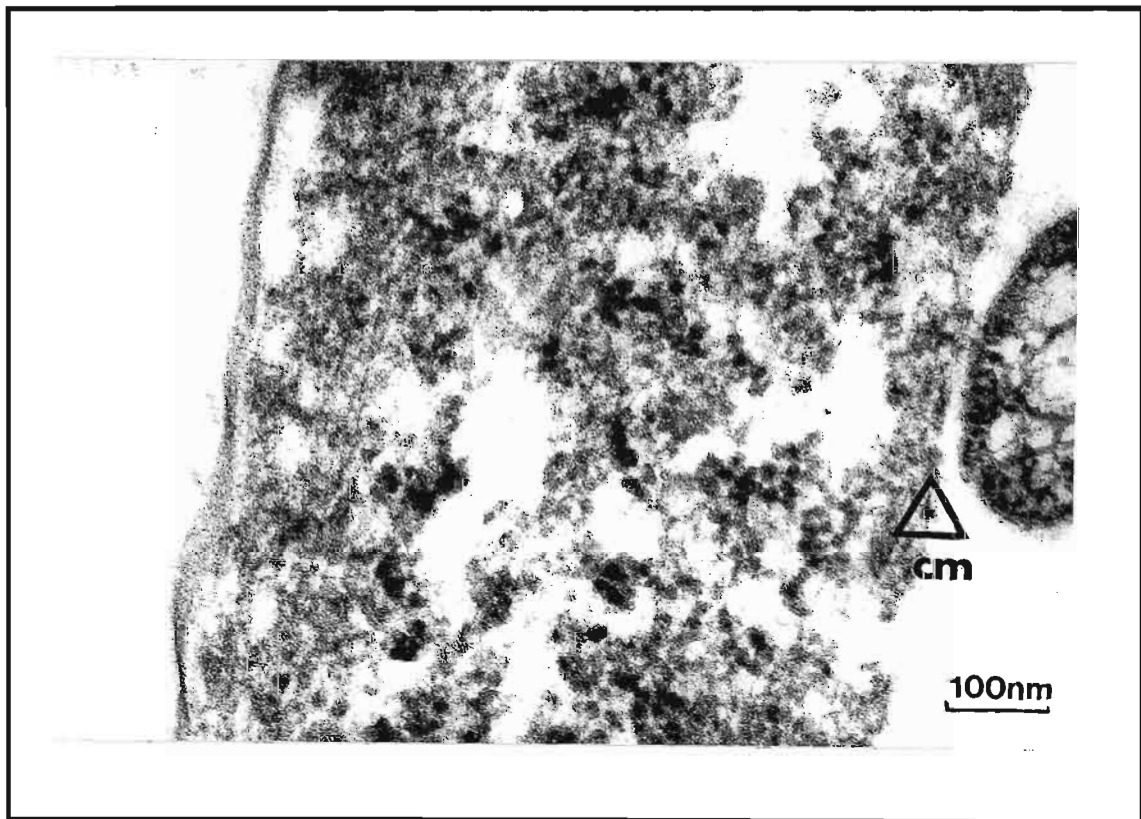


Fig 4.37 ICC electron micrograph showing the presence of label along the cell membrane(cm) of an AFB₁ treated cell.

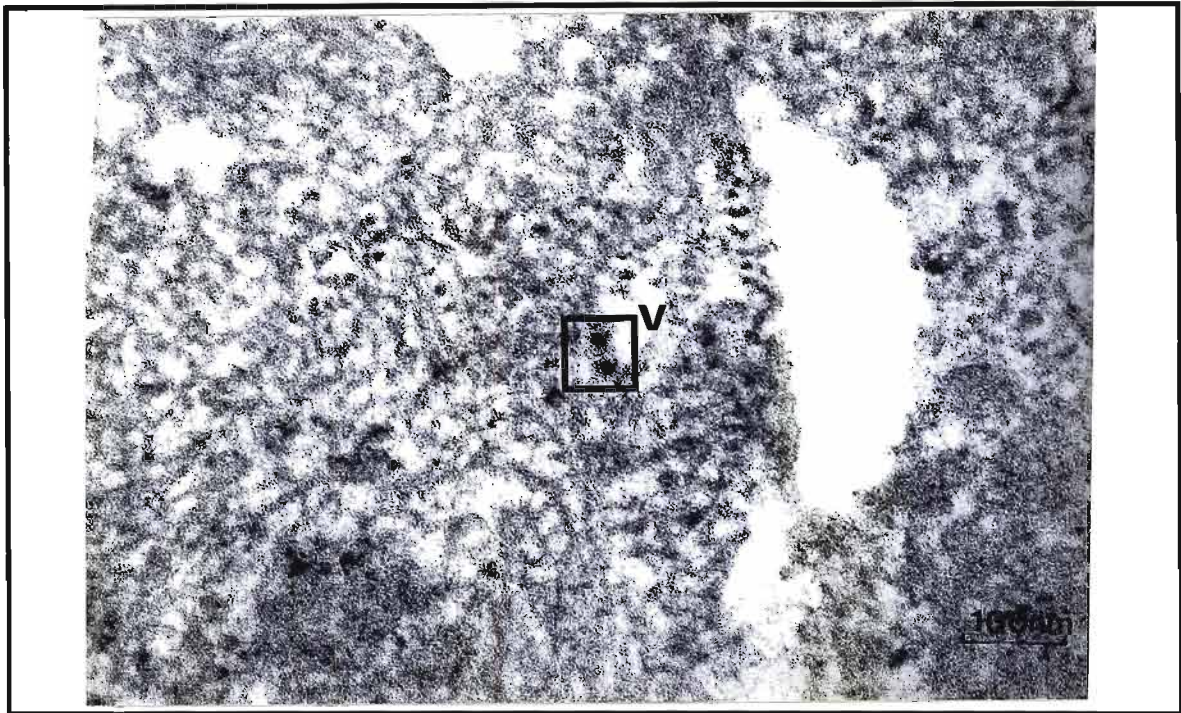


Fig 4.38 ICC electron micrograph showing the presence of label close to vesicles (v) in AFB₁ treated cells.

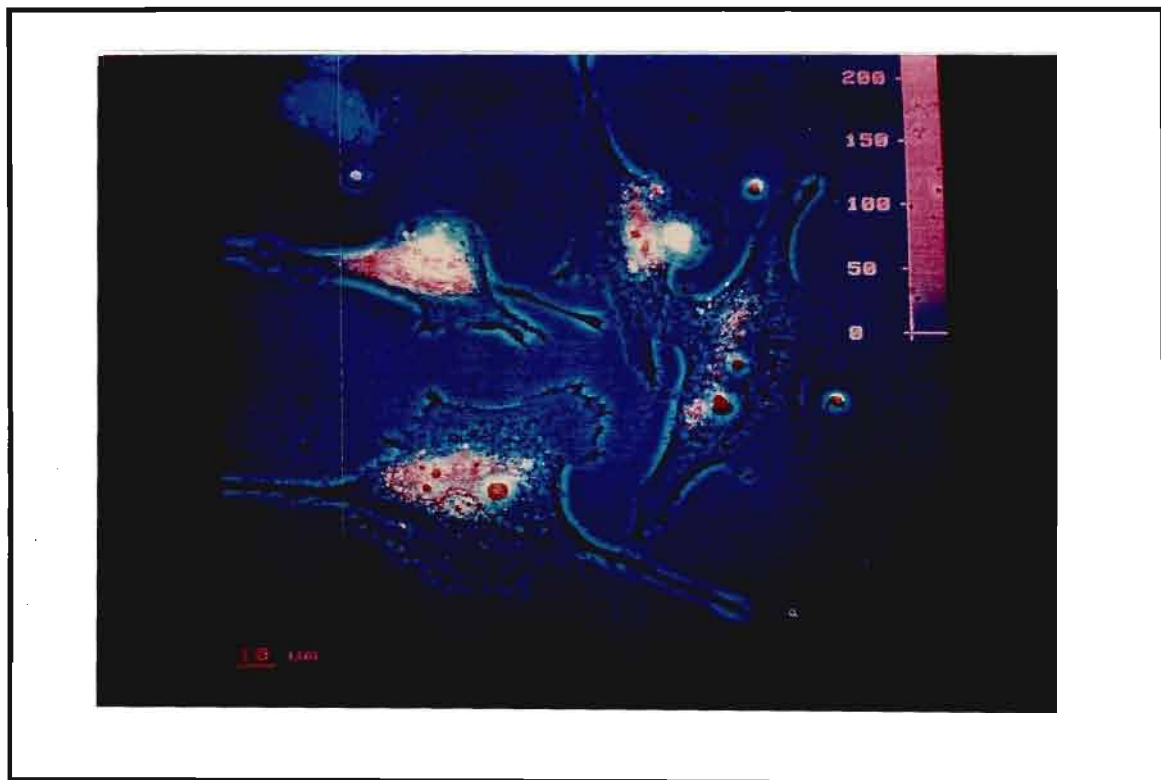


Fig 4.39 Confocal micrograph showing the localisation of fluorescent AFB₁ (12µg/ml) in the nucleolus (N) after a four hour incubation period. The toxin is represented by the pink stain. The greater the intensity of the colour the greater the amount of toxin.

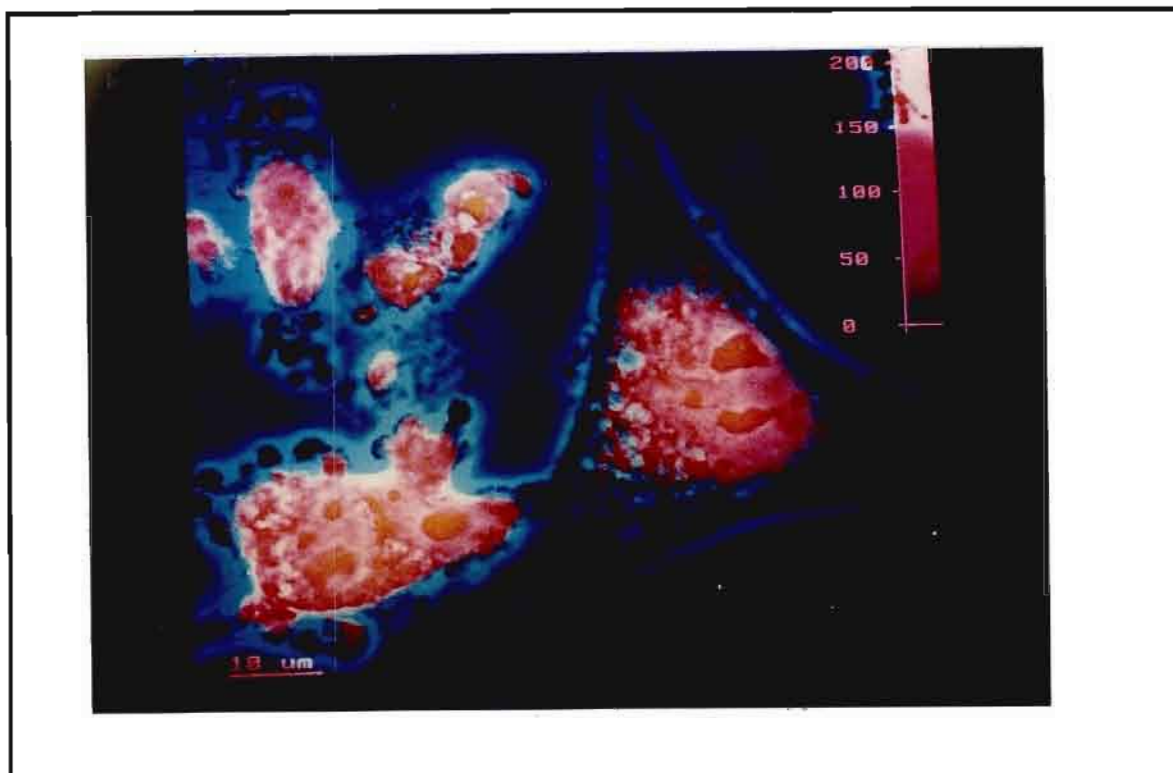


Fig 4.40 Confocal micrograph showing the presence of fluorescent label (AFB_1) in the rest of the nucleus after 8 hours.

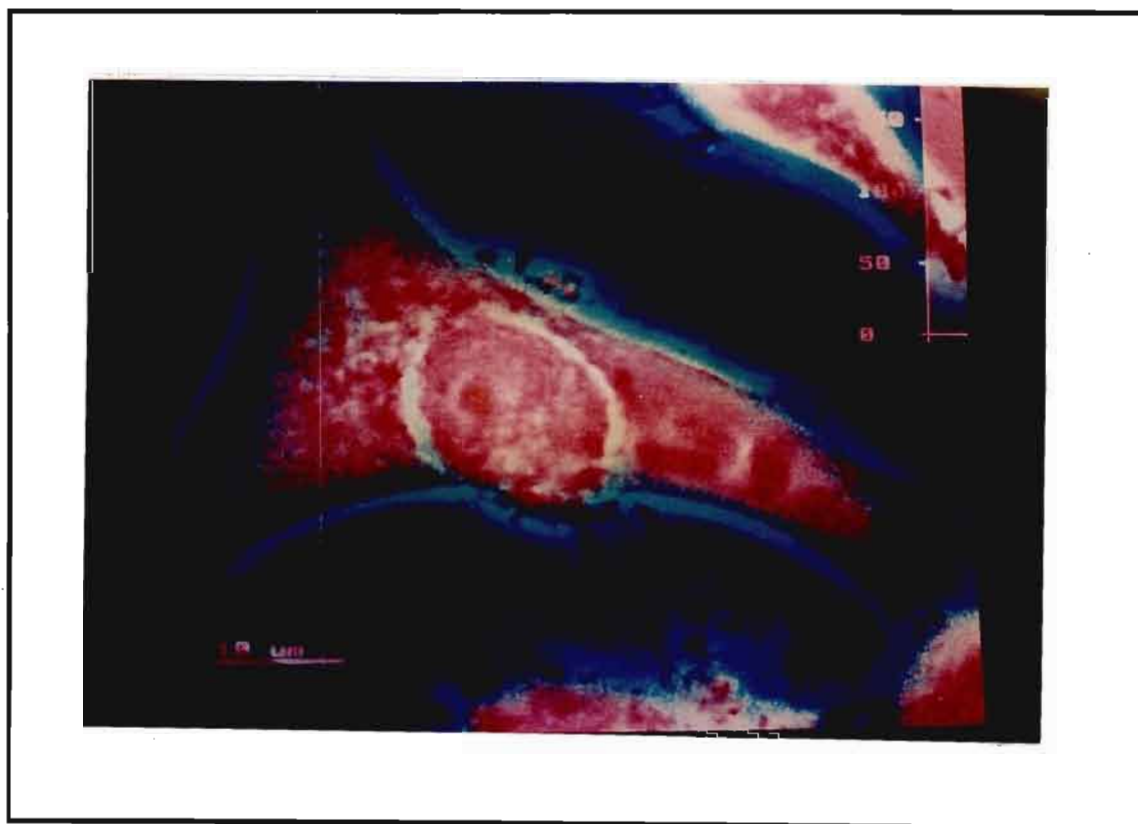


Fig 4.41 Confocal micrograph showing the presence of fluorescent label within the cytoplasm (C) after 12 hours.

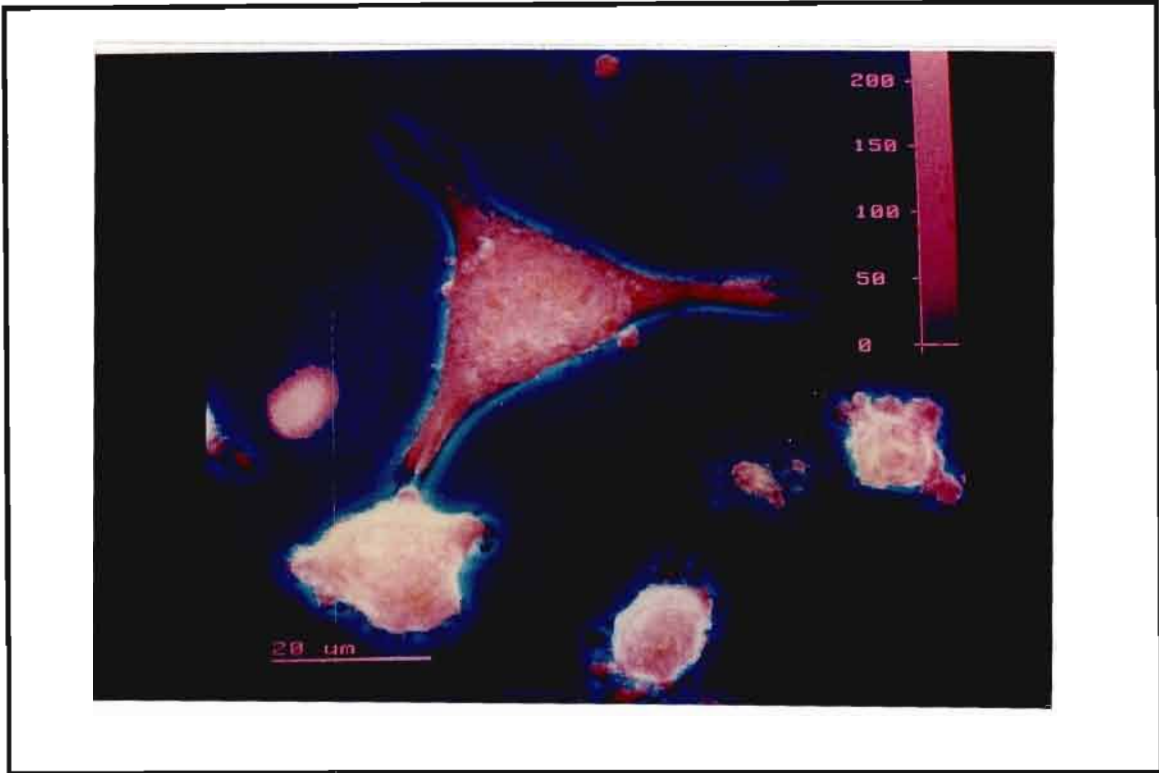


Fig 4.42 Confocal micrograph showing the label after 24 hours which spread throughout the cell.

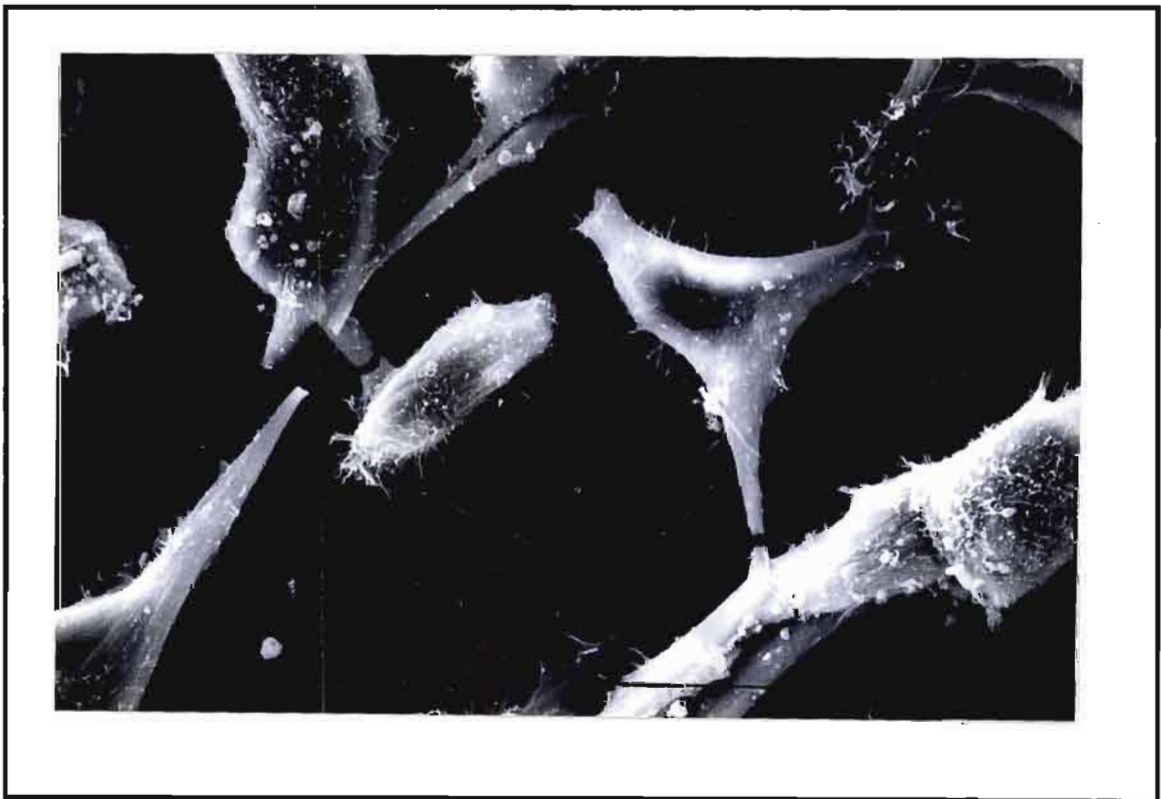


Fig 4.43 A scanning electron micrograph showing the cell surface processes of untreated HepG2 cells (C).

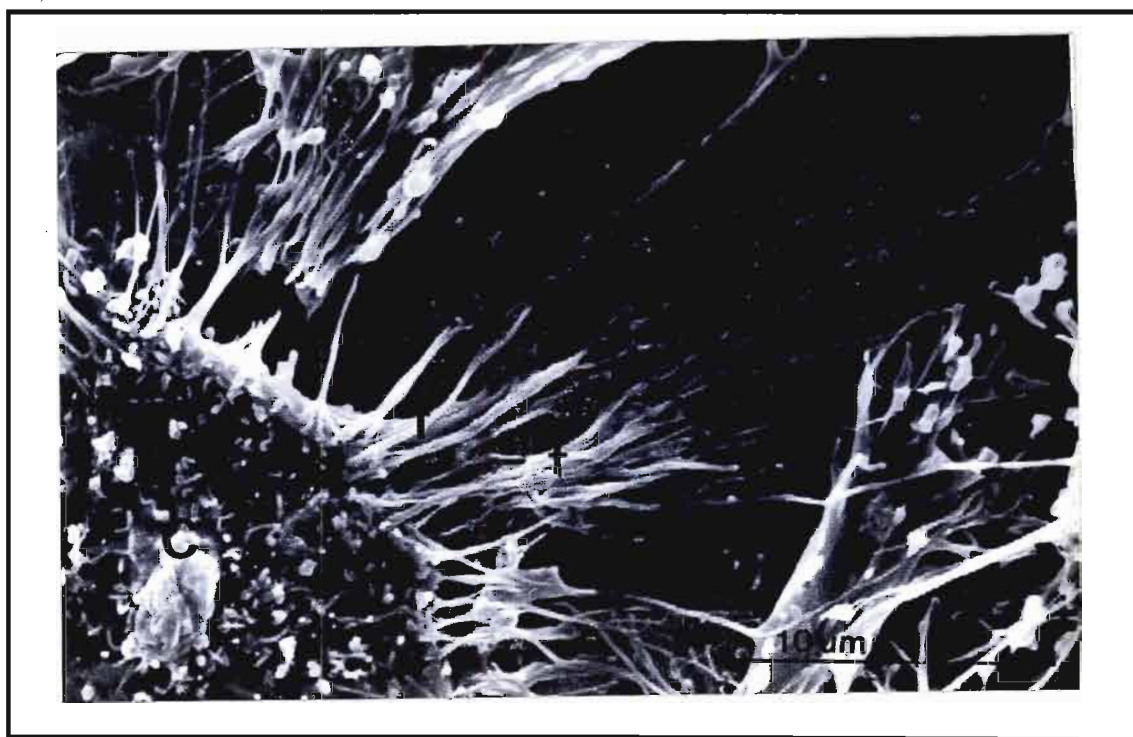


Fig 4.44 Scanning electron micrograph of the filamentous processes of untreated cells (f) which allow the cells(C) to attach to the substrate and to one another.



Fig 4.45 Scanning electron micrograph of a confluent layer of untreated cells at a higher magnification as they lie next to one another (C). The filamentous processes (f) are interconnected. in untreated cells.

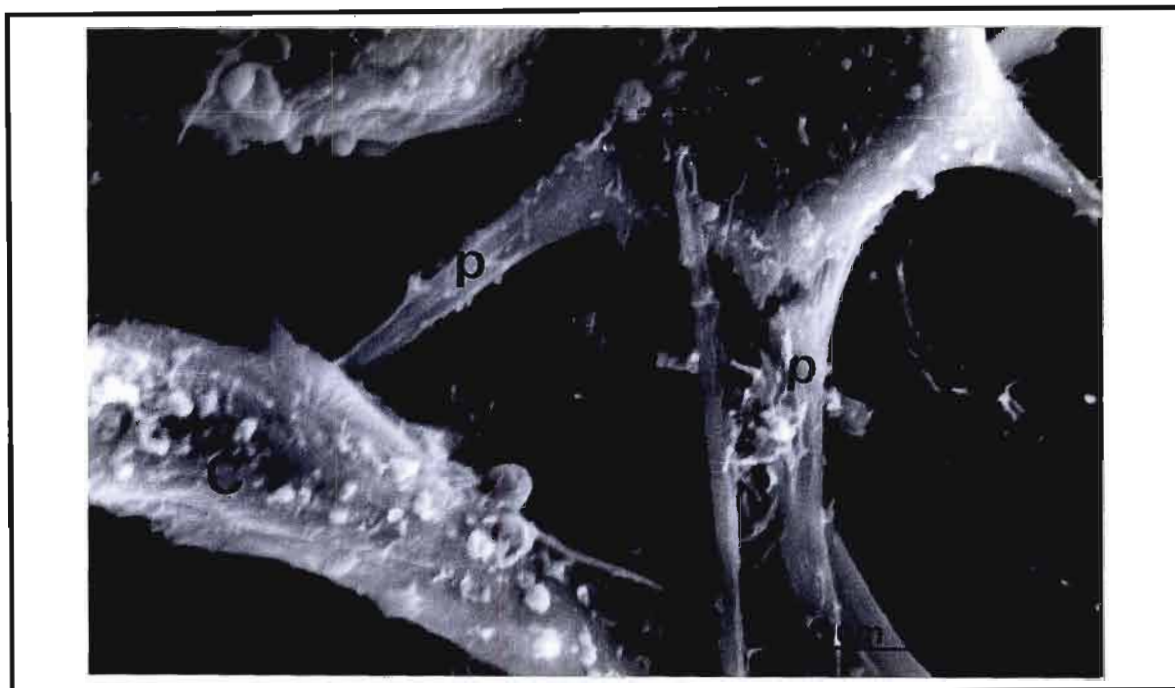


Fig 4.46 Scanning electron micrograph showing the interconnecting projections (p) of untreated cells (C).

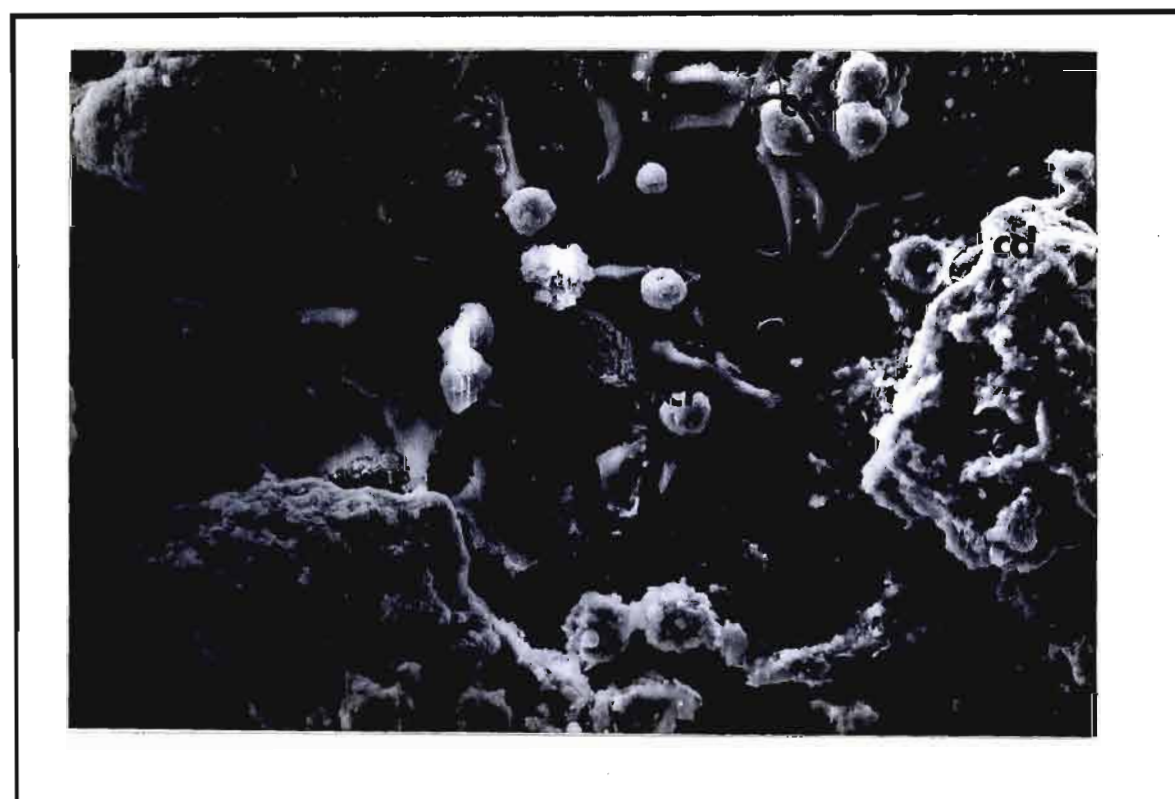


Fig 4.47 Scanning electron micrograph of AFB₁ treated cells which appeared rounded (cr), indicative of cell death and together with cellular debris (cd).

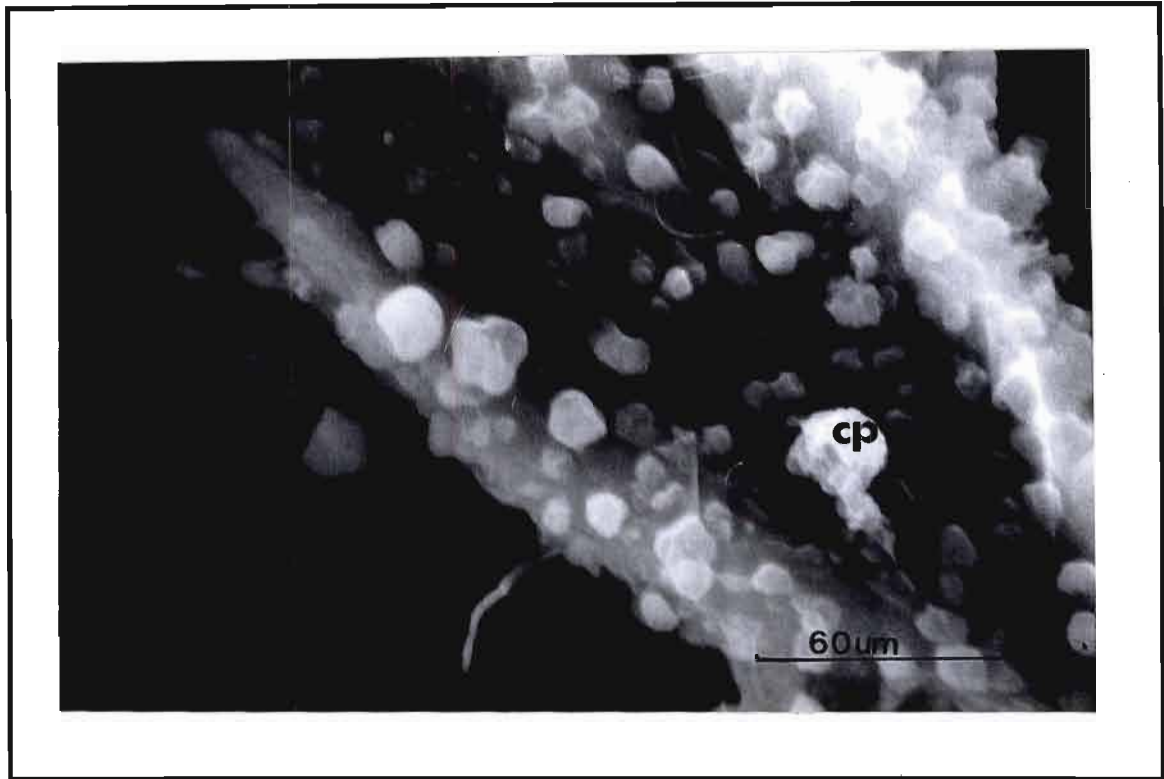


Fig 4.48 Scanning electron micrograph of a AFB₁ treated cell showing the presence of rounded cytoplasmic processes (cp) which would indicate a detachment from the substrate and other cells.

4.4 DISCUSSION

The use of cultured cancerous cell lines for electron microscopy has proven to be invaluable in the correlation of internal architecture to the environmental stress. The use of the HepG2 cell line in this investigation is attributed to its high rate of turnover and is representative of the target organ of the toxic insults. Many of the cellular alterations observed in toxin treated cells may be manifestations of cell injury. AFB₁ has been characterised as an indirect hepatotoxin that produces injury by interference with a specific metabolic pathway or process essential for cell integrity (Zimmerman and Ishak, 1987).

As compared to the untreated cells, both AFB₁ and AFB₁-8,9-epoxide treated cells displayed similar cellular alterations at the light microscopic level (Fig 4.1 to 4.7) which could be attributed to the extreme toxicity of these mycotoxins, which caused a rapid cell death and the subsequent detachment from the substrate. It is possible that because of the extreme toxicity of the AFB₁-8,9-epoxide (after a 24 hour incubation period), most of the cells died and detached from the substrate leaving only a few behind while the AFB₁ treated cells were not as severely affected by the toxin, thus remaining firmly attached to the substrate, displaying only slight cellular alterations at the light microscopic level. Cytoplasmic processes which are essential in cell anchorage on the substrate were shown by SEM to be altered. Cells that detach from the substrate have rounded processes while cells attached to the surface have numerous long filamentous processes attached to both the cell surface and to one another. The attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca²⁺ and Mg²⁺ ions. The loss of cell viability would imply a loss of production of these ions and glycoproteins required to facilitate cell adhesion (Freshney, 1983).

Lipid accumulation within the liver cells exposed to both AFB₁ and the AFB₁-8,9-epoxide as observed by light microscopy may be related to reduced protein synthesis and the subsequent impaired release of lipid as lipoprotein (Zimmerman and Ishak, 1987). It is possible that AFB₁ blocks the synthesis of proteins by binding to ribosomes and therefore prevents the mobilisation of fatty acid metabolism. The defective egress of lipid from the liver is defined as hepatic steatosis which occurs as a result of the defective

synthesis of the apoprotein moiety of the very low density lipoprotein (VLDL) needed to transport the lipid from the liver to the depots (Zimmerman and Ishak, 1987).

Microvesicular steatosis observed in AFB₁ and AFB₁-8,9-epoxide treated cells would suggest that these toxic insults attribute to the accumulation of lipid within the cell. It has been previously documented that there does exist a possible relationship between AFB₁ and fatty degeneration in Thai children (Bourgeois *et al.*, 1971). It was found that Reye's syndrome, a childhood neurologic disease consisted of systems including a pale fatty liver. Evidence from Thailand associated the high levels of AFB₁ in victims tissues with Reye's syndrome (Bourgeois *et al.*, 1971).

The presence of multinucleated cells in toxin treated groups may be attributed to its inability to undergo mitosis whilst still undergoing meiosis and is therefore implicated in decreased cell growth (Grossfield *et al.*, 1963). Legator (1965) showed that AFB₁ suppressed mitosis and DNA synthesis leading to an increase in giant cell formation and cell vacuolisation. Abnormal mitoses was also a feature observed only in toxin treated cells. Although it is known to be a common feature of cancerous cells, it was not observed in the untreated cells and therefore it can be postulated that the toxins induce DNA mutations.

The altered structural state of the mitochondria may be attributed to the toxin treatment. Previous studies show that mitochondria are one of the major cellular targets for AFB₁ during experimental carcinogenesis. It is documented that the c P-450 type of monooxygenase system in rat liver mitochondria is capable of activating the AFB₁ into an electrophilic reactive form which then covalently modifies mitochondrial DNA, RNA and protein. The *in vitro* effect of AFB₁ on oxidative phosphorylation in rat liver mitochondria showed an inhibition of oxygen uptake by mitochondria (Ramachandra, 1975). It has been shown that AFB₁ inhibits the electron transport chain between cytochrome b and c. This uncoupling is thought to be due to a gross change in the structure of mitochondrial membrane or a binding of uncoupling agents at specific sites thus inhibiting oxidative phosphorylation (Ramachandra *et al.*, 1975). In this study, mitochondrial membrane damage as indicated by swelling of mitochondria and the clearing of the mitochondrial matrix may be associated with both uncoupling and an inhibition of

oxidative phosphorylation in epoxide treated cells. Meldolesi et al. (1967) showed that there was a positive correlation between swollen mitochondria and low levels of ATP. It is widely accepted that there is a positive correlation between the metabolic activity of tissue and the number and size of mitochondria and the concentration of cristae. The paucity or absence of cristae in AFB₁-8,9-epoxide treated cells shown in this study would suggest low metabolic activity.

The alteration of the arrangement of cristae has been reported to be associated with a deficiency of cytochrome oxidase (Ghadially, 1982). Increased numbers of myelin figures observed in AFB₁ treated cells indicates increased mitochondrial turnover. This may be related to the toxic effects of AFB₁. It is possible that since AFB₁ elicits its effects on the mitochondria, the cell responds by repairing the damage with an increased production of mitochondria. The presence of large vacuoles in epoxide treated cells is possibly the remnants of degenerate mitochondria. Previous studies have shown that the administration of toxin results in the covalent binding of AFB₁ to liver mitochondrial DNA at concentrations 3 to 4 times higher than nuclear DNA and is accompanied by the pronounced inhibition of messenger ribonucleic acid (mRNA) and protein synthetic activities (Niranjan and Avadhan, 1982). Backer and Weinstein (1980) showed that an epoxide derivative of benzopyrene similar to the AFB₁-8,9-epoxide, modified mitochondrial DNA to a greater extent than nuclear DNA (more binding of the epoxide to mitochondrial DNA residues). Their work further showed that when cells were exposed to the AFB₁-8,9-epoxide, the high uptake by mitochondrial membranes and the high lipid to DNA ratio of the mitochondria results in extensive modification of mitochondrial DNA by this carcinogen. This suggests that lipophilic carcinogens have a high affinity for mitochondrial membranes which may explain the altered mitochondrial morphology observed in AFB₁ and AFB₁-8,9-epoxide treated cells. The presence of 5 to 10 nm gold probes in close proximity to and within the mitochondria is suggestive of the toxic effects elicited by the toxins resulting in altered mitochondrial architecture. The presence of toxin within the mitochondria suggests that it is one of the organelles of attack. It is possible that the occurrence of CP₄₅₀ systems within the mitochondria is suggestive of its role in biotransformation of AFB₁ into the AFB₁-8,9-epoxide.

In several models of experimental cell injury, changes in the ER have in fact been considered as constituting some of the earliest cytological changes (Trump *et al.*, 1965). The swelling of the rough ER accompanied by degranulation in hepatocytes has been documented in previous studies where experimental animals were exposed to various noxious agents. Such alterations are known to hinder protein synthesis. AFB₁ is known to inhibit protein synthesis, in a variety of ways, including the inhibition of RNA synthesis. The swelling of the ER may be linked to the storage of secretory products and/or the ingress of water due to an enzyme or mechanical defect in the rER (Ghadially, 1982). In this investigation, the most striking alteration in the rER was the decrease in the number of elongated profiles and a preponderance of short strands, distorted profiles, vesiculation or enlargement of the cisternae in both AFB₁ and AFB₁-8,9-epoxide treated cells. Another prominent feature was the presence of areas along the cisternae in which the membrane had been completely devoid of ribosomes. It has been shown that AFB₁ derivatives directly effect the functional activity of polysomes (collection of ribosomes) (Sarasin and Moule', 1973). From the results obtained it is evident that the AFB₁-8,9-epoxide treated cells showed more severely altered rER as compared to that of the AFB₁ treated cells. It is possible that since AFB₁ requires biotransformation into its derivatives to inhibit protein synthesis it does not immediately display a severe toxic effect while the synthetic AFB₁-8,9-epoxide has a pronounced effect on the ultrastructure of the rER negating its functioning in protein synthesis. It has been previously shown that the aflatoxin derived compounds inhibit *in vitro* amino acid incorporation by the polysomes, while AFB₁ is completely inactive *in vitro* (Sarasin and Moule', 1973).

In both the AFB₁ and AFB₁-8,9-epoxide treated cells the presence of areas of cytoplasmic lysis may be a consequence of the disintegration of swollen ER, rupture of lysosomal membranes or the action of lysosomal enzymes on pockets of glycogen. In addition the occurrence of focal cytoplasmic lysis within cytoplasmic blebs in epoxide treated cells is suggestive of the more pronounced cytotoxic effects of this derivative. Increased numbers of secondary lysosomes were also observed in treated cells. It is possible that AFB₁ and its epoxide are sequestered into the secondary lysosomes in an attempt to isolate and digest the toxins.

Both TEM and SEM showed the presence of numerous cytoplasmic blebs in toxin treated cells. Cytoplasmic blebbing on cell surfaces is an early reaction of many cells to injuries by cytotoxic compounds and is thought to occur as a result of microfilament disruption, prior to loss of cell viability (Eriksson *et al.*, 1989). Both AFB₁ and the AFB₁-8,9-epoxide presumably elicit blebs as a consequence of its disruption of metabolic processes necessary to maintain the integrity of microfilaments. It has been suggested that an inhibition of ATP production resulting in a lowering of the level of cellular ATP induces microfilament disruption (Eriksson *et al.*, 1989). It has been shown that the AFB₁-8,9-epoxide has a pronounced effect on the mitochondria which would result in an inhibition of ATP production. This may explain the subsequent formation of blebs. The presence of AFB₁ and AFB₁-8,9-epoxide within blebs was confirmed in this study by immunocytochemical localisation and SEM.

From the results obtained it is clear that the nucleolus is one of the sites of action of AFB₁ and the AFB₁-8,9-epoxide derivative. Irregular clumps of condensed chromatin, nucleolar segregation and nucleoli with an extensive fibrillar component have been observed in both AFB₁ and AFB₁-8,9-epoxide treated cells. In a previous study it was shown that a decrease in nuclear RNA content, microsegregation of nucleolar and an inhibition of RNA polymerase activity are all early effects of aflatoxin in liver (Edwards, 1971). It has been suggested that the inhibition of hepatocyte RNA polymerase activity following a dose of AFB₁, is due to an inhibitory effect of the toxin or a metabolite on some component of the nuclear chromatin. Nucleolar segregation induced by this toxin was extensively reviewed by Simard (1968) who suggested that nucleolar segregation reflects DNA binding and the subsequent inhibition of DNA-dependent RNA synthesis because of a loss of template activity of DNA.

AFB₁ treated cells showed the presence of peripheral chromatin which reflects an inhibition of protein synthesis. Epoxide treated cells displayed single or double distinctive fibrous centers which is also indicative of an inhibition of RNA synthesis (Ghadially, 1982). The presence of nucleoli with enlarged fibrillar component, is reported to be characteristic of an inhibition of nucleolar RNA synthesis which is known to be a consequence of the inhibitory effect of AFB₁ on the synthesis of the 60S subunit of

ribosomes (Moule, 1971). Moule (1971) has suggested that AFB₁ impairs the normal molecular processing of 45S RNA which would produce 40S and 60S subunits. It has been postulated that 45S RNA is associated with the fibrils of the nucleolus and the pathway of RNA maturation is from the template to the fibrillar component and then to the granular component of the nucleolus (Pong and Wogan, 1970).

AFB₂ caused no significant alterations in the hepatocyte nuclear and nucleolar fine structure. It has been reported that AFB₂ is at least 350 times less active as an inhibitor of RNA polymerase than AFB₁ within 3 hours of dosing and a slight microsegregation was reported in the liver cell nucleolus following a large dose (200mg/kg) of AFB₂ (Edwards, 1971). Edwards (1971) showed that AFB₁ binds more strongly to DNA in the form of the 8,9 epoxide while AFB₂ needs to be biotransformed into AFB₁ in order to exert any biological effect (Chapter 2).

There is good correlation between ultrastructural pathology and localisation of AFB₁ in organelles. The presence of label in the nucleus may represent the binding of the highly reactive epoxide to DNA forming the DNA adducts. It is now well established that the formation of the AFB₁-DNA adduct is implicated in tumour formation but the mechanism by which AFB₁ or its derivative is translocated into the nucleus is still unclear. However cytosolic receptors for lipophilic carcinogens such as AFB₁ have been shown in tumorous cells. Mainigi and Sorof (1977) showed that AFB₁ gives rise to relatively high levels of DNA adducts and a wide variety of protein adducts. They also postulated that AFB₁ translocates across the nuclear pore as a ligand-protein complex by binding to proteins destined for the nucleus (histones) thus increasing its uptake, activation (nuclear CP₄₅₀) and the subsequent damage to DNA leading to an increased risk of developing cancer. The nucleus is clearly one of the target organs of these toxin treatments. This is also reflected in the CLSM results which shows that the nucleus with the nucleolus in particular is the target site for AFB₁. It (CSLM) also shows a progressive sequence of movements of the toxin from the primary site (nucleolus) after 4 hour exposure period to the rest of the cell after 24 hours at 12.5µg/ml.

CONCLUSION

TEM, SEM and ICC showed that both AFB₁ and the AFB₁-8,9-epoxide induce cellular alterations which ultimately leads to cell death. However the severity of the cytotoxic effects was more pronounced in the synthetic AFB₁-8,9-epoxide treated cells. The synthetic AFB₁-8,9-epoxide was found to adversely alter the ultrastructural pathology of the mitochondria and nucleus. The cytotoxicity of the biotransformed AFB₁ into the highly reactive metabolite is confirmed in this study by its *in vitro* pathological effects on cellular ultrastructure.

The use of CLSM in localising these mycotoxins based on their fluorescence is a novel idea, which is still in its preliminary stages. Further work on the other mycotoxins will be carried out.

Chapter 5

A COMPARISON OF THE CYTOTOXIC EFFECTS OF Zea AND ITS EPOXIDE DERIVATIVE ON CELL MORPHOLOGY

5.1 INTRODUCTION

Zearalenone, despite its structural dissimilarities with oestrogen, induces effects consistent with those produced by excessive steroid as well as synthetic oestrogen i.e. anabolic and uterotrophic activities. It has been extensively documented that the mode of action of Zea involves the interaction with oestrogen receptors, translocation of receptor-Zea complex to the nucleus, combination with chromatin receptors and selective RNA transcription leading to altered biochemical effects (Reddy and Hayes, 1989). Over three hundred derivatives of Zea have been synthesised, some commercially as anabolic agents to improve growth rates and feed efficiency. Zearanol is one of the synthetic compounds used commercially as an anabolic agent (Goodman *et al.*, 1987).

Many of the reports pertaining to these compounds have relied on the use of animal models to assess oestrogenicity, therefore raising the complication of possible species differences in their hormonal activity. It has been discovered that oestrogen receptors do exist in the cytosol of hepatocytes. This discovery has stimulated research attempting to define a possible role of oestrogen receptors in the pathogenesis of hepatic disorders. Recent experimental evidence has suggested that the liver is the target organ for sex hormones (Eagon *et al.*, 1991). The oestrogen may play a role in the regulation of liver regeneration in that there is a strong temporal relationship between the increase in hepatic DNA synthesis and the total oestrogen receptor content of the liver (Eagon *et al.*, 1991). This would therefore imply that Zea would have an effect on the liver.

A zea epoxide synthesised in our laboratory has been assessed for its possible role in the metabolism of Zea. As with the AFB₁-8,9-epoxide which is highly reactive and unstable we have attempted for the first time to produce an epoxide similar to the AFB₁-8,9-epoxide to compare its possible oestrogenic properties with that of Zea (Chapter 3).

The purpose of this study was to investigate the possible oestrogenic activity of Zea and its epoxide derivative using HepG2 cells in culture. Transmission electron microscopy was employed to compare the ultrastructural effects of Zea and its derivative while immunocytochemistry was used in an attempt to localise the toxins in the cell.

5.2 MATERIALS AND METHODS

5.2.1 Mycotoxin treatment

Confluent flasks (25cm³) of HepG2 cells were exposed to Zea (12µg/ml) and its epoxide derivative (12µg/ml) for 24 hours. Following this exposure period cells were processed for TEM and ICC (as in Chapter 4). The microscopic techniques employed are summarised below.

5.2.2 Transmission electron microscopy & Immunocytochemistry

All ICC consumables were purchased from Sigma. The primary antibody used was a polyclonal (rabbit anti-Zea) (1:100 dilution), while the secondary antibody was goat anti rabbit IgG conjugated to a 5 to 10nm gold probe. Sections were viewed using a Joel-JEM 100S transmission electron microscope.

Table 14 A summary of the steps involved in TEM and ICC

	TEM	ICC
Materials	Cells grown in 25cm ³ culture flasks	Cells grown in 25cm ³ culture flasks
Fixation	1.5% gluteraldehyde	1.5% gluteraldehyde
Post fixation	Osmium tetroxide	-
Dehydration	70%, 90%, 100% alcohol	70%, 90%, 100% alcohol
Resin embedding	Araldite	Araldite
Ultramicrotomy	Copper grids	Nickel grids
Stain/Probe	Uranyl acetate /Lead citrate	IgG gold

5.3 RESULTS

5.3.1 Transmission electron microscopy

Distinct cellular alterations were observed in both Zea and Zea-epoxide treated cells. Ultrastructural alterations observed are summarised in table 14.

Table 15 A comparison of the ultrastructural alterations observed in Zea and Zea epoxide treated cells.

Zearalenone	Zearalenone epoxide
Endoplasmic reticulum(ER)	
Numerous cisternae of rough ER (rER).	Few cisternae of rER
Slight swelling of rER	Grossly swollen rER with a loss of ribosomes
Mitochondria	
Few swollen mitochondria	Few swollen mitochondria and occasional elongated forms
Slight change in cristae orientation- Some with cristae orientated parallel to longitudinal axis of mitochondria	Slight change in cristae orientation. Few cristae lying parallel to longitudinal axis of mitochondria
Often enveloped by ER	Often enveloped by ER
Nucleus	
Single nucleus with regular nuclear membranes	Multiple nuclei with invaginating nuclear membranes
Microsegregation of nucleolus	Absence of microsegregation
Very few small lysosomes	Several lysosomes
A few cytoplasmic blebs with lysis	Numerous cytoplasmic blebs with focal areas of cytoplasmic lysis
No cytoplasmic lysis	Areas of cytoplasmic lysis

The organelles most greatly affected by Zea and its epoxide derivative were the ER, nucleus and mitochondria. The Zea treated cells showed numerous rER often enveloping the mitochondria (Fig 5.1). The zea-epoxide treated cells displayed few rER and grossly altered rER which were swollen and showed a loss of ribosomes (Fig 5.2). Zea treated cells showed slightly swollen and vessiculated rER with many intact ER (Fig 5.3).

Nuclear alterations in Zea treated cells were an abundance of euchromatin and the microsegregation of the nucleolus which contained prominent fibrous centres (Fig 5.4). Epoxide treated cells showed the presence of multilobed nuclei and an absence of fibrous centers in the nucleoli (Fig 5.5).

In Zea treated cells mitochondria appeared normal with a slight disorientation of the cristae (Fig 5.6) while in epoxide treated cells mitochondria were elongated and showed a loss of cristae and membrane integrity (Fig 5.7). Both Zea and Zea-epoxide treated cells showed very few cytoplasmic blebs. In the Zea epoxide reaction however, a number of secondary lysosomes were present (Fig 5.8). Areas of cytoplasmic lysis were also observed (Fig 5.9).

5.3.2 Immunocytochemistry

Electron ICC results showed Zea and Zea epoxide to be localised in the nucleus, cytoplasm and within altered organelles. The following table illustrates the intensity of label in the altered organelles.

Table 16 Intensity of label in toxin treated cells

Organelles	Zearalenone	Zearalenone-epoxide
Nucleus	++	++
Cytoplasm	++	++
Swollen ER	++	++
Cytoplasmic blebs	+	+
Along cell membrane	+	+

+ few gold probes

++ several gold probes.

Gold labelled Zea and zea-epoxide was found on the nuclear membrane (Fig 5.10), in the nucleoplasm (Fig 5.10); in the nucleolus (Fig 5.11); in the swollen ER and in close proximity to it (Fig 5.12); through the nucleoplasm to the nucleolus (Fig 5.13); from the cytoplasm to the nucleus (Fig 5.14) and outside the cell, within cytoplasmic blebs (Fig 5.15).

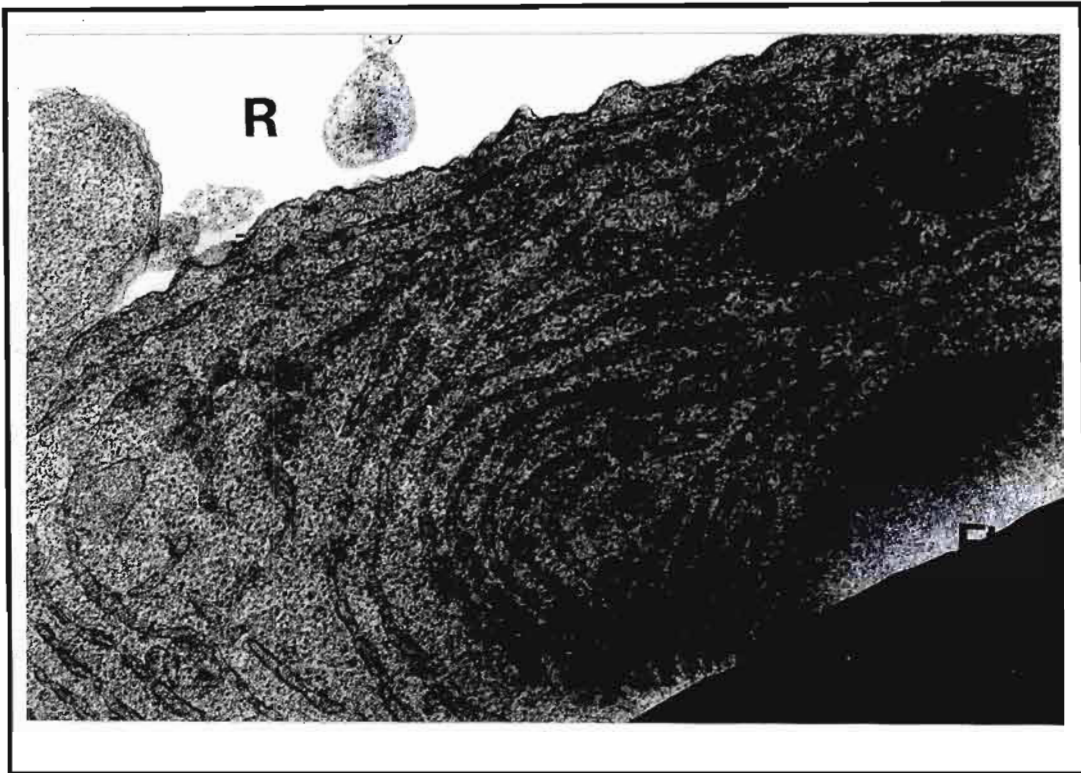


Fig 5.1 Electron micrograph of a Zea (12 μ g/ml) treated HepG2 cell showing the presence of numerous rough endoplasmic reticuli (rER), indicative of enhanced protein synthesis, often enveloping the mitochondria (M). The cell is sandwiched between the flask (Fl) and the resin (R).

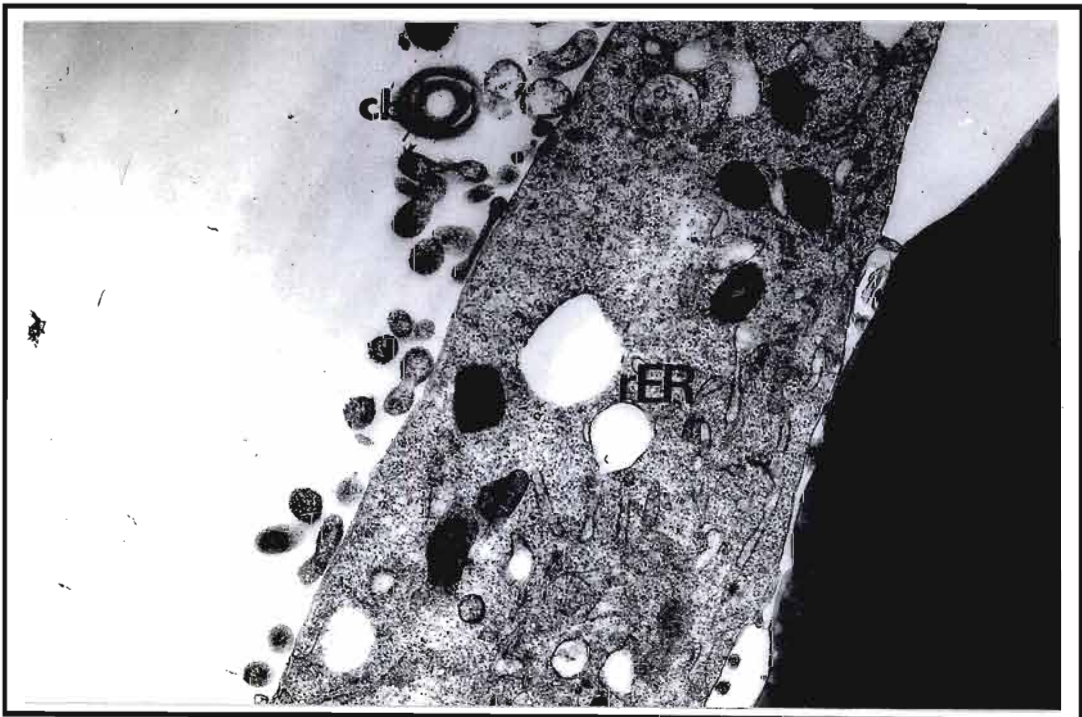


Fig 5.2 Electron micrograph of a Zea-epoxide (12 μ g/ml) treated cell showing grossly altered rER with a subsequent loss of ribosomes (r). There are also a few cytoplasmic blebs (Cb) accompanied with focal areas of cytoplasmic lysis (\uparrow).

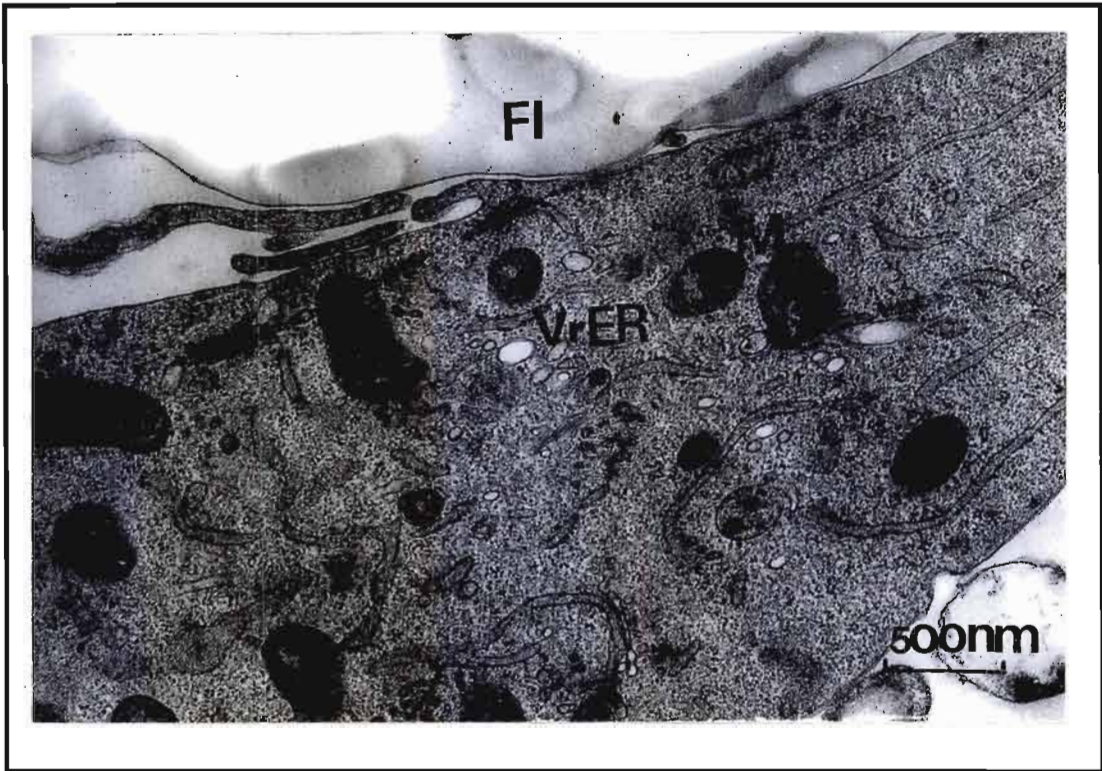


Fig 5.3 Electron micrograph of a *Zea* treated cell showing slightly swollen and vesiculated (V) rER, together with ovoid mitochondria (M). Flask (FL).

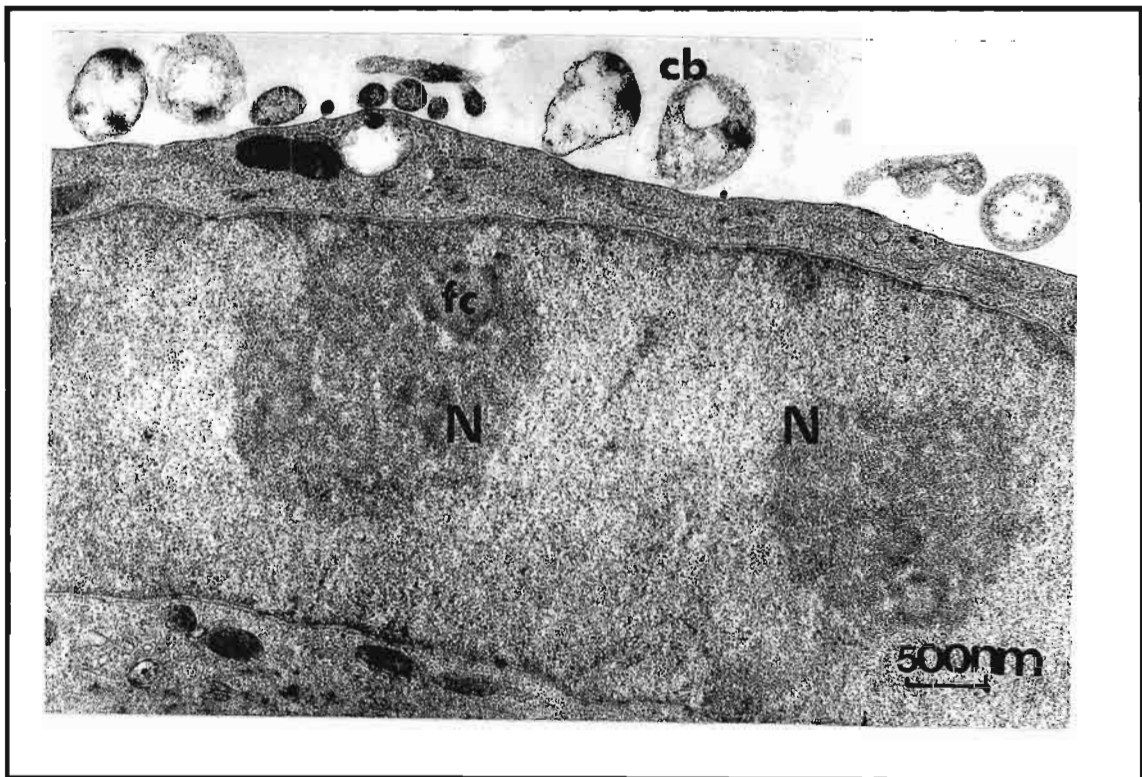


Fig 5.4 Electron micrograph of a *Zea* treated cell showing microsegregation of the nucleolus (N) and the presence of fibrous centers (Fc). Cytoplasmic blebs (Cb).

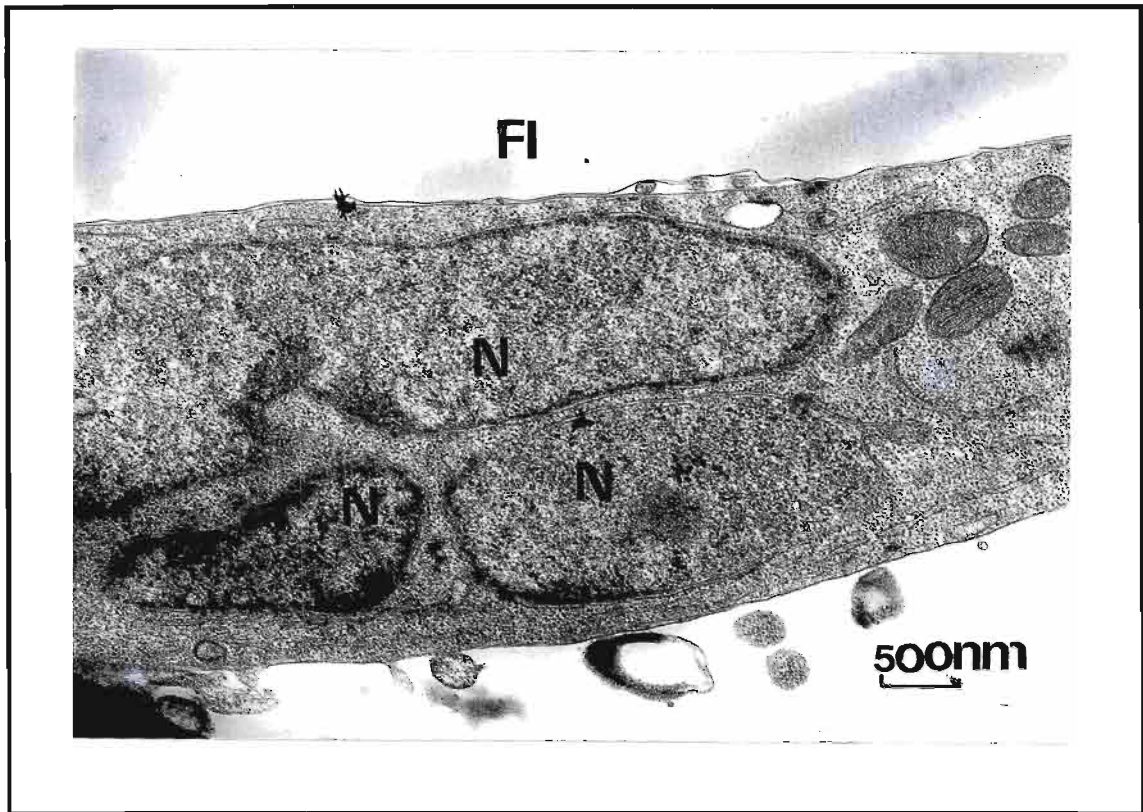


Fig 5.5 Electron micrograph of a Zea-epoxide treated cell exhibiting multi-lobed nuclei (N). Flask (Fl)

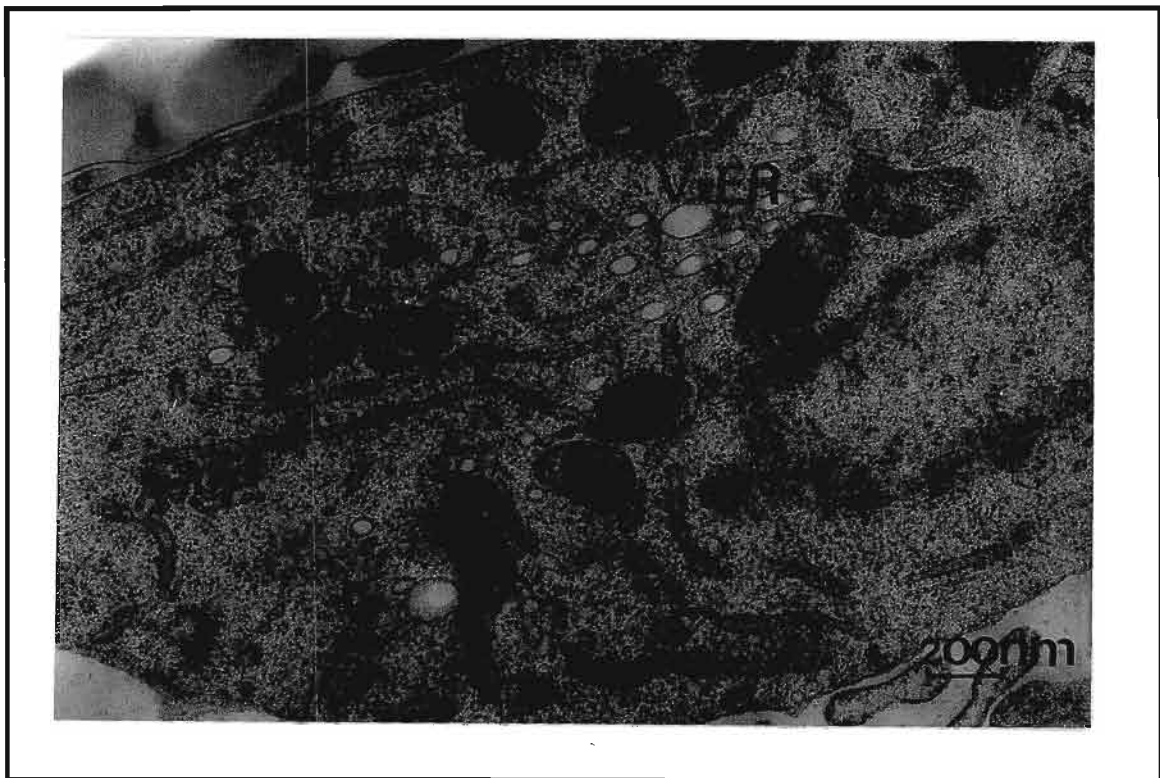


Fig 5.6 Electron micrograph of a Zea treated cell showing normal mitochondria (M) with slight disorientation of cristae and vesiculated rER.

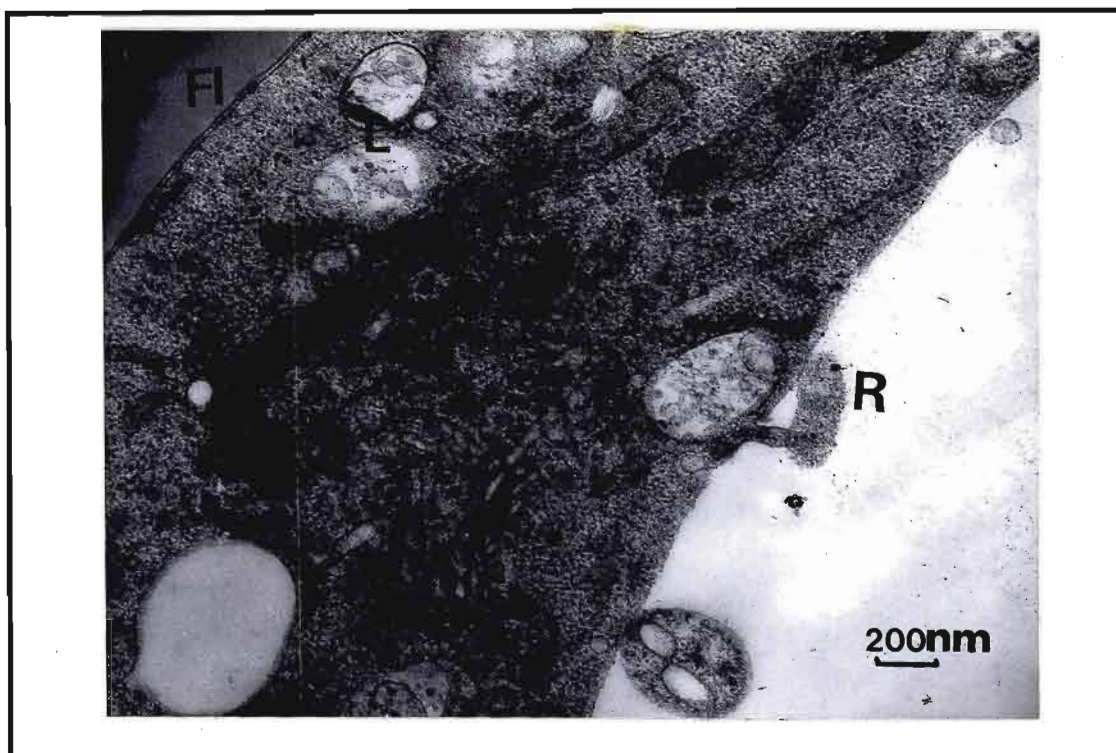


Fig 5.7 Electron micrograph of a Zea-epoxide treated cell showing elongation of mitochondria (M) and loss of membrane integrity. There are also secondary lysosomes (L) filled with proteinacious material. Flask (FI), Resin (R).



Fig 5.8 Electron micrograph of Zea treated cells showing the presence of a few cytoplasmic blebs (Cb), a desmosome(D) (tight junction between neighbouring cells), and numerous ovoid mitochondria (M).

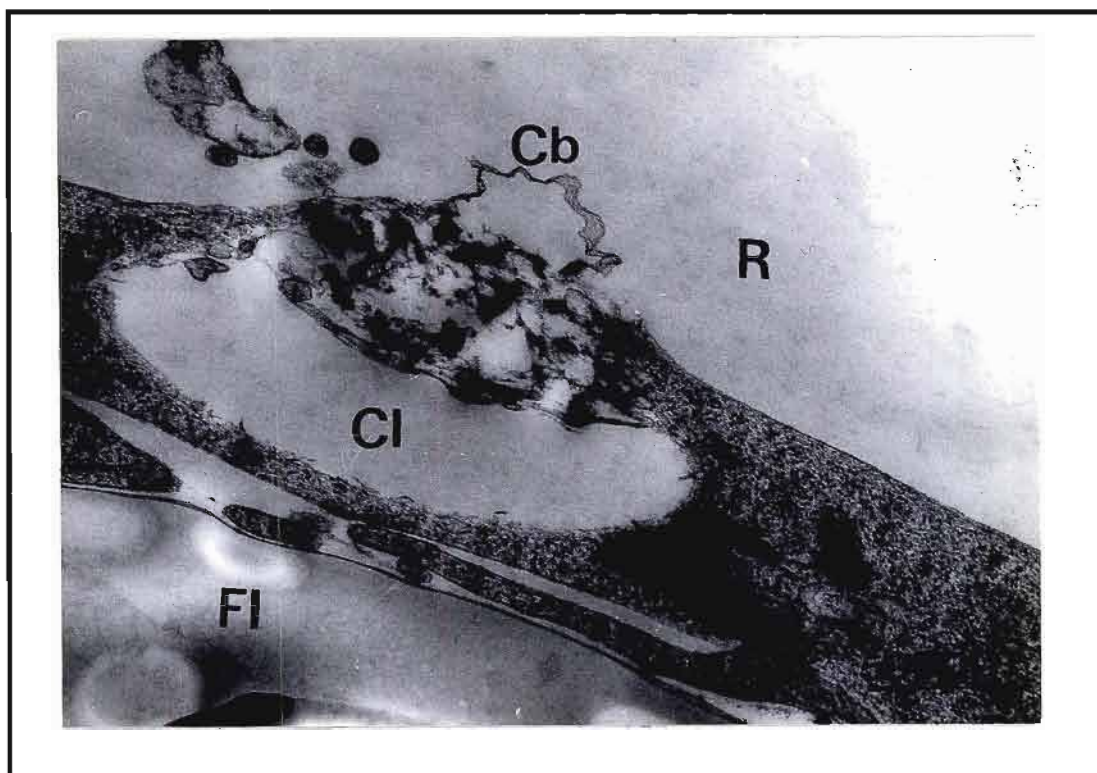


Fig 5.9 Electron micrograph of a Zea-epoxide treated cell showing the gross alteration of the cytoplasm into areas of lysis (Cl) and the subsequent formation of the cytoplasmic blebs (Cb). Flask (Fl), Resin (R).

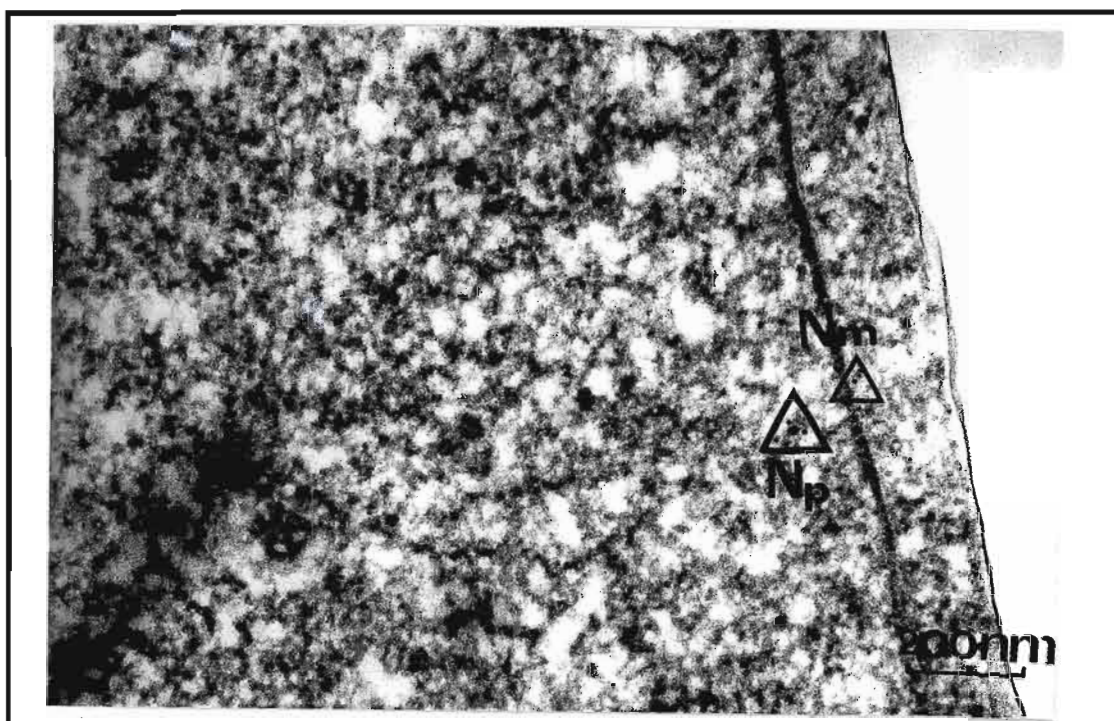


Fig 5.10 Immunocytochemical (ICC) electron micrograph of a Zea treated cell showing the presence of gold-labelled anti-Zea as 10nm gold probes along the nuclear membrane (Nm) and within the nucleoplasm (Np).

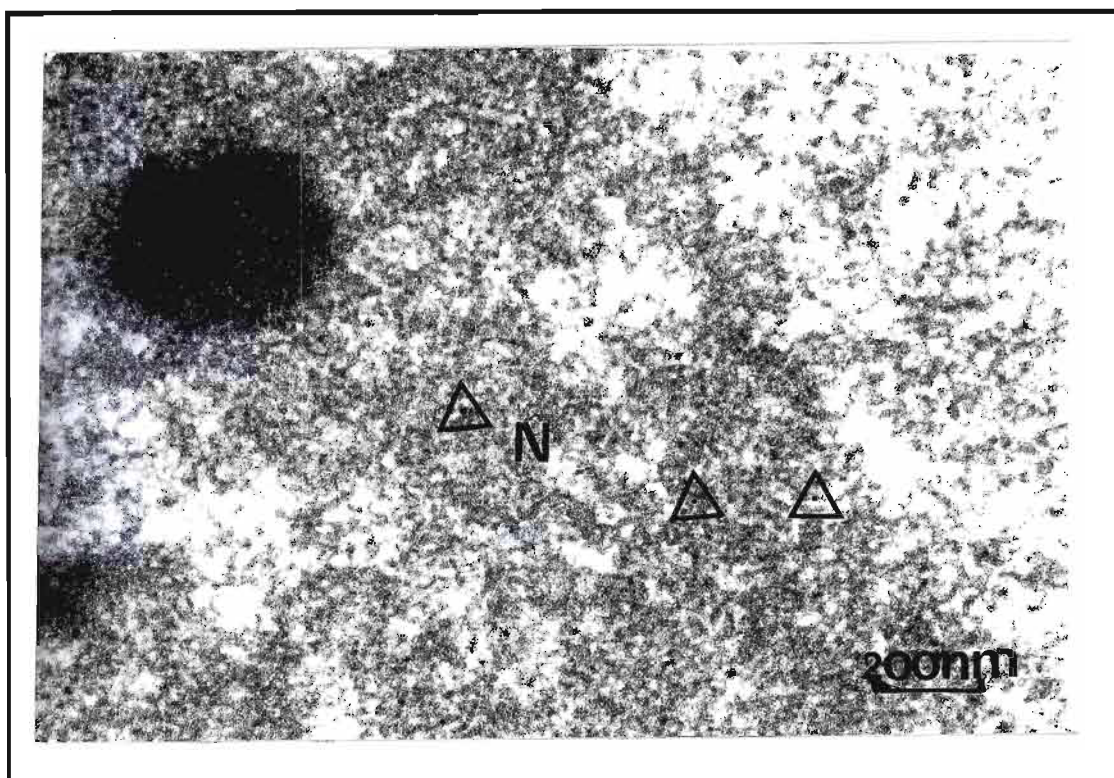


Fig 5.11 ICC electron micrograph of a Zea treated cell showing the presence of label within the nucleolus (N). The label is circled .

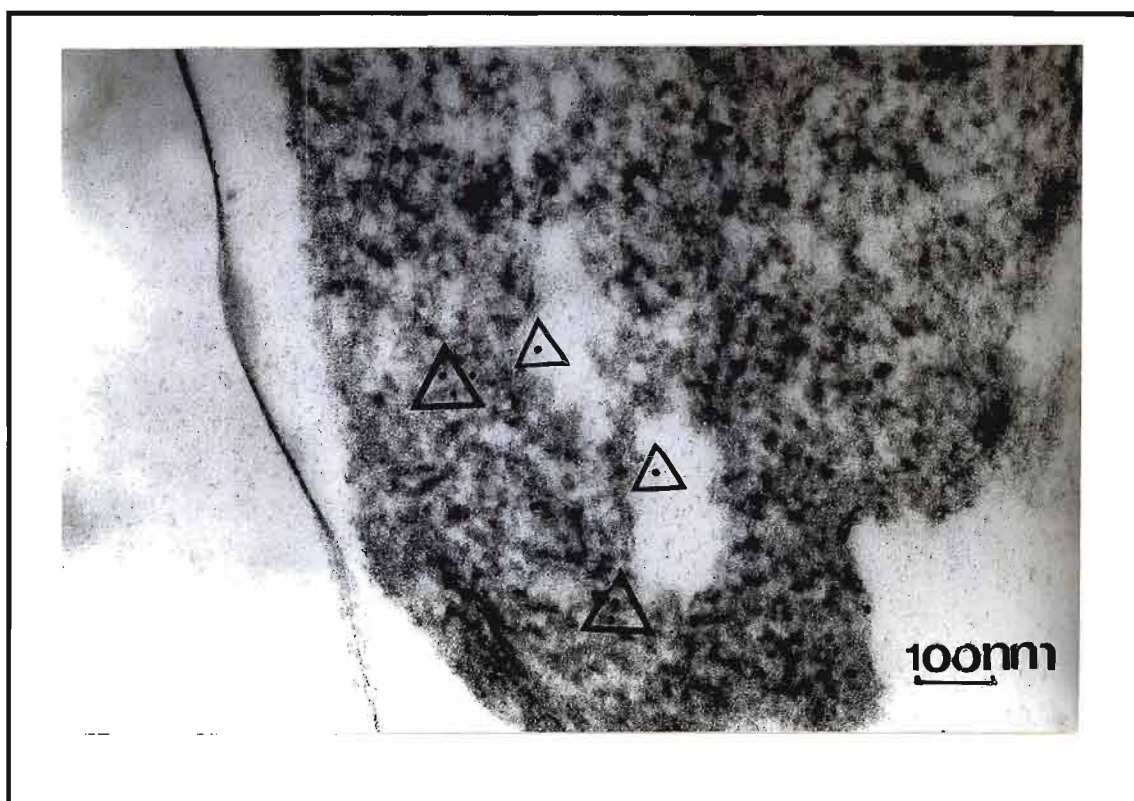


Fig 5.12 ICC electron micrograph of a Zea-epoxide treated cell showing the presence of label in the swollen Er and in close proximity to it.

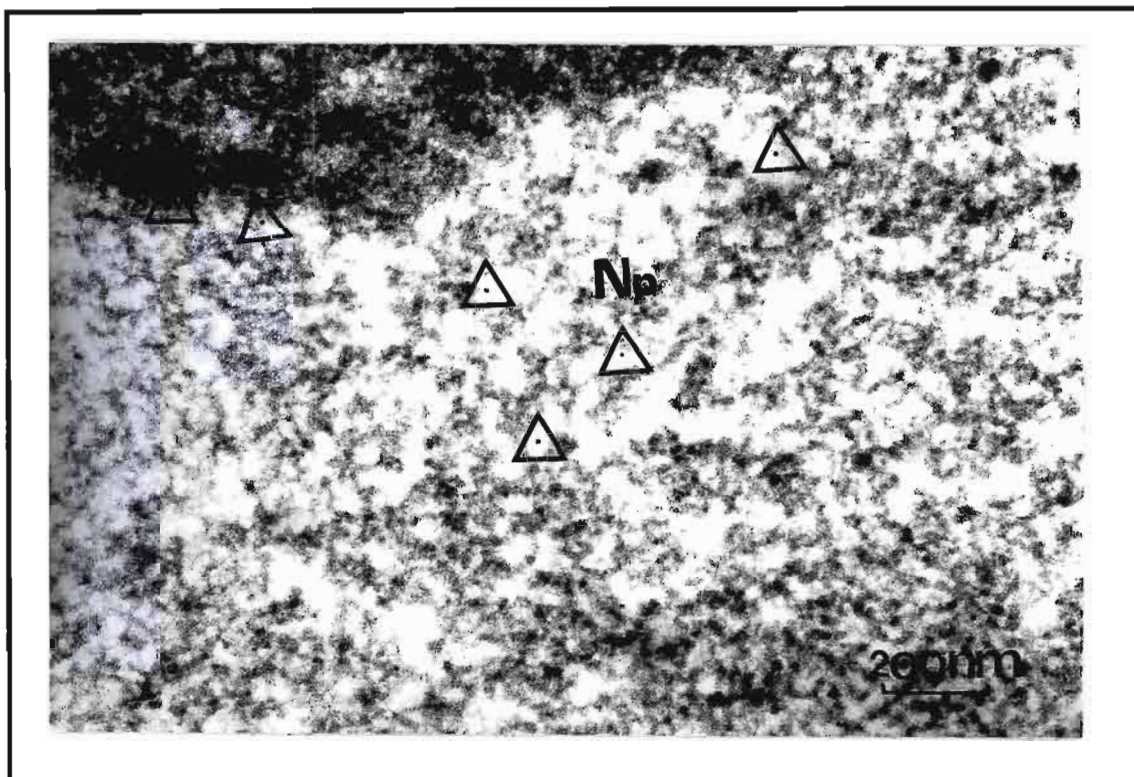


Fig 5.13 ICC electron micrograph of a Zea-epoxide treated cell showing the presence of label in the nucleoplasm (Np) and within the nucleolus (N).

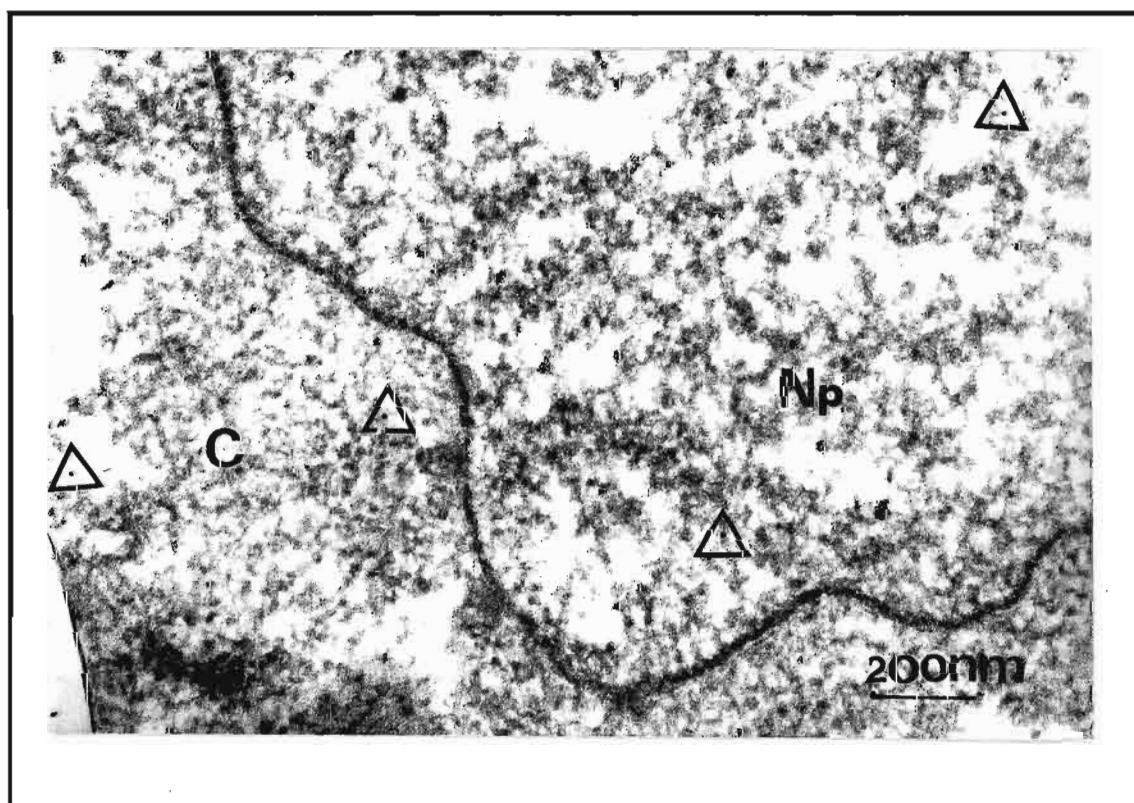


Fig 5.14 ICC electron micrograph of a Zea-epoxide treated cell showing the presence of label in the cytoplasm (C) and within the nucleoplasm (Np).

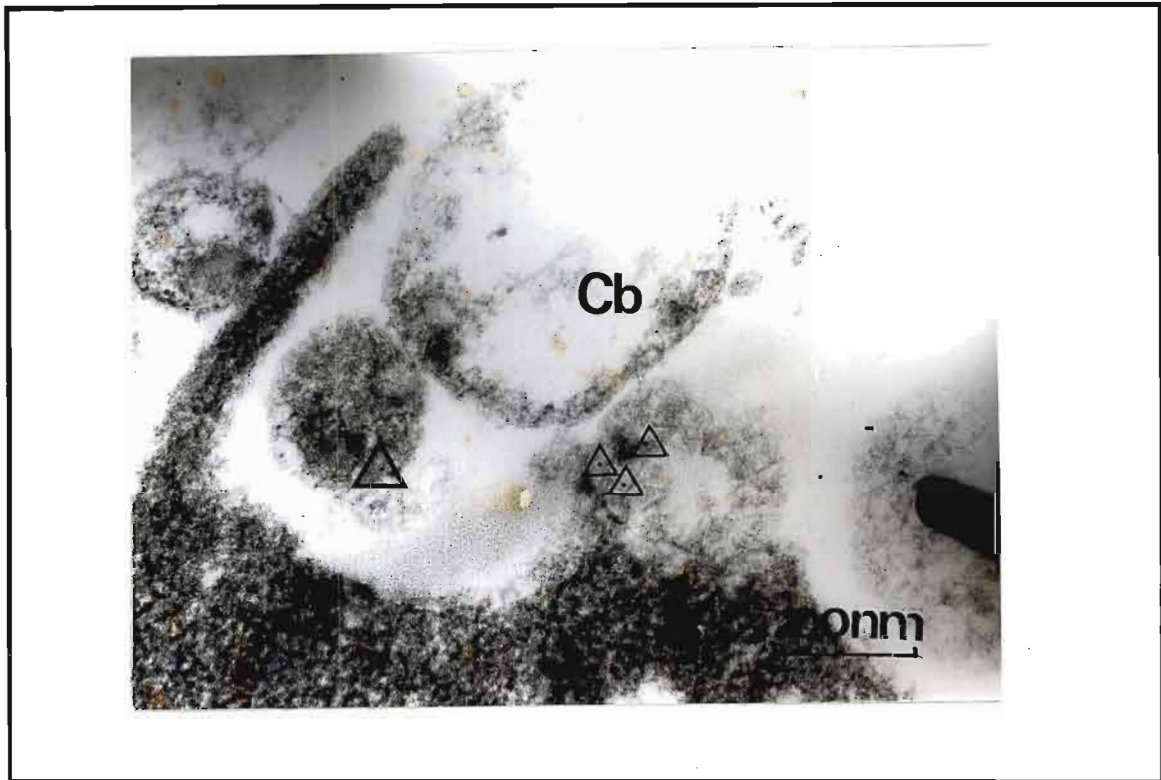


Fig 5.15 ICC electron micrograph of a Zea-epoxide treated cell showing the presence of label within cytoplasmic blebs (Cb).

5.4 Discussion

There have been many reports of compounds that mimic oestrogenic steroids particularly 17 β -oestradiol. These claims have been substantiated by studies of the hormonal effects of these compounds in well established animal or cell culture assays. A survey of literature on the topic of oestrogenic principles found in plants and fungi suggested a list of 15 naturally occurring nonsteroidal compounds which represent putative agonists for the oestrogen receptor (Miksicek, 1994). One of these compounds is Zea which was found to interact with human oestrogen receptors by competing with 17 β -oestradiol.

TEM showed a variety of ultrastructural alterations which is in keeping with various documented results on the oestrogenic activity of Zea. The nucleus was found to be one of the organelles most affected by both toxic treatments. The altered ultrastructure and the presence of abundant label in the nucleus would suggest a possible DNA adduct formation. The biological effect of oestrogens in target tissues depend on the presence of cytoplasmic receptors. The steroid-receptor complex translocates into the nucleus, binds to the DNA template and in turn stimulates cellular RNA and protein synthesis. The binding affinity of the receptor for oestrogenic molecules is extremely strong and specific. However nonsteroidal compounds like Zea bind to oestrogen receptors and exhibit either agonistic or antagonistic effects of oestrogenic function (Kiang *et al.*, 1978). From the results obtained, Zea displays an agonistic feature by promoting cell growth. A limited number of genotoxicity assays have been conducted with Zea. It was found that Zea and its oestrogenic metabolites showed a positive DNA damaging effect in recombination tests with *Bacillus subtilis* (Leszkomicz *et al.*, 1995). Its ability to cause DNA damage in both microbial and mammalian assays indicated that Zea is possibly weakly genotoxic. The epoxide derivative however displayed antagonistic effects of oestrogenic function by reducing cell growth.

The microsegregation of the nucleolus in both toxin treated cells suggest that the toxins inhibit DNA-dependent RNA synthesis (Ghadially, 1982). However the toxic effects of the Zea derivative is much greater than that of Zea. It probably binds to DNA, inducing mutations that limits cell growth. However in order for Zea to form an adduct it

must be metabolised to electrophilic compounds. These electrophilic compounds are the reduced products, α and β -zearalenol (Goodman *et al.*, 1987), the reduction of which is catalysed by microsomal and cytoplasmic fractions which is normally housed in the ER and the mitochondria.

The ER was also greatly affected by the toxic insults but the severity of the cellular alteration was greater in the epoxide treated group of cells. These cells showed various stages of swelling of the ER; ranging from slightly to grossly swollen. The increased swelling was associated with a loss of ribosomes. The disorganisation of the rER (dilatation, vesiculation and disaggregation of polyribosomes) in epoxide treated cells would imply that it disturbs protein synthesis since the ER is the site of protein synthesis. The phenomenon of degranulation was observed in both toxin treated cells but was severe in the epoxide treated group. It has been suggested that degranulation is an early, relatively mild lesion (Ghadially, 1982). However in epoxide treated cells drastic alterations such as vesiculation and dissolution of membranes of the rER were pronounced. This would suggest that the epoxide is highly toxic altering the architecture of the ER and subsequently protein synthesis. The Zea treated cells however displayed slight swelling which may be a consequence of Zea or its more oestrogenic derivatives like zearalenol (Kiessling *et al.*, 1978) but induced the production of numerous rER which may be indicative of enhanced protein synthesis. From Chapter 3 it was found that there is a significant difference between the toxic effects of Zea and its epoxide derivative with the latter being highly toxic at 12 μ g/ml. The close association between rER and mitochondria is necessary for the provision of ATP by mitochondria to rER actively synthesising secretory products (enzymes in this case).

The mitochondria were also affected following the toxic treatment. Zea treated cells displayed numerous mitochondria which could imply enhanced ATP production required by the active cells. Epoxide treated cells however displayed mitochondrial elongation and more severe alterations such as a loss of membrane integrity, disorientation and dissolution of cristae. Ghadially (1982) has suggested that the presence of disintegrating cristae is an indication of a noxious effect on the mitochondrion. The disorientation of the cristae is related to a deficiency of cytochrome oxidase in the mitochondrion which is essential in the production of ATP (Ghadially, 1982).

Mitochondrial damage is reported to suppress ATP production which in turn leads to a failure of the ATP-dependent Na^+ pump at the cell membrane, resulting in cellular compartments becoming flooded with water. Increased cellular alterations in the epoxide treated cells, therefore, may be attributed to insufficient ATP levels by defective mitochondria.

5.5 Conclusion

It is clear that the synthetic epoxide derivative of Zea does not display any oestrogenic properties in the liver associated with enhanced protein synthesis but displayed antagonistic features of oestrogen function. It was shown to be a highly toxigenic compound severely altering the essential organelles required for cell survival. Zea on the other hand displayed very little toxic effects on the cells in culture. Instead it appeared to promote protein synthesis in these human hepatoma cells which is in accordance with documented results on other cell lines with high oestrogenic receptor content (Goodman *et al.*, 1987). The Zea epoxide has not been isolated as a naturally occurring compound and it is unlikely, if it is a highly reactive and unstable compound. One can postulate that its inability to display oestrogenic properties may be attributed to a possible covalent linkage with the oestrogen receptors thus stabilising the compound. However the Zea-epoxide is a good model to compare with the activity of Zea.

CHAPTER 6

CONCLUSION

To date, much progress has been made in elucidating the biochemical and molecular mechanisms that underlie aflatoxin carcinogenesis. A substantial wealth of *in vitro* biochemical data that underlie aflatoxin metabolism suggests that humans are sensitive to the carcinogenic effects of aflatoxin. Clearly, the use of *in vitro* models has provided us with a greater understanding of aflatoxins and their cytotoxic effects on living systems.

The evidence that incriminates aflatoxins as human carcinogens includes: the production of liver cancer in laboratory animals in a dose dependent manner; the presence of this toxin in animal and human foodstuff; physical characteristics such as heat stability which suggests their presence in foods ready for digestion; their presence in areas of the world in which the incidence of liver cancer is high and finally correlative studies show a significant relationship (Linsell, 1979). The toxic properties of these toxins is also another important factor since a number of reports of acute toxicity in man, some of endemic proportions with high mortalities has come from Asia and Africa.

At present, the mechanism of AFB₁ hepatotoxicity is yet to be fully elucidated. Direct covalent binding between activated metabolism and cellular macromolecules such as DNA, RNA and proteins has been proposed as a major event in its hepatotoxicity. From the results obtained it can be concluded that the localisation of the mycotoxins in specific organelles such as the nucleus, endoplasmic reticulum and mitochondria is indicative of its sites of action and subsequent interference with cellular metabolism. The use of innovative techniques such as confocal laser scanning microscopy and immunocytochemistry to localise the toxins within the cell makes the results obtained reliable and valid.

The use of cells in culture to assess the cytotoxicity of the mycotoxins and their epoxide derivatives proved very effective together with the use of the MTT assay. The

results obtained suggested that both parent toxins (AFB₁ and Zea) were less toxic than their epoxide derivatives. These results correlated with the microscopy analyses which showed the severity of cellular alterations in the epoxide treated groups of cells. TEM showed the nucleus, ER and mitochondria to be the most affected organelles which are important in ensuring the normal functioning of the cell.

Zea has received little attention as compared to aflatoxins because of its speculative role in human disease. One cannot ignore the fact that it exists as a potential carcinogen and needs a more in-depth study of its biochemical mechanisms. This investigation forms the basis of future studies which includes the isolation of oestrogen receptors, characterisation of its binding to Zea and the subsequent biological effects, using *in vitro* assays. More investigations *in vitro* and *in vivo* are necessary to determine how Zea metabolism in different tissues leads to DNA adducts, the nature of modified bases and the nature of the compounds bound to DNA.

One cannot lose sight of the root of the problem of mycotoxins. Clearly a preventive measure would be improved husbandry, improved methods in harvesting, drying and storage of crops in defined areas. Such measures are necessary to protect the food supply from contamination of moulds which is an essential goal for protecting the health of humans and animals.

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APPENDIX

Appendix 1

Table 17 The constituents of the cell media (adapted from the Biowhittaker: Biotechnology products catalogue and technical manual, 1993).

Component	EMEM (mg/l)	HBSS (mg/l)	Earle's salts (mg/l)
Inorganic salts			
CaCl ₂ . 2H ₂ O	186.00	186.00	265.00
KCl	400.00	400.00	400.00
KH ₂ PO ₄	60.00	60.00	
MgSO ₄ . 7H ₂ O	200.00	200.00	200.00
NaCl	8.000.00	8.000.00	6.800.00
NaHCO ₃	350.00	350.00	2.200.00
Na ₂ HPO ₄ . 7H ₂ O	90.00	90.00	140.00
Other components			
Glucose	1.000.00	1.000.00	1.000.00
Phenol red	20.00	20.00	10.00
Amino acids			
L-Arginine-HCl	126.40		
L-cysteine	24.00		
L-Histidine-HCl.H ₂ O	42.00		
L-isoleucine	52.40		
L-Leucine	52.40		
L-Lysine.HCl	73.00		
L-methionine	15.00		
L-Phenylalanine	33.00		
L-threonine	47.60		
L-tryptophan	10.20		
L-tyrosine	36.20		
L-valine	46.80		
Vitamins			
D-CaPantothenate	1.00		
Choline Chloride	1.00		
Folic acid	1.00		
i-Inositol	2.00		
Nicotinamide	1.00		
Pyridoxine	1.00		
Riboflavin	0.10		
thiamine.HCl	1.00		

Appendix 2

The routine procedure in cell maintenance in a culture environment

At the beginning of each working session, hands were thoroughly washed and the laminar flow was swabbed down with a 70% ethanol solution. Before anything was introduced into the laminar flow it was swabbed down with the ethanol solution. Flasks containing growing cells were examined using an inverted microscope (Zeiss) and the condition of the cells were noted.

PAPERS AND PRESENTATIONS

PAPERS

Pillay D, Bux S, Chuturgoon A, Dutton MF. Effects of selected mycotoxins using human cell cultures. *Electron Microscopy Society of Southern Africa-Proceedings 1995*; 25:42.

Pillay D, Bux S, Chuturgoon A, Dutton MF. The cytotoxicity of Zearalenone and its epoxide derivative using Hep G2 cells. *Microscopy Society of Southern Africa-Proceedings 1996*; 26:74.

Pillay D, Bux S, Chuturgoon A, Dutton MF. The cytotoxic evaluation of Aflatoxin B₁ and the AFB₁-8,9-epoxide using the MTT assay. In Press.

PRESENTATIONS

Oral :

Pillay D, Bux S, Chuturgoon A, Dutton MF. The cytotoxicity of selected mycotoxins using human cells in culture. **Natal Biochemistry Symposium, University of Natal, Pietermaritzburg, October 1994.**

Pillay D, Chuturgoon A, Bux S, Dutton MF. Cytotoxicity testing using human cell lines. **The first annual Mini-Symposium of the Indigenous Plant Use programme, 1 November 1994.**

Pillay D, Bux S, Chuturgoon A, Dutton MF. The cytotoxic effects of Aflatoxin B₁ and the AFB₁-8,9-epoxide using HepG2 cells. **Natal Biochemistry Symposium, University of Zululand, Empangeni, October 1995.**

Pillay D, Bux S, Chuturgoon A, Dutton MF. The cytotoxic evaluation of selected mycotoxins using *in vitro* assays. **Faculty Research Day, Faculty of Medicine, University of Natal, Durban**, 6 September 1995.

Pillay D, Bux S, Chuturgoon A, Dutton MF. Effects of selected mycotoxins using human cell cultures. **Electron Microscopy Society of Southern Africa, University of the North, Aventura Resorts, Transvaal**, 4 December 1995.

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