E. METABOLISM AND BIOCHEMICAL EFFECTS OF AFLATOXINS

The biochemical nature of the toxic response elicited by aflatoxin B$_1$ is generally accepted to be as a result of metabolic activation (Campbell and Hayes, 1976) leading to interactions with nucleic acids and proteins. As the most potent hepatocarcinogen and hepatotoxin in several animal species, aflatoxin B$_1$ has been investigated much more than aflatoxins B$_2$, G$_1$ and G$_2$ which are considered less toxic. The metabolism of aflatoxin B$_1$ either to more or less toxic products, may occur through various pathways.

As with many foreign compounds, the principle enzyme system in aflatoxin metabolism is the cytochrome P-450-dependent mixed function oxidase (MFO) system, which is located in the endothelial reticular membrane system of the hepatocyte. The microsomal fraction, sometimes referred to as the S-9 fraction, of this system contains the MFO actively responsible for the Phase I reactions, so named since many of the products undergo further reactions.

Aflatoxin B$_1$ can be biotransformed in vivo by hepatic enzyme preparations into several metabolites (Fig. 1.2). The microsomal MFO may oxidize aflatoxin B$_1$, to aflatoxins M$_1$, B$_{2a}$, P$_1$, Q$_1$ and the aflatoxin B$_1$-epoxide while reductases in the cytoplasmic fraction can reduce aflatoxin B$_1$ to aflatoxicol. Though considered a detoxifying system, a few of the aflatoxin metabolites are still considerably toxic such as aflatoxin B$_1$ epoxide which has been considered an activated form of aflatoxin B$_1$. 
The hydroxylated products of the Phase I reactions are not the only metabolites of the MFO system. To enhance excretion, many of these products undergo Phase II reactions, in particular conjugation with endogenous compounds such as the active forms of glucuronic acid, sulphate, glutathione and methyl donors.

Aflatoxin M₁, the first aflatoxin metabolite identified, was initially observed in milk (Allcroft and Carnaghan, 1963). Toxicity data, as reviewed by Campbell and Hayes (1976), indicate that the acute toxicity of aflatoxin M₁ is equivalent to that of aflatoxin B₁ while its carcinogenicity, mutagenicity and cytotoxicity are considerably less. It was therefore suggested that this metabolite was not involved in causing the carcinogenic lesion, but possibly for the acutely toxic lesion.

Another product of the MFO system, aflatoxin B₂ₐ, has rarely been observed experimentally (Campbell and Hayes, 1976). Dann et al. (1972) have however observed aflatoxin B₂ₐ in the urine of rats administered aflatoxin B₁. It has been suggested that it represents a major metabolite (Gur too and Dahms, 1974; Patterson and Roberts, 1970) and may be rapidly removed either by protein adsorption to form a Schiff's base and/or by alcohols (Gur too and Campbell, 1974) during its extraction with organic solvents. Initially aflatoxin B₂ₐ was thought to be essentially non-toxic (Wogan, 1973) but it was later considered an activated form of aflatoxin B₁ responsible for the acute toxicity observed (Garner, 1976). However, the production of aflatoxin B₂ₐ has since been questioned (Neal et al., 1981). It was suggested that the earlier reports had mistakenly identified
aflatoxin \( B_{2a} \) for 2,3-dihydroxy - 2,3-dihydro - aflatoxin \( B_1 \) (\( AFB_1\)-dhd).

The reduction of aflatoxin \( B_1 \) to aflatoxicol has been shown to render it less toxic in ducklings (Detroy and Hesseltine, 1968) and \textit{Salmonella typhimurium} (Garner et al., 1972), though not in rainbow trout (Schoenhard et al., 1975). However, aflatoxicol still has carcinogenic acitivity (Schoenhard, 1974) and is the most potent mutagen of the aflatoxin \( B_1 \) metabolites (Wong and Hsieh, 1976). The possibility of a correlation between the metabolism of aflatoxin \( B_1 \) to aflatoxicol and species susceptibility to the acute and carcinogenic effects of aflatoxin \( B_1 \) has been reported (Edwards et al., 1975). According to this correlation, tests on human liver samples suggest that humans may be a relatively resistant species to both the acute and carcinogenic effects of aflatoxin \( B_1 \) (Hsieh et al., 1977).

The conversion of aflatoxin \( B_1 \) to aflatoxicol \textit{in vitro} has been shown to be catalyzed by a reversible reductase (Patterson and Roberts, 1972) which is NADH-dependent. This observed reversibility had led to the theory that aflatoxicol may serve as an intracellular reservoir of aflatoxin \( B_1 \), thus prolonging cellular exposure to aflatoxin \( B_1 \) and thereby enhancing its effects. The \textit{in vivo} biotransformation of aflatoxicol to aflatoxin \( B_1 \) was later also shown (Wong et al., 1979), proving furthermore the attractiveness of the 'intracellular reservoir of aflatoxin \( B_1 \)' theory.

Wong and Hsieh (1978) identified aflatoxicol as the major \textit{in vivo} metabolite in rat plasma when \( AFB_1 \) was administered either intravenously or orally. Concentrations were observed to be maximal
5 minutes after dosing. Recently it has been reported that in vitro interconversions of aflatoxin B₁ and aflatoxicol can be catalyzed by rat erythrocytes (Chang et al., 1985). It was thus suggested that since aflatoxin B₁ is carried in the blood from the GIT mucosa to target organs, transformation of aflatoxin B₁ in the hepatic tissues or target organ may be modified by the action of enzyme(s) present in the erythrocytes.

The recovery of aflatoxicol from rat plasma and not from mice and monkeys (Wong and Hsieh, 1980) which are resistant to the carcinogenic effects of aflatoxin B₁ supports the earlier proposed correlation (Edward et al., 1975) between species susceptibility to aflatoxin B₁ carcinogenicity and aflatoxicol-forming activity. Recently aflatoxicol has also been identified in human serum (Hendrickse et al., 1982), urine (Hendrickse, 1984) and liver (Lamplugh and Hendrickse, 1982). These were samples taken from PEM children.

The oxidation of aflatoxins B₁ to P₁ may be considered a detoxification step as aflatoxin P₁ is considerably less toxic (Buchi et al., 1973) as well as less mutagenic (Wong and Hsieh, 1976). Dalezios et al. (1971) reported it as the major urinary metabolite in monkeys given a single intraperitoneal injection of aflatoxin B₁. Aflatoxin, in this case represented 20 per cent of the injected aflatoxin B₁ of which 17 per cent was present as the glucuronide conjugate and 3 per cent as the sulphate conjugate. However, after oral administration of aflatoxin B₁, excretion as aflatoxin P₁ was considerably reduced, representing only 5 per cent of the administered dose of which 3.3 per cent was the glucuronide conjugate and 1.2
per cent the sulphate conjugate (Dalezios and Wogan, 1973).

Though in vitro studies with human liver microsomal preparations had demonstrated the production of aflatoxin P₁ (Merrill and Campbell, 1974b), in vivo studies were unable to demonstrate the excretion of aflatoxin P₁ and/or its conjugates (Merrill and Campbell, 1974a). From the available literature it appears that aflatoxin P₁ has not been detected as an in vivo metabolite in humans. Aflatoxin P₁ has however been reported to be a major metabolite in the mouse, in both in vitro (Hsieh et al., 1977) and in vivo (Wong and Hsieh, 1980) studies and as the mouse is considered relatively resistant to both toxic and carcinogenic effects of aflatoxin B₁, this does not correlate with human insensitivity.

Aflatoxin Q₁ considered a detoxification product of aflatoxin B₁ metabolism has been reported as the major in vitro metabolite in the hamster and monkey (Masri et al., 1974, Hsieh et al., 1977) as well as in humans (Buchi et al., 1974; Hsieh et al., 1977). Human liver in vitro preparations have been reported to metabolize aflatoxin B₁ rapidly to aflatoxin Q₁ (Moss and Neal, 1985). Thus if aflatoxin Q₁ production is a detoxification pathway then this suggests that this may reduce the susceptibility of an animal to aflatoxin B₁, hepatotoxicity. Though excretion of aflatoxin Q₁ has been reported in the urine of monkeys administered aflatoxin B₁ aflatoxin Q₁ was not observed as the free or conjugated form in the urine of humans known to have ingested between 11.7 and 30.1 ug aflatoxin B₁ over a 48 hour collection period (Campbell and Hayes, 1976). This in vivo production of aflatoxin Q₁ from aflatoxin B₁ has thus
not yet been established.

Aflatoxin $Q_1$ has been reported to be less toxic and less mutagenic than aflatoxin $B_1$ in the Ames Salmonella Mutagenicity Test and the Chicken Embryo Test (Hsieh et al., 1974; Gurtoo et al., 1978). However in Salmo gairdnerii (rainbow trout), known to be extremely susceptible to the acute and chronic effects of aflatoxins carcinogenicity has been observed (Hendricks et al., 1980). The observation that aflatoxin $Q_1$ has not been significantly detected in the mouse (Hsieh et al., 1977) as well as its carcinogenic effect in rainbow trout suggests that aflatoxin $Q_1$ might not be as innocuous as has been assumed. It has been reported that a significant amount of aflatoxin $Q_1$ is either bound strongly within the protein matrix and/or is metabolically degraded to yield fragments that do not absorb UV light (Rohrig and Yourtee, 1983) which would explain how it has not been detected as an in vivo metabolite.

A metabolite of great interest is the aflatoxin $B_1$-2,3-epoxide (sometimes called aflatoxin $B_1$-8,9-epoxide). The 2,3-vinyl ether bond of the aflatoxin molecule had been suspected as the possible determinant of its toxicity, carcinogenicity and biochemical effects due to covalent binding to nucleic acids and proteins to form the respective aflatoxin adducts. Swenson et al. (1973) produced the first direct evidence by showing that upon hydrolysis of aflatoxin $B_1$-RNA adducts the dihydriodiol, 2,3-dihydro-2,3-dihydroxy-aflatoxin $B_1$ (AFB$_1$-dhd) was produced. It was concluded that the 2,3-oxide of aflatoxin $B_1$ was the precursor of the dihydriodiol and therefore was the most likely candidate as the 'ultimate carcinogen'. This was
further supported by Garner (1973) who observed detectable amounts of the dihydriodiol in large scale incubation mixtures of liver microsomes and aflatoxin B₁. Mild acid hydrolysis of DNA and RNA adducts was shown to result in a derivative identified as aflatoxin B₁-dihydriodiol (AFB₁-dhd) (Swenson et al., 1974). The results indicated that 60 per cent of the nucleic acid adducts were derived from in vivo reactions with aflatoxin B₁-2,3-epoxide.

Attempts to isolate or synthesize aflatoxin B₁-2,3-epoxide have been unsuccessful, which suggests that the metabolite is highly reactive. Its formation can therefore only be postulated by the identification of AFB₁-dhd as well as the absolute structure of the primary adduct formed by the epoxide upon interaction with guanyl residues in DNA (Lin et al., 1977; Croy et al., 1978). There are thus three proposed routes for the degradation of the epoxide: (i) may be hydrated to form AFB₁-dhd (Colley and Neal, 1979); (ii) may be conjugated to form glucuronides sulphates (Campbell, 1977) or the glutathione (GSH) conjugate (Degen and Neumann, 1978); and (iii) due to its great reactivity may covalently bind to nucleophilic macromolecules such as DNA, RNA and protein (see Fig. 1.3). On examination of the first two deactivation steps, the results indicated strongly that the formation of AFB₁-dhd was the major detoxifying pathway rather than of AFB₁-GSH conjugate (Ch'ih et al., 1983). This agrees with earlier work by Emerole et al. (1979) which suggested that GSH was a less efficient nucleophile for aflatoxin B₁ metabolites. However, quantitation of aflatoxin B₁-DNA binding and aflatoxin B₁-GSH conjugation has been reported to indicate an inverse relationship
between these two processes (Lotlikar et al., 1984) i.e. inhibition of aflatoxin B₁-DNA binding was observed to occur with a concomitant increase in aflatoxin B₁-GSH conjugation.

The major aflatoxin-DNA adduct formed has been identified as 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxy-aflatoxin B₁ (AFB₁-N⁷-Gua) (Lin et al., 1977; Croy et al., 1978). The second most abundant adduct in DNA has recently been identified as 2,3-dihydro-2-(N⁵-formyl)-2′, 5′, 6′- triamino-4′-oxo-N⁵-pyrimidyl-3-hydroxy-aflatoxin B₁ (AFB₁ formamidopyrimidine) a derivative of AFB₁-N⁷-Gua formed in the intracellular environment (Essigmann et al., 1982). The absolute structures of these 2 adducts have been established as those in Fig. 1.3. In experiments with rats it was observed that AFB₁-N⁷-Gua was excreted in urine with 80 per cent of the total excretion of adducts occurring during a 48 hour period after dosing (Essigmann et al., 1982). Of the maximum amounts of adducts in the liver 30 - 40 per cent was excreted (Bennet et al., 1981). This work was extended to a study of human urine samples which showed an incidence of 7.4 per cent for the tentative presence of AFB₁-N⁷-Gua (Autrup et al., 1983). This therefore constitutes indirect proof of in vivo activation of aflatoxin B₁ and formation of the guanine duct by humans though in vitro human liver studies indicate that this may not occur to a large extent (Moss and Neal, 1985).

Hypothetically, all the aflatoxin that retain the 2,3-double bond are potentially capable of being activated through epoxidization. Essigman et al. (1982) has reported such formation of 2 adducts i.e. AFP₁-N⁷-Gua and AFM₁-N⁷-Gua, through the metabolic activation
of aflatoxins $P_1$ and $M_1$ respectively. It is generally thought that most DNA adducts are formed by attack of the $N^7$-atom of guanine on the 2,3-epoxide of aflatoxin $B_1$ or its metabolites.

It is now believed that AFB$_1$-dhd is the intermediate metabolite in the formation of AFB$_1$-protein adducts (Swenson et al., 1977; Ueno et al., 1980). The extent of formation of AFB$_1$-dhd has been shown to correlate with increased species susceptibility to the toxic and carcinogenic effects of aflatoxin $B_1$ in vivo (Neal et al., 1981). Through the formation of the AFB$_1$-protein adducts, protein synthesis is inhibited which therefore gives rise to the various biological effects observed in aflatoxin $B_1$ toxicity. Thus the presence of AFB$_1$-dhd indicates increased toxicity in the animal by aflatoxin $B_1$.

It has been shown that water-soluble AF conjugates account for a significant percentage of the total AF excreted in various animal species (Gregory et al., 1983; Raj & Lotlikar, 1984). Conjugation of the AF is possible if a hydroxyl group is present and then a form is presented which can be easily excreted. AF may be conjugated by glucuronic acid, sulphate or glutathione to form water-soluble conjugates. Levels of urinary water-soluble conjugates have been reported (Wong & Hsieh, 1980) to correlate with species susceptibility: more susceptible species such as the rat and monkey produced lower levels of conjugates than the mouse. Very few attempts have been made to detect the water-soluble conjugates in the urine of humans. Merrill and Campbell (1974a) and Campbell & Hayes (1976) failed in their attempts to detect conjugates of AFB$_1$ and AFQ$_1$, respectively, in the urine of humans who were known to have ingested AFB$_1$. It does appear that there is little information on water-soluble conjugates in serum samples from humans and even from animals.
F. AFLATOXIN TOXICITY IN ANIMALS

Acute and chronic toxic effects of the aflatoxins have been studied in great detail. Toxicity of the aflatoxins vary with aflatoxin $B_1$ being the most toxic followed by aflatoxins $B_2$, $G_1$ and $G_2$ in order of decreasing toxicity. The acute and chronic toxicity of aflatoxin $B_1$ has been emphasized on for experimental purposes, due to its greater toxicity.

The toxic effects of aflatoxin $B_1$, acute and chronic, vary between species as shown in Table 1.1. Acute toxicity of aflatoxins in animals is characterized by acute hepatitis, hemorrhagic disease and sudden death. In all species studied, the organ most severely affected is the liver, although other organs, particularly the kidney, show signs of damage (Newberne and Butler, 1969). It has been further observed that aflatoxin is the most potent hepatocarcinogen so far recognized. The variation in susceptibility to the carcinogenic effects of aflatoxin are shown in Table 1.2 (Linsell, 1982). Aflatoxin $B_1$ is the main hepatocarcinogen but $G_1$ and $B_2$ have been shown to cause cancers but with reduced potency. The potency of aflatoxin $M_1$ to induce liver tumors in rainbow trout is about 40 per cent that of aflatoxin $B_1$ (Smith and Moss, 1985). The general features of this toxicity is that the young appear to be more susceptible than mature animals (Newberne and Butler, 1969). Susceptibility is also influenced by sex (Newberne and Wogan, 1968), age (Wogan, 1966), environmental factors (Campbell et al., 1972)
<table>
<thead>
<tr>
<th>Species</th>
<th>LD$_{50}$(mg/kg Bodyweight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>0.3</td>
</tr>
<tr>
<td>Duckling (1 day old)</td>
<td>0.34</td>
</tr>
<tr>
<td>Cat</td>
<td>0.55</td>
</tr>
<tr>
<td>Pig</td>
<td>0.6</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>0.8</td>
</tr>
<tr>
<td>Dog</td>
<td>0.5 - 1.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.0 - 2.0</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1.4 - 2.0</td>
</tr>
<tr>
<td>Baboon</td>
<td>2.0</td>
</tr>
<tr>
<td>Chicken</td>
<td>6.3</td>
</tr>
<tr>
<td>Rat (male)</td>
<td>5.5 - 7.2</td>
</tr>
<tr>
<td>Rat (female)</td>
<td>17.9</td>
</tr>
<tr>
<td>Macaque (female)</td>
<td>7.8</td>
</tr>
<tr>
<td>Mouse</td>
<td>9.0</td>
</tr>
<tr>
<td>Hamster</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The LD$_{50}$ is the dose that will cause the death of 50 per cent of a statistically significant population. It is usually obtained by extrapolation from dose response experiments.
<table>
<thead>
<tr>
<th>Species</th>
<th>Dose</th>
<th>Duration of Observation</th>
<th>Tumor Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duck</td>
<td>30 µg/kg in diet</td>
<td>14 months</td>
<td>72%</td>
</tr>
<tr>
<td>Trout</td>
<td>8 µg/kg in diet</td>
<td>1 year</td>
<td>40%</td>
</tr>
<tr>
<td>Tree shrew</td>
<td>24 - 66 mg total</td>
<td>3 years</td>
<td>75%</td>
</tr>
<tr>
<td>Marmoset</td>
<td>5.0 mg total</td>
<td>2 years</td>
<td>65%</td>
</tr>
<tr>
<td>Monkey</td>
<td>100 - 800 mg total</td>
<td>Over 2 years</td>
<td>7%</td>
</tr>
<tr>
<td>Rat</td>
<td>100 µg/kg in diet</td>
<td>54 - 88 weeks</td>
<td>100%</td>
</tr>
<tr>
<td>Mice</td>
<td>150 µg/kg in diet</td>
<td>80 weeks</td>
<td>0%</td>
</tr>
</tbody>
</table>
and nutritional status (Newberne, 1976).

In the rat, aflatoxins appear to exert their effect on RNA polymerase as a consequence of covalent binding to DNA (Clifford and Rees, 1967; Pong and Wogan, 1970). It has since been reported that aflatoxin B₁ has to be metabolized to a reactive compound which inhibits RNA polymerase (Moule and Frayssinet, 1972). The mechanism of gene expression through transcription and translation suggests that alterations in RNA synthesis would be reflected by alterations in protein synthesis.

One of the features of aflatoxicosis is the presence of a fatty liver. Donaldson et al. (1972) demonstrated that while the lipid content of animal livers is increased the ability of the animals to synthesize lipids was decreased. This was demonstrated in ducklings, rats, humans and chickens. The three major classes of lipids transported in the blood from the liver where they are synthesized to other organs are triglycerides, phospholipids and cholesterol. These three classes have been reported to be decreased to the same extent and their levels in the blood are very sensitive to aflatoxin levels (Tung et al., 1972). This data was reconciled by the observation that aflatoxin affected lipid transport in the chicken to a greater extent than RNA content of the liver. It was later observed (Tung et al., 1975) that aflatoxin also affected levels of serum proteins. The most sensitive component was serum albumin which was decreased significantly at an aflatoxin consumption level of 0.625 μg/g of diet. A mixture of aflatoxins was used which was determined and found to consist of 71 per cent B₁, 8 per cent B₂, 16 per cent G₁ and 5 per cent G₂.

Experiments in primates have been carried out to determine
what the possible toxic effects are in a species closely related to man. Campbell and Stoloff (1974) in summarizing the experimental data available on primates have estimated that a daily dose of 0.05 mg/kg body weight would cause serious liver damage within a short period of time and 2.2 mg/kg body weight would result in death.

An interesting finding is that under certain circumstances aflatoxins may be beneficial. Aflatoxins have a well-defined anticoagulatory effect and this has been suggested as a possibility for therapeutic purposes. This feature is thought not be due to the generalized hepatotoxic effect but rather the result of aflatoxin competing with vitamin K (which is structurally similar) for the apoenzyme in production of prothrombin (Bababunmi et al., 1980).

It has recently been reported that infection of mice by Schistosoma mansoni reduces, in vivo and in vitro, the capability of metabolizing aflatoxin B$_1$ to the macromolecular binding species (Hasler et al., 1986). The possibility of schistosomiasis (bilharzia) enhancing the resistance of mice to the carcinogenic effects of aflatoxin B$_1$ was suggested. Hendrickse (1984; 1985) has reported that aflatoxin B$_1$, on the other hand, suppresses infection by the malaria organism Plasmodium berghei in mice. This protective effect of aflatoxin B$_1$ with regard to malaria is interesting, as the epidemiology of malaria and aflatoxin occurrence both concentrate in the tropic and sub-tropics.
G. AFLATOXIN TOXICITY IN HUMANS

Since the recognition of the acute, as well as chronic toxicity of the aflatoxins, much attention has been focused on the possible effects of these toxins on human health. Using toxicity data from experiments carried out in primates it has been estimated that a daily dose of 1.7 mg/kg would cause serious liver damage within a short period of time, while a single dose of 75 mg/kg would result in death (Campbell and Stoloff, 1974). The possible toxic effects of aflatoxins in man have been extrapolated from human incidents of aflatoxicosis. A summary of human incidents in which aflatoxins have been implicated or claimed to have an etiological role are summarized in Table 1.3.

There is therefore considerable evidence that aflatoxins are indeed dangerous to humans. Most of this evidence has centred around liver diseases as the liver appears to be the target organ for the toxic effects of aflatoxins. Thus aflatoxins are strongly associated with liver damage leading to diseases such as Encephalopathy and fatty degeneration of the viscera (EFDV) or Reye's Syndrome (Becroft and Webster, 1972), Indian Childhood Cirrhosis (Amla et al., 1971), outbreaks of acute heptatis (Krishnamachari et al., 1975; Ngindu et al., 1982) and quite recently Kwashiorkor (Hendrickse et al., 1982). Due to aflatoxin's carcinogenicity in many species of animals, it has also been incriminated strongly in the etiology of primary hepatocellular carcinoma (PHC).
<table>
<thead>
<tr>
<th>Year</th>
<th>Investigators</th>
<th>Country</th>
<th>Evidence for Aflatoxin consumption/involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1966</td>
<td>Payet et al.</td>
<td>Senegal</td>
<td>Groundnut meal 0.5 - 1.0 mg AFB$_1$/kg - chronic hepatitis</td>
</tr>
<tr>
<td>1970</td>
<td>Serck-Hanssen</td>
<td>Uganda</td>
<td>cassava 1.7 mg AFB$_1$/kg - death in one boy and siblings ill</td>
</tr>
<tr>
<td>1970</td>
<td>Alpert et al.</td>
<td>Uganda</td>
<td>aflatoxin contamination highest in areas with most incidence of hepatoma</td>
</tr>
<tr>
<td>1971</td>
<td>Amla et al.</td>
<td>India</td>
<td>groundnut meal 0.3 mg aflatoxin/kg - Indian Childhood Cirrhosis</td>
</tr>
<tr>
<td>1971</td>
<td>Bourgeois et al.</td>
<td>Thailand</td>
<td>rice 6 mg AFB$_1$/kg - EFDV/Reye's syndrome</td>
</tr>
<tr>
<td>1971 and 1976</td>
<td>Keen and Martin Peers et al.</td>
<td>Swaziland</td>
<td>areas with higher relative incidence of primary liver cancer have increased contamination of foods by aflatoxin - significant correlation</td>
</tr>
<tr>
<td>1971</td>
<td>Shank et al.</td>
<td>Thailand</td>
<td>AFB$_1$ found in 22/23 EFDV/Reye's syndrome cases</td>
</tr>
<tr>
<td>1972</td>
<td>Becroft and Webster</td>
<td>New Zealand</td>
<td>AFB$_1$-like substance found in 2 children who died of Reye's syndrome.</td>
</tr>
<tr>
<td>1972</td>
<td>Bosenberg</td>
<td>Germany</td>
<td>AFB$_1$ present in liver of patient who suddenly died with liver sclerosis</td>
</tr>
<tr>
<td>Year</td>
<td>Investigators</td>
<td>Country</td>
<td>Evidence for Aflatoxin consumption/involvement</td>
</tr>
<tr>
<td>------</td>
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<td>--------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1973</td>
<td>Peers and Linsell</td>
<td>Kenya</td>
<td>Statistically significant association between aflatoxin levels ingested and liver cancer cases.</td>
</tr>
<tr>
<td>1973</td>
<td>Van Niewenhuize et al.</td>
<td>Netherlands</td>
<td>12/55 fatal liver disease cases in a study of men who had inhaled aflatoxin-contaminated particles in a mill.</td>
</tr>
<tr>
<td>1974</td>
<td>Dvorackova et al.</td>
<td>Czechoslovakia</td>
<td>AFB, present in 2/3 cases of EFDV Reye's syndrome.</td>
</tr>
<tr>
<td>1974</td>
<td>Van Rensburg et al.</td>
<td>Mozambique</td>
<td>Highest known aflatoxin intake observed in area with highest primary liver cancer rate.</td>
</tr>
<tr>
<td>1975</td>
<td>Krishnamachari et al.</td>
<td>India</td>
<td>Maize - 2mg aflatoxin/kg; about 6mg aflatoxin ingested daily for 1 month - 106/397 were fatal cases of toxic hepatitis</td>
</tr>
<tr>
<td>1976</td>
<td>Bababunmi et al.</td>
<td>Nigeria</td>
<td>Increased levels of urinary aflatoxins in those with hepatomas and other liver diseases</td>
</tr>
<tr>
<td>1976</td>
<td>Deger</td>
<td>United States</td>
<td>Two men developed colonic carcinoma after spending 1 and 3 years, respectively scraping aflatoxin from TLC plates.</td>
</tr>
<tr>
<td>Year</td>
<td>Investigators</td>
<td>Country</td>
<td>Evidence for aflatoxin consumption/involvement</td>
</tr>
<tr>
<td>------</td>
<td>---------------</td>
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<td>---------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1976</td>
<td>Dvorackova</td>
<td>Czechoslovakia</td>
<td>AFB₁-like compound present in autopsy lung tissue from fatal alveolar carcinoma case who had worked with aflatoxin-contaminated groundnut meal</td>
</tr>
<tr>
<td>1976</td>
<td>Phillips et al.</td>
<td>United States</td>
<td>Biopsy liver sample from a man with carcinoma of the rectum and liver found to contain 520 ng AFB₁/g liver.</td>
</tr>
<tr>
<td>1976</td>
<td>Richir et al.</td>
<td>France</td>
<td>Autopsy livers from liver disease cases showed presence of AFB₁.</td>
</tr>
<tr>
<td>1978</td>
<td>Hogan et al.</td>
<td></td>
<td>AFB₁ present in the blood of two children with Reye's syndrome, during its acute phase.</td>
</tr>
<tr>
<td>1980</td>
<td>Onyemelukwe et al.</td>
<td>Nigeria</td>
<td>AFB₁ detected in autopsy liver samples from 4/5 liver cancer cases</td>
</tr>
<tr>
<td>1981</td>
<td>Dvorackova et al.</td>
<td>Czechoslovakia</td>
<td>AFB₁ observed in autopsy pulmonary tissue from 2 patients who had lung cancer. One patient had been exposed to aflatoxin-contaminated groundnut meal daily for 3 months.</td>
</tr>
<tr>
<td>Year</td>
<td>Investigators</td>
<td>Country</td>
<td>Evidence for aflatoxin consumption/involvement</td>
</tr>
<tr>
<td>------</td>
<td>---------------------</td>
<td>---------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>1981</td>
<td>Stora et al.</td>
<td>Czechoslovakia</td>
<td>5/15 liver cancer cases showed the presence of AFB$_1$ in biopsy liver samples</td>
</tr>
<tr>
<td>1982</td>
<td>Bulatao-Jaime et al.</td>
<td>Philippines</td>
<td>Study of dietary intakes of primary liver cancer cases showed aflatoxin load per day to be 440% that of the controls.</td>
</tr>
<tr>
<td>1982</td>
<td>Ngindu et al.</td>
<td>Kenya</td>
<td>Maize - 3.2-12.0 mg aflatoxin/kg caused outbreak of toxic hepatitis with 12/20 fatal cases.</td>
</tr>
<tr>
<td>1982</td>
<td>Yeh et al.</td>
<td>China</td>
<td>Correlation between aflatoxin intake and hepatocellular carcinoma observed.</td>
</tr>
<tr>
<td>1985</td>
<td>Van Rensburg et al.</td>
<td>Mozambique and Transkei</td>
<td>Correlation between highest levels of aflatoxin contamination and incidence of hepatocellular carcinoma still observed.</td>
</tr>
</tbody>
</table>
H. AFLATOXINS AND IMMUNITY

Though the main target for the toxic effects of the aflatoxins is the liver, various other organs have been known to be affected too. Evidence is now mounting which shows that the aflatoxins are able to exert an immunosuppressive effect in various animal species. It appears that immunosuppression is caused through the effects of aflatoxin on the thymus and consequently the cell-mediated immune system, the non-specific humoral pathways, and in a few cases, the humoral immune response (Pier et al., 1979).

Aflatoxin at moderate to high doses can cause an acute intoxication even leading to death while its consumption in low quantities over a period of time may result in the development of cancer. However, a third possibility also exists in that levels of aflatoxin, too low to cause an overt aflatoxicosis, could predispose the individual to infectious diseases through a depression of the native defence mechanisms as well as the actively acquired immune responses. Termed secondary mycotoxic diseases (Pier et al., 1977), these are of significance since the clinical symptoms relate to the infective agent rather than the underlying problem which, though not obvious, predisposes the host to the infection. The result is that significant economic losses are incurred in the rearing of livestock through reduced productivity, impaired resistance to disease or impairments in the development of acquired immunity to infectious diseases.
Resistance to Disease

The ability of the aflatoxins to inhibit protein synthesis (Clifford and Ress, 1966) suggested that the aflatoxins might possibly have an effect on antibody formation, and hence could interfere with the immune responses of animals. Siller and Ostler (1961) and Brown and Abrams (1965) had earlier reported Salmonella infections in field cases of aflatoxicosis providing the basis for later studies on the effects of aflatoxin on resistance to disease in various species of animals. A direct test of this hypothesis failed to show an interaction between aflatoxin at 5μg/g and oral doses of Salmonella gallinarum in chickens, i.e. there was no significant increase in mortality (Smith et al., 1969). However, Pier and Heddleston (1970) observed that 0.2 - 0.5μg/g aflatoxin B₁ impaired the acquired immunity of turkeys and chickens to Pasteurella multocida (fowl cholera) if the aflatoxin was administered during or after the immunization period.

Other workers have also reported increased susceptibility of various animal species to several diseases due to dietary aflatoxin. In poultry, aflatoxin-induced increased susceptibility (and/or mortality) has been shown to Eimeria tenella - cecal coccidiosis (Edds et al., 1973; Wyatt et al., 1975), to three species of Salmonella (Boonchuвит and Hamilton, 1975), to Candida albicans (Hamilton and Harris, 1971), to infectious bursal disease (Hamilton, 1982) and to Mareks disease virus (Edds et al., 1973). In swine, aflatoxin at low levels has been reported to interfere with the development of acquired immunity to Erysipelothrix rhusiopathiae vaccine.
(Cysewski et al., 1978), while increased susceptibility to Treponema hyodysenteriae has also been observed (Joens et al., 1981). Dietary aflatoxin has been reported to impair resistance to infections by Fasciola hepatica in calves (Osuna et al., 1977) as well as increase inflammation by Streptococcus and Staphylococcus mastitis in bovine (Brown et al., 1981).

In contrast, there have also been reports in which dietary aflatoxins have had no effect or, if anything, has increased resistance to disease. The acquired immunity of turkeys to Newcastle Disease virus was not impaired by the administration of aflatoxin (Pier et al., 1971), nor was increased susceptibility to Aspergillosis (Aspergillus fumigatus) observed in turkeys on a diet containing aflatoxin (Richard et al., 1973). Similarly, in hamsters, aflatoxin does not increase susceptibility to Mycobacterium paratuberculosis, in fact it actually appears to increase resistance to the disease (Larsen et al., 1975)

**Antibody Formation**

Despite some confusion in the literature (Pier et al., 1979) it appears that aflatoxin does have an effect on antibody production. Possibly these differences could be reconciled if it was realized that this was due to differences in doses, time intervals between aflatoxin administration and challenge by the disease, and the species used in the experiments. Susceptibility to the effects of aflatoxin has been shown to vary amongst species (Newberne and Butler, 1969) and it is possible that this could explain most of
the discrepancies observed.

One of the earliest workers in this area, Galikeev et al. (1968), postulated that aflatoxin caused an inhibition of immunogenesis, through its toxicity in the antibody producing cells. On injecting typhoid vaccine subcutaneously into mice, they found that the typhoid agglutinin titers were reduced if it preceded aflatoxin administration. However, if the aflatoxin was administered before the vaccination or if the aflatoxin and vaccine were injected simultaneously, minimal antibody production occurred nor were there any significantly lowered titers.

In general, aflatoxin does not cause suppression of antibody production except when high doses are involved. The antibody titre formed as a primary immune response, has been found to be depressed, while also causing a delay in peak titer, following administration of 0.625 - 10µg/g aflatoxin in chickens (Thaxton et al., 1974). Administration of 2.5 - 10 µg/g aflatoxin (B₁ - 71 per cent, B₂ - 8 per cent, G₁ - 16 per cent, G₂ - 5 percent) to chickens has been reported to induce a significant decrease in immunoglobulin (IgG values but not in IgM values) (Tung et al., 1975). Another group of workers (Giambrone et al., 1978) have also observed a depression of IgG and IgA values at aflatoxin levels of 2.5 µg/g, in otherwise healthy chickens. In pigs, suppression of antibody has been observed at dietary aflatoxin levels of 0.4 mg/kg/day (Joens et al., 1981).

The difficulties in integrating isolated reports is illustrated by the finding that when paratyphoid infections were associated
with aflatoxicosis in chickens (at levels of 10 µg/g), there was a significant increase in specific agglutinating antibodies, compared to the levels observed in uncomplicated paratyphoid infections (Boonchuvit and Hamilton, 1975). This was contrary to the earlier finding that aflatoxins dramatically suppressed antibody production against a non-infectious agent (Thaxton et al., 1974). However, it was also found that though the antibody levels were low the first nine days, by the twelfth and fifteenth day after the antigen injection, the antibody titer was higher in the chickens fed 10 µg/g aflatoxin, than in the controls. It is interesting to note that at an aflatoxin level of 0.625 µg/g, Tung et al. (1975) observed an actual increase in IgG and IgM values and it was only at slightly higher doses that the decrease occurred, though the depression of IgM was not as significant as it was for IgG.

In view of the experimental evidence it appears that in general aflatoxin does not decrease the antibody titre. The decreases observed have been partially explained by Tung et al. (1975). The progenitor cells of IgG and IgM are believed to originate in the bursa of Fabricius (Morgan and Glick, 1972) and aflatoxin has been observed to cause its severe regression in both chickens (Smith and Hamilton, 1970) and turkeys (Hamilton et al., 1972). Surgical bursectomy has been reported to cause decreased IgG but not IgM values (Glick et al., 1956; Larner et al., 1971).

Complement

Suppression of complement activity has been observed by many
workers. Pier and Heddleston (1970), in their study on the effects of aflatoxin on immunity in turkeys, observed immunosuppression though antibody levels were normal. It was suspected at the time that therefore aflatoxin probably suppressed a humoral substance other than the antibodies. A study was thus undertaken (Thurston et al., 1972; Richard et al., 1975) to determine the effects of aflatoxin on complement activity in guinea pigs. Administration of 0.03 mg of aflatoxin B₁ for 20 days significantly decreased complement activity in 0.5 kg guinea pigs (Thurston et al., 1972); while 0.05 mg aflatoxin B₁ caused a significant decrease in complement activity within a week (Thurston et al., 1974). A single dose of 0.3 mg aflatoxin B₁ administered to guinea pigs resulted in a significant depression of complement activity after 72 hours (Richard et al., 1975). Guinea pigs fed a single dose of 0.3 mg aflatoxin B₁ and their serum complement activity assayed a week after having been dosed were observed to have differences in the activities of the individual complements (Richard et al., 1978) C₂, C₃, C₄, C₆, and C₈ were significantly lowered in at least one of the two guinea pigs used in the experiment. The complement components C₂ - C₇ belong to the β-globulin group while C₈ is a γ₁-globulin (Muller - Eberhard, 1968; 1970; 1971). Thurston et al. (1974) observed that the earliest change in serum proteins in guinea pigs given aflatoxin B₁ was a decrease in the α₂-globulin and β-globulin fractions. The complement deficiency observed has been largely in the fourth component, C₄ (Thurston and Richard, 1979).
Interferon

There is one report that interferon, an antiviral humoral substance, has been observed to have been affected by aflatoxin (Pier et al., 1971). A lag in interferon production, during the first 24 hours, has been reported in turkeys fed 0.5 µg/g aflatoxin B₁ and then challenged with the Newcastle Disease virus.

Phagocytosis

In aflatoxin-treated animals, a significant reaction is that of reduced phagocytosis. Michael et al. (1973) demonstrated a significant reduction in the efficiency of fixed macrophages, derived from the reticuloendothelial system, in removing colloidal carbon from the bloodstream of chickens fed aflatoxin at 1.25 µg/g of feed. Reduced phagocytosis by free (alveolar) macrophages (Richard and Thurston, 1975) has been demonstrated in rabbits given daily doses of 0.03 - 0.09 mg aflatoxin B₁ for 2 weeks. The polymorphonuclear series of leukocytes are yet another group that show depressed phagocytic properties when aflatoxin is administered (Chang and Hamilton, 1979a) as do the precursor monocytes (Chang and Hamilton, 1979b). Besides a reduced rate of phagocytosis and extent of ingestion, there are several other ways by which impairment of phagocytes can occur. In heterophils (Chang and Hamilton, 1979a) and monocytes (Chang and Hamilton, 1979b) there is impairment in both the directed (chemotactic) and undirected (spontaneous) locomotion. In certain chickens exposed to aflatoxin there appears to be a retardation of the formation of phagolysosomes as well as
the destruction of ingested microorganisms (Chang and Hamilton, 1979 a). It could therefore be concluded that an impairment of resistance to disease occurs either through a depression of phagocytic activity or a defect in the mechanisms by which the macrophages participate in the immune response.

**Cell-Mediated Immunity**

Certain aspects of cell-mediated immunity have been observed to be impaired by the administration of aflatoxins. Impaired immunogenesis, in aflatoxin-treated turkeys and chickens, on being vaccinated for fowl cholera (*Pasteurella multocida* bacteria) has been attributed to an impairment of the cell-mediated immunity (CMI) response to the vaccine (Pier et al., 1979). *In vivo* studies done on guinea pigs have shown that various aspects of CMI are affected by aflatoxin and this included depression of delayed cutaneous hypersensitivity (DCH) and lymphokine activity (Migration inhibition factor (MIF) and phytohemagglutinin (PHA) - stimulated lymphoblastogenesis) (Pier et al., 1979). This was the first demonstration of an *in vivo* effect of aflatoxin on the CMI of thymus-derived lymphocytes and it was corroborated by studies performed on aflatoxin-treated chickens (2.5 μg/g) in which deficiencies in delayed cutaneous hypersensitivity and graft versus host reactivity was observed (Giambrone et al., 1978). The *in vivo* effect of aflatoxin on CMI responses has also been demonstrated in turkeys, in which DCH was depressed though a PHA
skin test was normal (Schultz, 1983). For neonatal pigs born to aflatoxin-treated sows, it has been demonstrated that the suppressive effects of aflatoxin on CMI can be transferred through transplacental migration, as an \textit{in utero} effect (Pier \textit{et al.}, 1985)

The depression of DCH in guinea pigs, by aflatoxin, has been shown to be dose-dependent with a significant decrease at 0.04 mg aflatoxin B$_1$ /kg/day (McLoughlin, 1982). In the same study, adoptive transfer of DCH by peritoneal exudate cells (PEC) was reduced (substantially but not significantly) in aflatoxin-treated PEC recipients and when the PEC was obtained from aflatoxin-treated animals. However, there was no difference in the absolute T and B cell populations in the peripheral blood from aflatoxin-treated and normal guinea pigs. This therefore demonstrated that aflatoxin could cause an impairment of DCH response as well as adoptive transfer of DCH without affecting the T and B cell populations of the peripheral blood. The implications appear to be that CMI suppression may be caused by (1) the activity of suppressor cells which do not alter the T and B lymphocyte numbers in the peripheral blood; (2) changes in the function of macrophages that relate to antigen transfer; or (3) differences in lymphokine activity (Pier and McLoughlin, 1985).

It has recently been shown that the purity of the aflatoxins used in experiments determine the immunosuppressive effect observed. Using chemically purified aflatoxins B$_1$ and a broad range of immunological parameters, immunosuppression could not be demonstrated in swine or turkey pouls at concentrations of up to 4000 $\mu$g/kg
(Schultz et al., 1982; Schultz, 1983). Chemical recovery of aflatoxin from the feed was reduced due apparently to binding of the aflatoxin to the feed. To prevent this binding to the feed, purified aflatoxin $B_1$ was administered in capsules at a concentration of about 200 $\mu g$/kg dietary aflatoxin. Depression of DCH in response to mycobacterial antigens was observed but a PHA skin test was normal, as were other immunological parameters (Schultz, 1983).

The above results bring up three important questions: (1) Is aflatoxin $B_1$ the immunosuppressive factor or is the presence of one or more of the other aflatoxins essential? (2) Is it possible that purified aflatoxin $B_1$ is bound in the intestine as it was bound in the feed, thus reducing its biological activity? (3) Since intradermal injections of PHA produced no response while injections of mycobacterial antigens showed significant depression of DCH, in aflatoxin-treated animals, could it not be that the problems are in the processing of antigens rather than in the lymphocyte populations, lymphokine production or other lymphocyte functions? (Pier and McLoughlin, 1985).

It has been observed that addition of aflatoxin to cell cultures of human lymphocytes reduces their ability to respond to PHA stimulation (Savel et al., 1970). A significant inhibition occurred at 5 $\mu g$/ml of aflatoxin $B_1$, and at levels of 50 $\mu g$/ml the investigators found that the inhibition could be reversed if the exposure time to the aflatoxin was less than 20 hours. This has been the only demonstration of an inhibition of an immune response using human cells. The sensitivity of other species, in their response to aflatoxin, has been confirmed in vitro in bovine lymphocytes (Paul et al., 1977) and in vitro in guinea
pigs (Pier et al., 1977). However, the source and purity of aflatoxin B₁ in the studies on human (Savel et al., 1970) and bovine (Paul et al., 1977) lymphocytes was different from the purified aflatoxin B₁ that suppressed only DCH in turkeys (Schultz, 1983) or the partially purified aflatoxin which caused immunosuppression in turkeys (Piet and Heddleston, 1970), guinea pigs (Pier et al., 1977) and swine (Cysewski et al., 1978). It therefore seems probable that different aflatoxin preparations may vary in their biological activity and that small amounts of impurities contribute to the apparent CMI-based immunosuppressive activity of aflatoxin B₁.

To study the action of various purified aflatoxin species Yang (1983) performed in vitro tests on porcine lymphocytes. Aflatoxins B₁, B₂a, M₁, M₂, and G₁ induced dose-dependent suppression of lymphoblastogenetic responses to PHA. Maximum suppression was observed by aflatoxin G₁ at 1 μg/ml, aflatoxin B₂a at 5 μg/ml and aflatoxins B₁, M₁ and M₂ required 10 - 25 μg/ml for suppression. Aflatoxins B₂, G₂, G₂a and P₁ showed no suppressive effect even at 25 μg/ml (Pier and McLoughlin, 1985).

With each new bit of information the effects of aflatoxin on the immune response become more complex. Many of the effects appear to be contrary to each other which tends to create confusion as to whether certain immunological parameters are suppressed or not. To summarize the available information at present, aflatoxin has been shown to exert its effect on both native resistance and acquired resistance. The native defense mechanisms affected
include a lag in interferon production, decreased complement activity, impaired phagocyte response and decreased phagocytosis and are important in that they impede replication as well as aid in clearing the system of the invading parasite. The major effect of aflatoxin on actively acquired immunity is through its effect on the T-cells, whose responses are collectively known as the CMI reactions. The B-cells do not appear to mediate aflatoxin-induced immunodeficiency except at very high doses of aflatoxin, and even then only part of the globulin spectrum is affected.

As there has only been one study on human cells, it is not known how far the animal experiments are applicable to the complex human immune system. However, it is likely that aflatoxins will have some deleterious effect on disease-resistance, as has been suggested by Pier and McLoughlin (1985). Contamination of foods by low levels of aflatoxin could therefore pose a serious hazard to human health.

I. AFLATOXIN STUDIES IN ZAMBIA

Various studies have been carried out with regard to the incidence of aflatoxins in Zambia. The staple food of the Zambian population is predominantly maize and this is eaten with a 'relish'. The relish is usually a green leafy vegetable, or beans, which may be cooked in oil with onion and tomato. If groundnuts are available they are ground and added to the leaves.
To investigate the level of aflatoxin contamination of foods in Zambia, a 'plate food' study was carried out in the Eastern province of Zambia (see map Fig. 2.1). This area was chosen since it could be considered a representative sample of the rural population and groundnuts are grown on a wider scale here. Analysis of 200 samples gave an aflatoxin incidence of 3 per cent with levels ranging from 1 - 44 μg/kg (Lovelace and Salter, 1979).

Once the level of contamination was known it was of interest to determine the aflatoxin incidence in the human population exposed to aflatoxin in their foods. Urine samples were obtained from 219 patients which included those with liver pathology (Lovelace et al., 1983). Aflatoxin M₁ was the only aflatoxin detected at an incidence of 3 per cent, identical to that obtained in the plate food study. A significant seasonal prevalence was observed, with most positive samples obtained in the rainy season.

Since the previous plate food study had been carried out in the dry season, another similar study was carried out in the rainy season (Njapau, H., pers. comm.). The aflatoxin incidence was observed to be higher in the rainy season, 7 per cent as compared to 3 per cent in the dry season. However, the levels of contamination were not really different as the range of aflatoxin concentration detected was 1 - 50 μg/kg. A survey of food samples was also performed in the rainy season and the results are given in Tables 1.4(a) and (b). Germinated maize samples were highly contaminated with levels up to 150 μg/kg and an incidence of 73 per cent. This may be considered serious since the germinated maize, chimela, is
### Table 1.4 (a)

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

(Njapau, H. pers. comm.)

<table>
<thead>
<tr>
<th>COMMODITY</th>
<th>NUMBER OF SAMPLES</th>
<th>NUMBER CONTAMINATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soaked maize (phale)</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Shelled dry maize</td>
<td>31</td>
<td>8 (26%)</td>
</tr>
<tr>
<td>Germinated maize (chimela)</td>
<td>11</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>Institutional nshima (maize)</td>
<td>43</td>
<td>3 (7%)</td>
</tr>
<tr>
<td>Institutional beans</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Village meals</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>211</td>
<td>19 (9%)</td>
</tr>
</tbody>
</table>

### Table 1.4 (b)

**MAIZE PRODUCTS OBTAINED FROM EASTERN CO-OPERATIVE UNION, CHIPATA**

(Njapau, H. pers. comm.)

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>NUMBER OF SAMPLES</th>
<th>NUMBER CONTAMINATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast meal</td>
<td>14</td>
<td>6 (43%)</td>
</tr>
<tr>
<td>Roller meal</td>
<td>11</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Maize bran</td>
<td>10</td>
<td>3 (30%)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>35</td>
<td>12 (34%)</td>
</tr>
</tbody>
</table>
used to prepare a sweet beer, thobwa, which is consumed by children as well as adults.

A study of 136 urine samples and 34 liver samples from patients with liver pathology and controls at the University Teaching Hospital, Lusaka has recently been completed (Dil, 1986). This showed an aflatoxin incidence of 3.7 per cent in urine and 14.7 per cent in liver. Aflatoxins $B_1$, $M_1$ and aflatoxicol were detected in the urine samples while only aflatoxin $B_1$ was detected in the liver samples. Sampling in the Eastern province gave an aflatoxin incidence of 15.4 per cent with a mean level of 16.5 ng/100 ml (see Table 1.5). The difference between rural and urban populations was found to be significant. A seasonal trend for the aflatoxin incidence was also observed with significantly more positive samples observed during the rainy season.

Table 1.5 AFLATOXIN ANALYSIS OF URINE AND LIVER SAMPLES FROM LIVER PATHOLOGY AND CONTROL CASES IN ZAMBIA (Dil, 1986)

<table>
<thead>
<tr>
<th></th>
<th>Total Number of samples</th>
<th>Incidence of positive samples</th>
<th>Mean aflatoxin concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine samples from Lusaka</td>
<td>136</td>
<td>3.7%</td>
<td>9.1 ng/100 ml</td>
</tr>
<tr>
<td>Urine samples from Katete</td>
<td>26</td>
<td>15.4%</td>
<td>16.5 ng/100 ml</td>
</tr>
<tr>
<td>Autopsy liver samples from Lusaka</td>
<td>34</td>
<td>14.7%</td>
<td>2.3 ng/100 ml</td>
</tr>
</tbody>
</table>
A study of the breastmilk samples from mothers in the Katete area showed levels of 10 - 100 ng/100 ml (Bayley, A.C., pers. comm.), due to excretion of aflatoxin $M_1$. 
II PROTEIN - ENERGY MALNUTRITION

INTRODUCTION

Protein - energy malnutrition (PEM) is a term that, in recent years, has been used to describe a range of spectrum of clinical disorders afflicting children who live on less than adequate diets. A nutritional disease, PEM can occur in any part of the world, though a concentration does seem to exist in the developing countries where socio-economic factors play a major role. It was originally termed 'protein - calorie malnutrition' by Jellife (1959) to cover not only the entire clinical spectrum of marasmus, kwashiorkor and intermediate forms but also mild and moderate subclinical stages. It was later adopted by the FAO/WHO Joint Expert Committee on Nutrition (1962). In conforming to new trends in nomenclature, the word 'calorie' was then replaced by 'energy'.

The term, P.E.M., suggests two important concepts. The first is that an inadequate intake of protein and/or energy is a causative factor. The second is that there is a range of clinical disorders caused by either protein or energy malnutrition, or the interaction between them and quite often a well-defined distinction between the various disorders is lacking. The two extreme forms are presented as kwashiorkor, primarily due to a protein deficiency, and marasmus, caused by a protein as well as an energy deficiency, and these form either end of a spectrum.

When it was introduced, the expression 'PEM' seemed useful since intermediate forms had begun to be recognized which were
variations from the classical picture of either marasmus or kwashiorkor. However, as with many new concepts, its purpose was misunderstood and there began a tendency to use the term 'PEM' indiscriminately as a convenient diagnosis term with insufficient attention to individual differences. What is even more regrettable is a growing tendency to actually treat it as a single disease. as has been personally observed in Zambia. It was for this reason that McCance and Widdowson (1966) wrote a paper to re-emphasize the fundamental difference between kwashiorkor and marasmus and it was suggested that the term be allowed to drop.

CLASSIFICATION OF PEM

It was to dispel some of the confusion surrounding the diagnosis of PEM that the Wellcome Trust (1970) held a working party in Jamaica to establish some sort of diagnostic criteria. The Wellcome classification (Table 1.6 uses the degree of body wasting and the presence or absence of edema as the principal diagnostic features.

Table 1.6 The Wellcome Classification on PEM

<table>
<thead>
<tr>
<th>Weight (percentage of standard *)</th>
<th>Edema</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 - 60</td>
<td>Present</td>
<td>Underweight</td>
</tr>
<tr>
<td>60</td>
<td>Kwashiorkor</td>
<td>Marasmic kwashiorkor</td>
</tr>
<tr>
<td></td>
<td>Marasmus</td>
<td></td>
</tr>
</tbody>
</table>

*Standard = fiftieth centile Boston value (Stuart and Stevenson, 1959)
Children with edema weighing between 60 and 80 per cent of their expected weight for age were classified as having kwashiorkor. However, it was recognized that it was possible for some children to present edema with all the other clinical features of kwashiorkor and thus be so classified even though their body weight was in excess of 80 percent of the standard weight for age. Those without edema and who weighed less than 60 per cent of the standard weight for age were considered to have marasmus. In those children with edema and a weight less than 60 per cent of the expected weight for age, the diagnosis was marasmic kwashiorkor. Children without edema but who weighed between 60 and 80 per cent of the expected weight for age were classified simply as being underweight.

This classification, as with all others has its advantages and disadvantages with the main virtue being in its simplicity. The main disadvantages are that the patient's age has to be known and the chronicity of the disease is not taken into consideration. However, a clear distinction is made between marasmus and kwashiorkor.

Various systems of classification of PEM have been proposed with the Gomez classification, based on weight for age, as one of the earliest (Gomez et al., 1956). This is as follows:

Weight for age: 75 - 90% of standard - 1st degree of malnutrition

61 - 75% of standard - 2nd degree of malnutrition

≤ 60% of standard - 3rd degree of malnutrition

*Standard = fiftieth centile Boston value (Stuart and Stevenson, 1959)

Limitations are immediately seen in this system of classification:
(i) the age of the patient needs to be known; (ii) the chronicity of the disease is not taken into consideration; and (iii) it does not identify kwashiorkor.

This system of classification has been modified by several people and more elaborate schemes proposed that take into account the clinical signs of malnutrition as well as anthropometric measurements. Waterlow (1972) stresses that anthropometric signs, in particular weight and height, give a good assessment of nutritional status which is independent of age and age-related external standards. This therefore, provides a means of determining the chronicity of the disease with the use of appropriate height and weight data (Table 1.7). Thus both of the limitations posed by the Wellcome classification and two of those posed by the Gomez classification are not limitations in this case. A major drawback, as in the latter classification, is that kwashiorkor is not identified.

Table 1.7 Classification of PEM (Waterlow, 1972)

<table>
<thead>
<tr>
<th>%Weight for Height</th>
<th>80</th>
<th>80 - 119</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht. for age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic (1) malnutrition</td>
<td></td>
<td>Stunted but not (2) actual malnutrition</td>
<td>Stunted but (3) obese</td>
</tr>
<tr>
<td>90%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACUTE (4) malnutrition</td>
<td>NORMAL (5)</td>
<td>OBESE (6)</td>
<td></td>
</tr>
</tbody>
</table>

Action required: (1) Long term socio-economic development
(2) & (5) Nil
(3) & (6) Nutrition education
(4) Emergency rehabilitation
It was therefore decided that in this study the Wellcome classification would be followed. This scheme is simple enough to be used in a busy hospital and most importantly, it classifies both kwashiorkor and marasmic kwashiorkor.

**HISTORY**

Marasmus is a nutritional disorder, of which the cause and clinical features have been widely recognized. A term that might almost be synonymous with starvation, nutritional marasmus can develop in people at any age. However, this discussion will be limited to its occurrence in young children. Marasmus was the name given by Austrian and German workers, during the Second World War, to describe the condition resulting from starvation or semi-starvation which they recognized could also be found in young children. It was observed, though, that this nutritional disease was not necessarily associated with infective diarrhoea (McCance and Widdowson, 1966)

On the other hand, kwashiorkor is a nutritional syndrome which was recognized relatively recently. The first article describing kwashiorkor in Ghana was by Cicely Williams (1933) in which she stressed that the disease was primarily due to an inadequate intake of protein and not the well-known vitamin deficiency, pellagra. Dr. Williams (1935) adopted the word 'kwashiorkor' from the Ga tribe who live around Accra, Ghana, a name used to describe the 'sickness the older child gets when the next one is born'.
However, it was not until the 1950's that the concept of primary protein malnutrition was recognized and accepted, for to many doctors, the etiology seemed more likely to be that of infectious diseases or multiple vitamin deficiencies.

As clinicians became more familiar with the characteristic features of kwashiorkor and marasmus, it was realized that quite often a mixed syndrome was also presented. This puzzled the clinicians and was termed marasmic kwashiorkor (McCance and Widdowson, 1966). Intermediate forms of PEM which displayed features of both kwashiorkor and marasmus were thus classified.

**CLINICAL FEATURES OF KWASHIORKOR**

Kwashiorkor is predominantly a condition of children just past the weaning stage. This is usually in children within the age range of 10 - 48 months, the age being dependent on when the child is weaned. These children are apathetic, very irritable, weak and inactive, have the characteristic edema of the extremities and, quite often, an enlarged liver. Fig. 1.4 shows a case of kwashiorkor with the generalized edema, skin lesions and the misery so constantly observed. Kwashiorkor represents a clinical syndrome in the literal sense of the word i.e. a group of features occurring at the same time which are characteristics of the condition. Most cases present a few of these features but not all of them.

Though a definition of kwashiorkor has not reached general agreement, one characteristic feature which has been universally
accepted as the criterion for diagnosis is edema. This may vary in severity from a mild puffiness around the eyes and slight swelling of the hands and feet to generalized edema. The general puffiness in the early stages quite often mislead the mother into thinking the child is extremely healthy. Commonly observed at the involve the face, trunk and generally determined by gravity associated with ascites or.

Though the edema is a which occupies the gross edema are quite often severe.

Kwashiorkor which are, which may be extensive in pigmentation are the usual dermatosis, which is more common on the trunk, patches of skin area or thin, hypopigmented the thin, shiny skin is stretched. Ulcerations commonly occur where the skin is pulled such as at the groin and buttocks and this is especially aggravated by the use of nappies which constantly rub against the skin. In severe cases, the ulcerations may resemble that of second-degree burns. Angular stomatitis is another skin lesion quite often observed in cases of kwashiorkor.

Changes in the hair are variable since it is usually dependent on the duration of the illness. In cases where the

Fig. 1.4 Kwashiorkor
accepted as the criterion for diagnosis is edema. This may vary in severity from a mild puffiness around the eyes and slight swelling of the hands and feet to generalized edema. The general puffiness in the early stages quite often mislead the mother into thinking the child is extremely healthy. Commonly observed at the extremities, the edema may also involve the face, trunk and genitalia with the site being partly determined by gravity (Alleyne et al., 1977). It is not associated with ascites or fluid collection in other body cavities. Though the edema is a clinical manifestation of excess body water which occupies the extracellular fluid space, children with gross edema are quite often severely dehydrated.

Skin changes are frequently seen in kwashiorkor which are reversible on recovery. Hypopigmentation, which may be extensive or in patches, together with areas of hyperpigmentation are the usual skin changes. In 'flaky - paint' dermatosis, which is more commonly seen on the extremities than on the trunk, patches of skin desquamate thus exposing a raw area or thin, hypopigmented skin (see Fig.1.4 ). Due to the edema, the thin, shiny skin is stretched tautly over the limbs. Ulcerations commonly occur where the skin is pulled such as at the groin and buttocks and this is especially aggravated by the use of nappies which constantly rub against the skin. In severe cases, the ulcerations may resemble that of second-degree burns. Angular stomatitis is another skin lesion quite often observed in cases of kwashiorkor.

Changes in the hair are variable since it is usually dependent on the duration of the illness. In cases where the
disease has developed rapidly the changes may be minimal or altogether absent. However, when the process has been of long duration, hair which is normally dark and curly becomes lighter and straighter. The hair also becomes dry, thin and easily plucked which results in sparseness of hair with the scalp clearly visible.

Hepatomegaly is a feature of kwashiorkor quite often reported but it appears to be restricted to a few areas of the world (Alleyne et al., 1977). Enlargement of the liver was also described by Williams (1933) in her original descriptions of kwashiorkor. Palpating the abdomen, the edge of the enlarged liver may be felt as being rubbery hard.

The irritability and misery observed in kwashiorkor children is a striking characteristic of the condition. Quite often these children are anorexic and have little interest in food. This is a feature which is not really stressed but is an important characteristic as the mothers are convinced that the children are satisfied.

CLINICAL FEATURES OF MARASMUS

Marasmus is primarily a disease of infancy and is usually the result of very early and abrupt weaning, when what follows is a diet of dilute milk formulae prepared in unhygienic conditions. Severe dietary inadequacy coupled with recurrent infections usually leads to this condition. Characteristically these children have wizened faces giving them an 'old man' appearance, there is gross wastage of muscle and subcutaneous tissues and significant stunting of growth with edema being absent.
Probably the most striking feature of a marasmic infant is its emaciated appearance with an almost complete absence of subcutaneous tissue (see Fig. 1.5). The ribs are clearly visible and the shrunken buttocks and limbs appear under folds of skin. In comparison with the rest of the body, the head tends to appear very large. The marked reduction in subcutaneous tissue and muscle is relatively greater than that of height.

The skin and hair changes are not as obvious as they are in kwashiorkor and in some cases may not be present at all. The skin is frequently dry and consequently has a dull appearance. Patches of hypopigmentation and scaling may also be observed. The hair in some cases can be sparsely distributed on the scalp, soft, dry and a lighter color than normal.

Hepatomegaly is another abnormality which is less frequently found in marasmus than in kwashiorkor. When it is observed the liver edge is only just palpable. In cases where the liver is enlarged it has been reported that fatty infiltration had occurred (Alleyne et al., 1977).

The apathy and misery of kwashiorkor may be observed in marasmus too, though irritability and fretfulness are more common features in the early stages. However, the disposition may vary from case to case. Severe cases of marasmus are quite often listless, silent and remain in apathetic immobility for long periods in a probable attempt to conserve energy. In most marasmic infants anorexia is rarely a problem and in fact the children usually have very good appetites.
CLINICAL FEATURES OF MARASMIC KWASHIORKOR

Children diagnosed as cases of marasmic kwashiorkor are those representing the intermediate forms of severe PEM (Alleyne et al., 1977). They therefore display one or more features of both end stages of kwashiorkor and marasmus. The emaciation of marasmus is observed to those found in kwashiorkor frequently ulcerating with its ribs clearly visible subcutaneously and typical fatty deposits in the legs. A palpable, a barely minimal

**Fig. 1.5** Marasmus

ETIOLOGY OF KWASHIORKOR AND MARASMUS

On the basis of the description of Williams (1933), kwashiorkor and marasmus were differentiated by what was later to become known as the 'classical' theory. By 1950 it was accepted that kwashiorkor was caused by a protein deficiency and relative excess of energy while marasmus was caused by a deficiency of both
CLINICAL FEATURES OF MARASMIC KWASHIORKOR

Children diagnosed as cases of marasmic kwashiorkor are those representing the intermediate forms of severe PEM (Alleyne et al., 1977). They therefore display one or more features of both ends of the PEM spectrum i.e. kwashiorkor and marasmus. The emaciated appearance so characteristic of marasmus is observed together with the presence of edema of the extremities. Skin and hair changes observed are similar to those found in kwashiorkor. Hypopigmentation and desquamation leading to ulcerations of the skin, features of kwashiorkor are frequently observed too. Fig. 1.6 shows such a child with its ribs clearly visible and folds of skin at the groin signifying loss of subcutaneous tissue and at the same time edematous legs with the typical hypopigmentation and desquamation of the skin. A palpable, fatty liver is quite often detected as well. The marasmic kwashiorkor infant is usually listless and apathetic. In most cases these children are anorexic and have a barely minimal interest in food.

ETIOLOGY OF KWASHIORKOR AND MARASMUS

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protein and energy (McCance and Widdowson, 1966). This simple
difference in the etiologies of the two syndromes appeared
settled since there was circumstantial evidence which justified
it on dietary grounds. In areas where kwashiorkor was the predominant
form of PEM, there was no shortage of food but the diet consisted
mainly of starchy staples, such as tapioca and cassava and the
protein at the
Fig 1.6 Marasmic kwashiorkor

It was indicated that the
observe
It was observed that the
develop
It was observed that the
energy intake of
tive or quantitative
differences between those children
Marasmic (Gopalan, 1988).
ery intake of
ner than high. The absence
argued of a definite relationship
between children
energy requirements were
recurrent infections and increased protein requirements necessary
for catch-up growth were not taken into consideration, nor were
irregularities of growth which also increase the need for protein.
protein and energy (McCance and Widdowson, 1966). This simple difference in the etiologies of the two syndromes appeared settled since there was circumstantial evidence which justified it on dietary grounds. In areas where kwashiorkor was the predominant form of PEM there was no shortage of food but the diet consisted mainly of starchy staples such as banana and cassava and the protein intake was significantly lower than what was recommended at the time (Alleyne et al., 1977).

Evidence then began to emerge which indicated that the differences were not all that clear-cut. In India it had been observed that essentially there was no qualitative or quantitative difference in dietary protein:energy ratios between those children developing kwashiorkor and those becoming marasmic (Gopalan, 1968). It was also stated that, if anything, the energy intake of children developing kwashiorkor was low rather than high. This absence of a demonstrable difference in dietary backgrounds of marasmic and kwashiorkor children was reported elsewhere too (Alleyne et al., 1977). Furthermore, the earlier minimum protein requirements were reconsidered and it was decided that in many countries, children were in actual fact well above this requirement as estimated by the FAO/WHO Expert Committee (1973) (Waterlow and Payne, 1975). However, this has since been re-evaluated as it did not allow for the increased requirements of protein in the developing countries where the conditions are not always favourable (Waterlow, 1984). Children in these areas are quite often subjected to recurring infections and increased protein requirements necessary for catch-up growth were not taken into consideration, nor were irregularities of growth which also increase the need for protein.
Furthermore, it has been suggested that synthesis of specific proteins such as albumin which is significantly lost in some infections, requires more protein than the average required for growth in body protein mass.

Nevertheless experimental evidence has shown that it is possible to induce the biochemical and pathological features of kwashiorkor in animals, such as the infant baboon, on restricted amounts of diets low in protein (Whitehead, 1980). It is difficult though to get experimental animals to eat enough of a low protein diet to show signs of edema and a fatty liver, the characteristic features of kwashiorkor. An exception is the pig in which it is possible to show the development of both edema and a fatty liver (Widdowson, 1968). Experiments with rats have also confirmed the 'classical' theory in that plasma albumin concentrations were found to be lowest when the diet contained the greatest excess of energy (Lunn and Austin, 1983).

Even so, criticism of the classical theory increased and evidence was brought to show that protein deficiency depends on many other factors in the developing countries (McLaren, 1974). Instead the problem was regarded as being one of quantity rather than quality of food (Waterlow and Payne, 1975). It is to be accepted that when looked at in a universal context, the lack of nutrients generally shows more of an energy gap than a protein gap. However, that does not explain the protein deficiency observed in areas where kwashiorkor is the predominant form of PEM. It therefore appears that though the evidence in favor of the classical theory is incomplete as well as inconclusive (Waterlow, 1984), the arguments
it carries cannot be discarded lightly.

MARASMUS - AN ADAPTATION TO ENERGY DEFICIENCY

The severe body wasting, so characteristic of marasmus, is generally accepted as the metabolic adaptation of the body to a drastically reduced nutrient intake. The adaptation induced is mediated by changes in the hormonal pattern and until carried to excessive limits, may be considered as a normal physiological process. Metabolic adaptation to a decrease in both energy and protein is necessary to ensure that homeostasis is maintained. Consequently there occurs a gradual wasting of muscle and subcutaneous fat which can be spared to protect more essential tissues and organs.

Whitehead and Alleyne (1972) postulated a process by which this adaptation might occur (Fig.1.7). Glucose, an essential energy source of the brain, may be synthesized from alanine which, in turn, is formed through the transamination of the amino acids provided by the catabolism of muscle protein. Essential amino acids resulting from muscle protein breakdown enable the liver to synthesize the necessary proteins required for homeostasis, such as serum albumin and β-lipoprotein. In marasmus the concentration of β-lipoprotein in the serum, which is essential in the transportation of triglycerides from the liver to the various fat deposits, is maintained. This probably explains the absence of a fatty liver in this condition. In fact the absence of any serious abnormality in these children is an indication of the remarkable adaptation they show to a diet insufficient in both protein and energy.
Fig. 1.7 Basic adaptation to energy deficiency (Whitehead & Alleyne, 1972)

Fig. 1.8 Basic disturbance
KWASHIORKOR - A FAILURE TO ADAPT TO PROTEIN DEFICIENCY?

To explain his findings, Gopalan (1968) postulated that kwashiorkor was essentially a failure to adapt to a poor diet. The term 'dysadaptation' was thus introduced in respect to kwashiorkor. He suggested that the biochemical mechanisms which usually ensured the protection of tissues such as the liver, at the expense of less essential muscle tissue during a deficit of energy, did not function in some children and consequently they developed kwashiorkor. The precise mechanisms involved were not known but it was suspected that it might have something to do with an impaired hormonal control.

A failure to maintain homeostasis, as apparent by the development of edema and fatty liver are the most significant features of kwashiorkor. In an attempt to reconcile the two opposing views i.e. the classical theory and Gopalan's findings, Whitehead and Alleyne (1972) proposed an interpretation of the situation in an area of Uganda where kwashiorkor predominates (Fig. 1.8 ). It was suggested that the high carbohydrate content of the diet stimulated the release of insulin which caused a preferential distribution of the limited amino acid supply in favor of muscle rather than liver and thus decreased tissue catabolism. However, due to the shortage of dietary protein, inadequate amounts of amino acids were available for essential protein synthesis and neither was this gap sufficiently filled from muscle breakdown. This, it was suggested, was the reason for the decreased albumin and $\beta$-lipoprotein synthesis, with the former resulting in edema.
and the latter in a fatty liver. In summary, it was postulated
that kwashiorkor was thus the result of a failure to adapt which
was probably due to the dietary carbohydrate inhibiting the tissue
catabolism processes, via the effects of insulin.

Another hypothesis to reconcile the classical theory with
that of Gopalan's was that by Waterlow (1974). Energy and protein
requirements differ between individuals, and a child with a high
protein requirement does not necessarily have a high energy
requirement, or vice versa. It was suggested that with children
on a given diet, marginal in both energy and protein, the limiting
factor was energy for those with a high energy requirement and
protein for those with a high protein requirement. As a result,
the former would develop marasmus and the latter would develop
kwashiorkor.

In a study of preschool children living in an area of Uganda
where kwashiorkor predominates, Lunn et al. (1973) found that their
fasting plasma insulin levels were higher than those of children
who had recovered from malnutrition, as well as higher than those
of 'normal' pre-school children from a rural population in Gambia
where the predominant form of PEM is marasmus (Whitehead et al.,
1977). Corresponding plasma cortisol and growth hormone levels
were not elevated. This therefore agreed with the hypothesis
that the failure to adapt was through the effect of increased
insulin levels. A similar hypothesis has been produced with a
different emphasis by Jaya Rao (1974). It was agreed that in marasmus
the maintenance of high plasma cortisol levels ensured that the
metabolic integrity of the body was preserved. However, it was
proposed that children developed kwashiorkor through their inability to maintain high plasma cortisol levels through the failure of the adrenal cortex to respond adequately. This view has failed to receive support from studies of investigators elsewhere. These different proposals have not been conclusively proved and the debate continues. Each view has its own supporters with experimental evidence to back their hypothesis. It thus appears that it would be best to accept each idea as a contribution to the understanding of the pathogenesis of kwashiorkor in a universal context. However, it should be realized that differences can occur between individuals as well as between countries.

The development of features of kwashiorkor such as hypoalbuminemia and edema are still under debate too. According to the classical theory the chain of events is simplified: a protein deficiency causes hypoalbuminemia which in turn results in edema. The two steps of this chain have been challenged as being an over-simplification of the actual series of events.

Edema is caused by the accumulation of fluid in the extravascular spaces and thus any mechanism that aids in the movement of fluid from the vascular into the interstitial spaces would enhance the development of edema. It has been observed that a fall in plasma protein levels in protein-deficient rats was matched by a reduction in the colloid content of interstitial fluid, thereby maintaining a transcapillary colloid osmotic balance which prevented excessive water filtration (Fiorotto and Coward, 1979). In normal animals the plasma: interstitial fluid concentration ratio is about two and while this adaptation to hypoproteinemia is effective over a
wide range of plasma protein concentrations, there is a limit (Coward and Lunn, 1981). Once the limit is reached e.g. when plasma concentrations are halved, control over the distribution of the extravascular fluid is lost. This brought about the idea that levels of plasma albumin would have an effect on the development of the edema observed in kwashiorkor. The contribution of albumin to the total colloid osmotic pressure of plasma is greater than 50 percent, larger than any other plasma protein concentration and thus a change in albumin levels would have a serious effect on the plasma colloid content (Coward and Lunn, 1981).

It should be realized that other factors can contribute to the development of edema in malnutrition. Defects in the sodium pump have been suggested to result in the development of edema, due to a retention of sodium and possibly a deficiency of vanadium (Patrick, 1980). The role of zinc in the sodium pump has been thought to contribute to edema formation as well (Patrick and Golden, 1977; Golden and Golden, 1981). Reduction in cardiac output and impaired renal function have also been postulated in the promotion of fluid retention but these cannot be the primary causes as the defects have been observed in both marasmus and kwashiorkor (Waterlow, 1984).

Results of experiments with patients on a restricted diet indicated that the disappearance of edema had no correlation with the levels of serum albumin (Golden et al., 1980). It was therefore suggested that the association between hypoalbuminemia and edema was not causal. However, this in itself does not appear to be
conclusive evidence since the disappearance of edema without hyperalbuminemia does not signify that the appearance of edema did not occur as a consequence of hypoalbuminemia. While in the Gambia, Lunn et al. (1979) had observed that edema could also occur in otherwise marasmic children. The only similarity between these marasmic kwashiorkor children and the classical kwashiorkor children of Uganda was the presence of hypoalbuminemia. It therefore appears that hypoalbuminemia is the most proximate cause for the edema observed in kwashiorkor and marasmic kwashiorkor (Waterlow, 1984).

The etiology of hypoalbuminemia is unclear too. Apart from the decrease caused through a lowered intake of protein and the influence of hormones, which has been discussed earlier, albumin loss can occur through the gut. Dosseter and Whittle (1975) have shown that the sharp fall in serum albumin levels could be due to gut loss. Measles has been associated with the onset of kwashiorkor in children. The intestinal wall which is already thin in an undernourished child, in combination with the effect of measles on all epithelial tissue, including the mucosa of the digestive tract, could possibly allow for the significant loss of albumin observed (Whitehead, 1980).

Recent experiments in rats (Lunn and Austin, 1983) have shown that hypoalbuminemia occurs mostly as a consequence of the metabolic response to excess dietary energy. Plasma albumin concentrations were thus observed to be lowest when the dietary energy was greatest. This rat model provides further support for the classical theory.
There can be little doubt that a variety of routes may be followed in the pathogenesis of kwashiorkor with the eventual development of edema possibly through hypoalbuminemia. Many details of the mechanism are still missing but it does appear to be certain that albumin synthesis in the liver and consequently serum albumin levels, are affected by dietary protein intake. A number of environmental factors can interact with diet and therefore it is difficult to determine exactly which form of PEM will manifest itself. Certainly the type of diet that the child is given plays the major role but definitely not exclusive, since various infections are quite often implicated in the pathogenesis of PEM (Alleyne et al., 1977).

PEM IN ZAMBIA

PEM is endemic in Zambia as is evidenced by the fact that it is a common cause for hospital admission. In 1977, PEM was the sixth commonest in-patient diagnosis in Zambian hospitals (Hone, 1986). Studies at St. Francis' Hospital, Katete showed a 22.8% incidence of PEM in admitted children, in 1981 (Hone and Fermor, 1985) while in 1984 it was the second most common in-patient diagnosis (Hone, 1986). PEM is a serious problem at the University Teaching Hospital, Lusaka as well (Bhatt, 1987, pers. comm.) as admissions appear to be on the increase (see Table 1.8)
Table 1.8

PEM ADMISSIONS AND MORTALITY RATES IN ZAMBIA (Bhatt, 1987 pers. comm.)

<table>
<thead>
<tr>
<th>Year</th>
<th>PEM Admissions</th>
<th>Mortality rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>16035</td>
<td>14.7</td>
</tr>
<tr>
<td>1982</td>
<td>18496</td>
<td>16.6</td>
</tr>
<tr>
<td>1983</td>
<td>21442</td>
<td>17.2</td>
</tr>
<tr>
<td>1984</td>
<td>22438</td>
<td>18.2</td>
</tr>
<tr>
<td>1985</td>
<td>26914</td>
<td>18.8</td>
</tr>
</tbody>
</table>

A recent survey (Voorhoeve, 1985) of the general health of Zambian children showed that the mean weight for age of children till the age of two was below that of international standards but that after two there was a remarkable catch-up growth. Between the ages of two and five, 75 per cent were healthy and 23 per cent undernourished of which 7 per cent were seriously malnourished. This percentage is almost double the 4 per cent serious malnutrition which has been quoted for Africa by King (1983).

In Zambia, ignorance is not so much the problem as are social and economic factors (Khan and Gupta, 1977). It has been observed that the role of breastfeeding or large families are not major etiological factors in the development of PEM. However, infections such as gastroenteritis and measles tend to precipitate many of the admissions.
The decline in the economy of the country has been mirrored in the steady increase of malnutrition in children, the most susceptible group in the community. Table 1.8 shows this steady increase in PEM admissions as well as PEM deaths (Bhatt, 1987 pers. comm.). This increase tends to reflect on the nutritional status of the community i.e. lower incomes lead to poor and inadequate diets which result in malnutrition. However, it should be taken into account that the population is increasing rapidly with 50 per cent of Zambians under the age of 15 years and as such the reported increase in number of malnutrition cases might not be such a significant increase in percentage of the total population.

A study of malnourished children at the UTH, Lusaka (Khan and Gupta, 1977) showed that kwashiorkor was the least common (24 per cent) form of PEM. The majority presented as marasmic (35.5 per cent) or marasmic kwashiorkor (40.15 per cent) cases. Significant difference in the age ranges of the children in the different PEM categories was not observed. Almost all the children were in the 1 - 3 year age range, irrespective of the form of PEM presented.
III AFLATOXINS & PEM

A. INTER-RELATIONSHIPS OF AFLATOXINS WITH NUTRITION

The achievement of optimum health and disease resistance in an animal is mainly a function of nutrient intake and nutrient status. The aflatoxins have been known to interact with nutrition and this is an indirect toxic effect of the aflatoxins. Vitamin, lipid and protein aspects of nutrition are most influenced by aflatoxicosis as has become evident from animal experiments.

The role of protein, its type and level, has been studied in quite considerable detail in relation to an animal's susceptibility to aflatoxin toxicity. The existing evidence is controversial. Aflatoxins have been shown to cause a marked inhibition of protein synthesis (Clifford and Rees, 1967; Tung et al. 1975). Thus the toxicity of aflatoxins at different levels of protein nutrition was studied. It was observed that in short-term experiments, susceptibility to fairly high (acute) doses of aflatoxin was increased when accompanied by decreased dietary protein (Madhavan et al., 1965; Sisk and Carlton, 1972). Increased protein levels however were shown to protect chickens from a growth inhibitory level of aflatoxins (Smith et al., 1971).

On the other hand, decreased dietary protein levels increased resistance of monkeys fed low levels of aflatoxin over an extended period of time i.e. a chronic dose (Madhavan and Gopalan, 1968). This was further confirmed by Wells et al. (1975) who observed that
low levels of dietary protein was associated with a marked decrease in both the incidence and severity of aflatoxin-induced tumors. It has also been observed that the proportion of aflatoxins which covalently bind to rat liver nuclear DNA is greatly reduced in rats on a low protein diet (Preston et al., 1976). The activity of the MFO was shown to be reduced by a low protein diet (Adekunle et al., 1977) and this it was thought was what caused the decreased tumor incidence. It has recently been reported that aflatoxin B<sub>1</sub> initiates a toxic lesion whatever the state of protein intake is but that the level of protein intake after its administration determines whether or not these lesions eventually develop and grow (Dunaif and Campbell, 1987). Thus a high protein intake appears to stimulate the emergence and further growth of presumptive preneoplastic foci in rat liver.

In conclusion, an increased dietary protein level can decrease the toxicity of aflatoxin and at the same time increase its carcinogenicity. This is important when the results are extrapolated to the effect of dietary protein on the toxic effects of aflatoxin. In developing countries where protein deficiency is common, this nutritional state may actually be protective when considering the low, chronic doses of aflatoxin that are more commonly found rather than high acute doses.

Smith et al. (1971) observed that the fat content of a diet had a profound effect on aflatoxicosis. A mortality sparing effect was observed in chickens on a high lipid diet
with aflatoxin present, regardless of the degree of unsaturation. However the growth sparing effect of a high lipid diet with aflatoxin was observed to be proportional to the degree of unsaturation, the lower the unsaturation the less the protection and thus the increase in growth inhibitory effect of aflatoxins. In rats however a saturated fat-containing diet has been shown to exert a protective effect against the severity of liver tumors induced by aflatoxins (Wells et al., 1974). This anomaly could be due to the fact that chickens do not develop liver tumors.

Deficiencies of vitamins A, D or riboflavin have been shown to make animals more sensitive to the toxic effects of aflatoxin (Hamilton, 1977). However, a thiamine deficiency has been shown to exert a protective effect against aflatoxicosis (Hamilton et al., 1974). An explanation put forward is that aflatoxin inhibits lipid transport from the liver while thiamine deficiency stimulates lipid metabolism. The interaction of aflatoxins with vitamins E and K has not as yet been clearly demonstrated (Hamilton, 1977). Recent investigations have revealed that the interaction of aflatoxicosis and vitamins are complex. Classification of vitamin deficiencies into those that exacerbate aflatoxin's effects, those that protect against aflatoxin and those without any detectable effect depends on whether the animal is in an iso, hypo, or hypervitaminotic state, species, age, macronutrients and environmental factors such as exposure to light (Hagler and Hamilton, 1982).
Carbohydrate metabolism in relation to aflatoxicosis has not been studied in great detail. It has been reported, though, that during aflatoxicosis there are alterations in the enzymes that regulate glycogen catabolism and neogenesis (Newberne, 1974). It has also been observed that during aflatoxicosis there is a suggestion of increased glycolysis (Jayanthi Bai et al., 1977). The same study showed that aflatoxins B₁ and G₁ in both the liver and kidney increased the hexose monophosphate (HMP) shunt as did aflatoxin M₁ in the kidney.
B. AFLATOXIN IN RELATION TO PEM

As has been discussed earlier in Section II, kwashiorkor remains a disease syndrome of obscure pathogenesis. Gopalan's postulated concept of dysadaptation (Gopalan, 1968) is not particularly helpful since it only describes the course of events but does not explain the cause. Furthermore, criticisms of the classical theory (McLaren, 1974) meant that even etiology of kwashiorkor was not clear-cut. Consequently many hypotheses have been brought forward to explain this syndrome.

A preliminary report of findings in Sudan (Hendrickse et al., 1982) suggested that a difference existed in the metabolism of aflatoxin between the various categories of PEM. In the initial study of 252 Sudanese children it was observed that the frequency of aflatoxin detection and concentration found in sera was higher in children with kwashiorkor and marasmic kwashiorkor than in the other groups of marasmus and controls. In particular the frequency was significantly higher in the kwashiorkor group as compared to that observed in the control group. Though the frequency of aflatoxin detection in urine was found to be greatest in the kwashiorkor group, the average concentration was the lowest. Two alternative explanations were brought forward
to explain these findings. Firstly that this was possibly a
reflection of greater exposure to the aflatoxins in the kwashiorkor
group; or secondly, that children in the kwashiorkor group,
due to derangements in the liver, were unable to transport and
excrete the aflatoxins.

Differences in the aflatoxin detected was also observed
in the various categories. Aflatoxicol was the metabolite
detected most frequently in the sera of kwashiorkor and marasmic
kwashiorkor children. However, aflatoxicol was not detected
in any of the urine samples screened. The occurrence of aflatoxicol
in the sera of only kwashiorkor and marasmic kwashiorkor was
suggested as signifying a fundamental difference in the metabolism
of aflatoxin in these two groups. Its absence in urine samples
was not explained.

These preliminary findings were part of an extended study
by Professor R.G. Hendrickse of the Liverpool School of Tropical
Medicine and his colleagues. It sought to investigate the relationship
between aflatoxins and PEM, in particular kwashiorkor. These
results undoubtedly do show that children with kwashiorkor are
more susceptible to aflatoxins but it was not possible to
determine from the data whether this was a causal role relationship
or if kwashiorkor caused the increased susceptibility. Close to
600 samples have so far been analyzed (Coulter et al., 1986a).
Reports on the progress of the extended study have shown results
which are basically in agreement with the original findings of
Hendrickse et al. (1982). However earlier reports (Hendrickse et al.,
1982; 1983) showed that urinary aflatoxins were less often present
in kwashiorkor as compared to other groups, whereas later results showed that the excretion of aflatoxin in urine was similar in all the groups (Hendrickse, 1984; 1985). Recent results show that urinary aflatoxin are in fact more frequently detected in marasmic kwashiorkor and marasmus children (Coulter et al., 1986a). Another difference found during the course of their investigations was the detection of aflatoxicol in urine from children in all groups, including controls in whose sera aflatoxicol had not been detected (Hendrickse, 1984). Analysis of sera from Zimbabwe has also shown a higher frequency of aflatoxin in kwashiorkor (48 per cent) and marasmic kwashiorkor children (75 per cent) (Hendrickse, 1984) than controls.

Following the preliminary findings, analyses of autopsy liver samples of PEM children from Nigeria and South Africa were performed (Lamplugh and Hendrickse, 1982). Only aflatoxin $B_1$ was detected in all three kwashiorkor samples analyzed, aflatoxicol and aflatoxin $B_1$ were detected in the three marasmic kwashiorkor samples while no aflatoxins were detected in the marasmic sample. These results appeared to support the findings in urine and serum samples which showed an association between aflatoxin and kwashiorkor, though the numbers were rather small to draw conclusions. A later report (Hendrickse et al., 1984) which included results of more autopsy samples however showed similar findings. Recently aflatoxins have been detected in 22 autopsy samples from kwashiorkor children in Ghana (Apeagyei et al., 1986). Aflatoxin $B_1$ was detected in the livers of 20 children while in
the remaining two, aflatoxicol was detected. It is interesting to note that fatty change and parenchymal necrosis showed no consistent correlation with the concentration of aflatoxin $B_1$ detected in the livers. However, it was observed that the liver samples with the lowest level of aflatoxin $B_1$ (62 pg/g) showed only mild fatty change. Another interesting finding is that the two samples in which aflatoxicol was detected had some of the highest degree of fatty change observed, though in neither was parenchymal necrosis observed.

Due to the difficulty of obtaining autopsy samples in Sudan, percutaneous needle liver biopsies were obtained instead (Coulter et al., 1986 b) from children with PEM as well as those with miscellaneous liver diseases. Aflatoxins were detected in 31 per cent of the kwashiorkor biopsies but in none of the marasmic or marasmic kwashiorkor biopsies. Interestingly aflatoxin was detected in a high percentage (42 per cent) of biopsies from children with chronic liver disease. The differences noted were that aflatoxins $B_1$, $B_2$ and aflatoxicol were detected in kwashiorkor cases while only aflatoxins $G_1$, $G_2$ and $M_2$ in the cases of liver disease. It was also reported that a very high concentration of aflatoxicol (68.9 ng/g) was detected in an 8-week-old infant with neonatal hepatitis, who appeared to have been exclusively breastfed.

These findings appear to be the only report of the detection of aflatoxins in liver biopsies. The results obtained are therefore of considerable interest in that it gives a certain insight into
the metabolism of aflatoxins in living subjects. Detection of aflatoxin B₁ and aflatoxicol in the kwashiorkor children is in keeping with the earlier findings of autopsy samples. However, the total absence of aflatoxin B₁ in any of the liver samples from children with chronic liver disease is surprising since aflatoxin B₁ is the aflatoxin that has been predominantly found in autopsy samples in previous reports. Aflatoxin G₁, which was found in highest concentrations, is believed to be more toxic to the kidney than the liver (Butler, 1969).

The presence of the high levels of aflatoxicol detected in the infant is pertinent to this discussion since it indicates that either in utero transfer of aflatoxins occurred or the breastmilk consumed had been contaminated with aflatoxin. Aflatoxins have been shown to transfer across the porcine placenta, one of the most complex (epitheliochorial) with six layers separating the maternal and fetal blood (Pier et al., 1985). Therefore one can expect aflatoxins to transfer in utero in any placentate species including humans. The other alternative is a more likely possibility as an earlier study had detected aflatoxin M₁ and/or M₂ in one-third of breastmilk samples from Sudanese mothers (Coulter et al., 1984).

These findings of this group are the only ones the writer is aware of on the association of aflatoxins with kwashiorkor. Analysis of urine, serum, liver and even fecal samples (Hendrickse, 1985) have indicated that aflatoxin metabolism in kwashiorkor children is different from that of normal children. An impaired
metabolism is clearly shown in that the ratio aflatoxins $M_1 : M_2$
in both urine and serum, was lower in kwashiorkor children
than in controls (Coulter et al., 1986a). This implies that
transformation of aflatoxin $B_1$ to the less toxic aflatoxin $M_1$
is less efficient in the former.

Analysis of human samples was corroborated with food studies
in the Sudan (Hendrickse, 1984). It was found that a variety
of commonly eaten foods were contaminated with aflatoxins though
the level of contamination has not been reported. The incidence
of aflatoxins in raw foods was found to be as high as 8 per cent.
A 'food on the plate' study carried out showed a fairly high incidence
of aflatoxin (46 per cent). It therefore appears that the people
of Sudan are definitely exposed to a high incidence of aflatoxin
in food. However, this does not appear to be reflected in the
incidence of aflatoxins in control children sampled which should
give an indication of the level of contamination that occurs
in the general population. Whether aflatoxins are causally related
to the etiology of kwashiorkor is a question that has as yet
not been clearly answered.
C. PROPOSED STUDY OF CORRELATION OF PEM AND AFLATOXINS IN ZAMBIA

In view of the findings of Professor Hendrickse and his co-workers in Sudan, Ghana, Nigeria, Liberia, South Africa and Zimbabwe, there was clearly a need for a similar investigation to be carried out in Zambia. Aflatoxins have been detected in food samples which include 'plate food' samples. (Lovelace and Salter, 1979; Njapau, pers. comm.). Aflatoxin incidence in these samples and consequently exposure of the general population to aflatoxins has been shown to vary significantly with a higher incidence in the rainy season. Seasonal prevalence of PEM admissions has also been observed both at the University Teaching Hospital (UTH) Lusaka (Fig. 1.9) and St. Francis' Hospital, Katete (Fig. 1.10), with a peak incidence occurring between November and April, the rainy season in Zambia.

Studies on breast milk samples have shown aflatoxin M₁ contamination at a level of 10 - 100 ng/100ml (Bayley, pers. comm.) which indicates that young children may be exposed to aflatoxins through their mothers. Put together, all these findings indicated that it was necessary to investigate the possible association between the exposure of aflatoxin at an early age and the development of PEM, in particular kwashiorkor. As Professor Hendrickse suggests, a situation can be envisaged in which, after an initial insult to the liver, impaired ability to handle and excrete aflatoxins created a vicious cycle in which aflatoxin accumulates in the system as the ability to
Fig. 1.10. P.E.M. INCIDENCE AT ST. FRANCIS' HOSPITAL, KATETE.
metabolize and eliminate these toxins declines.

It was therefore decided to carry out a one-year survey in which urine and serum samples would be collected from PEM children and appropriate controls. The PEM cases chosen would be characterized as kwashiorkor, marasmic kwashiorkor or marasmic to determine any possible differences between the groups in the metabolism of aflatoxins. The urine samples would be analyzed by TLC for aflatoxins while the serum samples, due to the small volumes that are obtained from such children, would be analyzed by HPLC. Serum total protein and albumin would also be determined as additional confirmation of the initial diagnosis made on admission of the patient. The one-year survey was to determine if there was a correlating seasonal prevalence between aflatoxin incidence and the type of PEM manifesting in a child. Any correlations between the total protein and/or albumin levels in the blood and the presence of aflatoxins were also hoped to be observed.

Studies have shown that conjugation reactions in animals play an important role in the excretion of aflatoxins. However, water-soluble conjugates of aflatoxin metabolites have not been observed in patients known to excrete high levels of free aflatoxin metabolites in urine (Dil, 1986) and it was therefore decided not to test for conjugates in urine. Instead conjugates were to be tested in the aqueous phase of serum samples. Conjugates in human serum have not been previously reported and so it was hoped that if detected, this would provide information on the amount of aflatoxin ingested and insight into the mechanism of detoxification available in children with PEM.
Since aflatoxin contamination had been shown in food samples from the Eastern Province it was decided to carry out concurrent studies at UTH, Lusaka and St. Francis' Hospital, Katete. This it was hoped would show if there were any differences in the incidence of aflatoxins in PEM between a rural and an urban area.
CHAPTER TWO

METHODOLOGY
I SAMPLING

A. SELECTION OF SUBJECTS

Patients were selected within 24 hours of admission to the Pediatrics Wards of the University Teaching Hospital, Lusaka or St. Francis' Hospital, Katete. The University Teaching Hospital (U.T.H.), as the only hospital in Lusaka and for a considerable area around Lusaka, was chosen for the purpose of obtaining a good representative sample from the Lusaka area. This area is within a radius of approximately 60 km and includes some rural area. However, most of the patients were from the city (i.e. urban area), though mostly from the low-income areas. Similarly, St. Francis' Hospital is the only hospital in Katete and serves many of the surrounding villages, at least with a radius of about 100 km and sometimes more. Though there are health centres in each town and a hospital in Lusaka and Chipata the only hospital in between is St. Francis' in Katete. The map (Fig. 2.1) shows the location of the two hospitals included in this study.

The sex, age and weight of each child was recorded and an initial diagnosis of kwashiorkor, marasmic kwashiorkor or marasmus made by the assisting doctor, based on the Wellcome Classification (Lancet, 1970). Cases of nephrotic syndrome were at times misdiagnosed as early kwashiorkor cases due to
Fig. 2.1
PROVINCES AND COMMERCIAL CROPS IN CENTRAL AND EASTERN PROVINCES

LEGEND
International Bound. ————
Provincial Boundary ————
Provincial Centres —■■
Main Road ————
the presence of edema and so it was therefore necessary to monitor some of the questioned kwashiorkor cases to ensure that this did not happen. Control patients were age-matched children (as far as possible) admitted for minor illnesses whose weight was over 80 per cent of the Boston fiftieth centile value (Stuart and Stevenson, 1959). All the children underwent clinical examination, which included palpation of the abdominal area to observe possible enlargement of the liver and/or spleen, extent and position of edema as well as changes in the skin and hair. A questionnaire was filled for each patient with details of their personal history and clinical examination included (Fig. 2.2).

B. SAMPLE COLLECTION FROM MAY 1985 – MAY 1986

It was intended that both a blood and urine sample would be obtained from each patient selected for the study. Unfortunately, owing to practical constraints most children had only blood or urine samples taken. Collection of more than one urine sample within the 24 hour period, from the malnourished children, proved practically impossible. However, one urine sample was considered sufficient to show the presence or absence of aflatoxins as it has been shown that in monkeys, mice and rats aflatoxin levels in the chloroform extractable portion drop sharply after 24 hours (Wong and Hsieh, 1974). Both the urine and blood samples were obtained within 24 hours of admission.
AFLATOXIN - P.E.M. PROJECT

Name: ---------------------------------
Age: ----------- Sex: M F Ward: ---------
Weight: ------------------ Height: --------
Address: ---------------------- Hospital No.: --------
Date of Admission: ---------

Provisional Diagnosis: ---------

1st Urine Sample: ------------ Code No.:  
(date and time taken)

2nd Urine Sample: ------------ Code No.:  
(date and time taken)

Blood 2-10 ml: -------------- Code No.:  
(date and time taken)

Examination:

Liver  Yes  No  ------  Cm  RCM
Oedema  Yes  No

Diagnosis Confirmed:  Yes  No

Laboratory Report: ----------------------------------------

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COMMENT: ----------------------------------------

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e.g. of Code No.:

1st urine sample

JK  /  8  /  4  /  85  /  A
Initials  date  month  year  1st urine

2nd urine sample

JK  /  8  /  4  /  85  /  B
Blood sample  JK  /  8  /  4  /  85  /  S

Fig. 2.2  Data sheet for urine and serum sample collection.
It was hoped to collect ten ml of blood from each child but this was not always possible and so a range of 4 - 10 ml was collected. A minimum of four ml was maintained to ensure that sufficient serum could be obtained. The blood was drawn by venipuncture from the femoral vein into a sterilized plain bottle. It was allowed to clot at room temperature for two hours, centrifuged and the serum, thus obtained, was stored at -20°C until analyzed.

At the UTH, the help of the mothers was employed in the collection of the urine samples from their children as the nurses were preoccupied with their other duties. Children over one year were usually able to have their urine collected voluntarily but for the rest a urine bag was employed for collection. However, as the mothers are not permitted to stay in the wards for very long many urine samples were lost and this meant fewer samples were obtained from UTH. Urine collection at St. Francis' Hospital was possible entirely through the use of urine bags and since this is a much smaller hospital care was taken to ensure that very few samples were lost. All the urine samples were stored at -20°C until they were analyzed. The samples from Katete were stored at -20°C and transported to Lusaka, packed in dry ice to keep the samples frozen.
II ANALYTICAL METHODS

A. Apparatus

1. U.V. Spectrophotometer, Shimadzu UV - 120, Shimadzu Co., Kyoto, Japan
2. Extrelut columns (4 x 15cm), E. Merck, Darmstadt, Germany
3. Filter paper (12cm), Whatman Qualitative No. 1, Whatman Ltd, Maidstone, England
4. SEP-PAK silica cartridges (No. 51900), Waters Associates Inc, Milford, MA 01757, U.S.A.
5. Rotary Evaporator, Buchi RE 120, Buchi Laboratoriums - Technik AG, CH - 9230, Flawil/Schweiz.
6. Edwards vacuum pump
7. Techne Dri Block DB-1
9. Hamilton Microliter Syringes, Hamilton Bonaduz, Switzerland.
10. TLC aluminium sheets silica gel 60 (without fluorescent indicator) pre-coated, 0.2mm layer thickness (10 x 10cm, handcut from 20 x 20cm plates), E. Merck, Darmstadt, Germany.
11. U.V. detector, Camag, Germany
12. Camag Unispray, Muttenz/Schweiz und Berlin (West)
13. Liquid Chromatography - Model LC-4A (Shimadzu HPLC, Shimadzu Co., Kyoto, Japan), with Model SIL-IA manual LC injector with septum. Spectrophotometric UV detector model SPD - 2AS (Shimadzu Co., Kyoto, Japan)
14. Bondapak C18 (2.0mm x 30cm)) stainless steel column, Waters Associates Inc. Milford MA 01757, U.S.A. Particle size/shape 10μm/irregular silica Nominal pore size (range) - 125 (50-300μm)
15. Kem-O-Mat 2, Coulter Electronics Ltd., England
B. **Reagents**

1. Solvents - Analar grade solvents (BDH Chemicals, Poole, England) were used throughout. The solvents used for TLC development were distilled prior to preparation of solvent systems. Distilled chloroform with 0.7 percent ethanol was used whenever chloroform was required.

2. Aflatoxin standards were from Sigma Chemical Company, Poole, England and Makor Chemicals Limited, Jerusalem, Israel.


4. Silica Gel G 60, 100 - 200 mesh (E. Merck, Darmstadt, Germany). Activated by drying in oven at 105°C for 1 hr. Once cooled, 1 ml of water was added to 100g activated silica gel and thoroughly mixed. The silica gel was then stored in a dessicating to equilibrate overnight before use.

5. Sodium sulphate, anhydrous, BDH Chemicals, Poole, England.

6. Trifluoroacetic acid (TFA) was stored in a tightly closed bottle in the cold room at 7°C.
C. Determination of Aflatoxin Concentration

The concentrations of the aflatoxin stock solutions was determined spectrophotometrically and the appropriate dilutions subsequently carried out. The spectrophotometer was initially calibrated with 3 solutions of potassium dichromate ($K_2Cr_2O_7$) in sulphuric acid ($H_2SO_4$). The three solutions were prepared as follows:

a) Dissolve 78 mg. $K_2Cr_2O_7$ in 1.0 L 0.009M $H_2SO_4$ to give a concentration of 0.25 mmol/L

b) Dilute 25 ml of (a) to 50 ml with 0.009M $H_2SO_4$ to give a concentration of 0.125 mmol/L

c) Dilute 25 ml of (b) to 50 ml with 0.009M $H_2SO_4$ to give a concentration of 0.0625 mmol/L

The absorbances of solutions a, b and c were determined at maximum absorption, near 350 nm, against 0.009M $H_2SO_4$ as solvent blank. The molar absorptivity coefficient ($\epsilon$) at each conc. was calculated using the Beer-Lambert equation:

$$\epsilon = \frac{A}{C \times L}$$

where $C$ = conc. in mmol/L

$A$ = absorbance at $\lambda$ max

$L$ = pathlength of quartz - face cell in m

Taking the average of the three molar absorptivities, the correction factor (CF) for the instrument used was determined by substituting in the equation:

$$CF = \frac{3160}{\epsilon}$$
where $\varepsilon =$ average molar absorptivity

$$3160 = \text{literature value for } \varepsilon \text{ of } K_2Cr_2O_7$$

If the correction factor did not fall within the range of 0.95 - 1.0, the instrument or the technique was checked, the cause eliminated and the procedure repeated. The same set of cells used in calibration were also used for the determination of aflatoxin concentrations.

As all the standards were dissolved in chloroform (with 0.7 percent ethanol), it was used as the solvent blank. The absorbances of the aflatoxin solutions were measured at wavelength of maximum absorption, close to 350nm. Concentrations of these solutions were then determined using the formula:

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

where $A =$ absorbance

$CF =$ correction factor of instrument

$MW =$ molecular weight of aflatoxin

$\varepsilon =$ molar absorptivity of aflatoxin

The aflatoxin solutions were then returned to their original vials. Normal exposure to UV light during the measurement of absorbances does not appear to result in any observable conversion to photoproducts (AOAC, 1976). Any glassware that was known or suspected to have come into contact with aflatoxin solutions, either in the spectrophotometric determination of concentration or during the course of the experiment, were soaked in 20 per cent $H_2SO_4$ overnight before being washed thoroughly.
D. URINE SAMPLES

a) Extraction of Aflatoxins

The extraction of the aflatoxins present was carried out using the method developed by Hsieh et al. (1981) for aflatoxin M₁ in dairy products which has been successfully used in our laboratory for the other aflatoxins in urine samples (Dil, 1986). Some modifications were made in the procedure to enhance recoveries. As it was not always possible to obtain as much as 20 ml of urine from the malnourished children, it was decided to use volumes between 10 and 20 ml and carry out the procedure as for 20 ml (the volumes used were noted). The recoveries for aflatoxins B₁ and M₁ by this method were approximately 75 percent and approximately 80 percent for aflatoxicol.

An extraction column was set up using an Extrelut tube with 14 g Chemtube Hydromatrix (an inert, hydrophilic matrix) as packing material. A glass funnel containing 4 - 5 g anhydrous sodium sulphate in a fluted 12 cm Whatman No. 1 filter paper was placed in a 250 ml round-bottomed flask and the funnel beneath the Extrelut column. The measured volume of sample was poured into the column and allowed to be absorbed onto the matrix for 10 minutes. The aflatoxins were eluted with 20 ml chloroform–acetone (9:1,v/v) and this extraction step was repeated twice. After each addition the solvent was allowed to run through but the column was not allowed to run dry. Pooling the extracts in the flask, the eluate was then evaporated to near dryness on a rotary
evaporator, at 40°C.

Sep-Pak silica cartridges were used to clean up the sample extract. The residue in the flask was washed with 3 ml diethyl ether - hexane (3:1, v/v) and the wash passed through a Sep-Pak cartridge with the help of a 10 ml syringe fixed onto the longer (inlet) stem of the Sep-Pak. The eluate was discarded and the step repeated once more.

The remaining residue was dissolved in 2 ml chloroform - acetone (4:1, v/v) and passed through the Sep-Pak cartridge into a 10 ml vial (with a teflon-lined screw cap). Repeating this step twice, the eluates were pooled in the vial and evaporated to dryness on a Dri-Block at 40°C under a steady stream of nitrogen gas. Redissolving the residue in 100 μl of chloroform (distilled with 0.7% ethanol added as stabilizer,) the sample extract was ready for thin layer chromatography (TLC). Higher (greater than 0.7%) ethanol concentrations are thought to elute more interferences from columns (Stubblefield and Shotwell, 1981) and it was therefore decided to use distilled chloroform, with 0.7% ethanol added, throughout the procedure. Chloroform was chosen as the solvent for spotting as it has been shown that aflatoxin M₄ dissolves better in chloroform than in benzene - acetonitrile (98:2, v/v) (Purchase and Altenkirk, 1973) and it was expected to observe aflatoxin M₄ in human samples since it is a metabolite of aflatoxin B₁.
b) Analysis of Aflatoxins by Thin layer chromatography

Two-dimensional chromatography was carried out on all the urine sample extracts to clear as much of the background interferences as possible. Merck pre-coated TLC plates were used, after activation at 105°C for one hour. Once reactivated, the TLC plates were stored in a dessicator so that at the time of usage all the plates were of the same activity, thus reducing any possible variations.

Extract and standard spots were applied on a TLC plate as indicated in Fig. 2.3. Care was taken to ensure the standard and extract solutions were applied slowly to keep the spots concise (less than 0.5 cm in diameter) and in diffused light to prevent oxidation. Microsyringes used for spotting were cleaned out thoroughly with chloroform after spotting each time, thus avoiding contamination of sample by standards or vice versa. The following aliquots of each were spotted in the indicated positions: A, 20μl sample extract; B, and C, 5 μl standard solution (10 ng aflatoxicol, 10 ng aflatoxin B₁ and 10 ng aflatoxin M₁). A mixture of the three aflatoxins were applied in the two reference channels and the relative positions of the other aflatoxins was kept in mind during the general screening. If the presence of any other aflatoxins was suspected (other than aflatoxicol, aflatoxin B₁ and aflatoxin M₁) another plate was run with the other aflatoxins in the reference channels.
Fig 2.3 Schematic Representation of Thin Layer Chromatogram

Direction 1 Chloroform: Acetone: Methanol (87:10:3, lined tank)

Direction 2 Diethyl ether: Methanol: Water (92:6.5:1.5, lined tank)

A = Sample spot
B & C = Mixture of Aflatoxin Standards
B' & C' = Aflatoxin B₁ Standards
Various solvent systems were experimented with to determine the best for the separation of aflatoxicol, aflatoxin $B_1$ and aflatoxin $M_1$. The solvent system eventually decided upon gave good resolution of the aflatoxins as well as clear, well-defined spots. Due to the variability in laboratory temperatures, it was found that better resolution was obtained when the two developing tanks (for the first and second direction) were placed in the cold room at 7°C and development carried out there. To avoid the diffusing of the applied spots it was ensured that there was a distance of 0.5 - 1.0 cm between the solvent level in the developing tank and the spotting place on the TLC plate. As a saturated tank was required for development in either direction, the solvent was left in the tank after development to maintain saturation but this was discarded before developing another plate and fresh solvent used.

In the first direction, the plate was developed in the dark using a solvent system of chloroform - acetone - methanol (87 : 10 : 3, v/v/v) in a saturated tank (Van Egmond pers comm., 1987). After development, the plate was dried in the dark for 15 minutes. Development in the second direction was carried out in a solvent system of diethyl ether - methanol - water (92 : 6.5 : 1.5, v/v/v) in a saturated tank and in the dark (Dil, 1986). The plate was examined under ultraviolet light (UV), at 365 nm wavelength and any fluorescence observed was compared with that of the mixture of standards in the reference channel. The relative position of some standards are shown in Fig. 2.4.
Fig. 2.4 Relative Positions of some Aflatoxin Standards by TLC

1 = Aflatoxin B₁
2 = Aflatoxin B₂
3 = Aflatoxin G₁
4 = Aflatoxin G₂
5 = Aflatoxicol
6 = Aflatoxin M₁
7 = Aflatoxin P₁

(Solvent used: Diethyl ether: Methanol: Water (92:6.5:1.5, lined tank))
Many confirmatory tests have been developed for aflatoxins and the principle is usually based on the addition of a hydroxyl group to the ether end of an enol ether unit under the catalytic influence of a strong acid, thus making it more polar. In 1964, Andrellos and Reid developed confirmatory tests for aflatoxin $B_1$ by treating it with formic acid thionylchloride, acetic acid thionylchloride and trifluoroacetic acid, respectively. The resultant products were separated by TLC and then observed under UV light. Addition being at the isolated double bond, the fluorescence spectrum is not significantly altered but an alteration in chromatographic behaviour does occur. It was found that the same tests were equally applicable to aflatoxin $G_1$ (Andrellos and Reid, 1964). Due to the simplicity of the method based on the trifluoroacetic acid (TFA) - catalyzed derivatization of aflatoxin $B_1$ and the presence of the same unsaturation in the furan ring of aflatoxin $M_1$, the method was successfully applied to aflatoxin $M_1$ (Trucksess, 1976).

For the purpose of this study it was decided to spray the plates with 25% sulphuric acid (Przybylski, 1975), which changes the fluorescence of the aflatoxins from blue or blue-green to yellow. This was the confirmatory test for the presence of aflatoxins in the sample extracts during the screening process. However, other compounds are changed by sulphuric acid as well and so this test only really confirms the absence of aflatoxins in the sample extract when a color change does not occur. On the other hand, if the $R_f$ value of the standard and fluorescent
spot in the sample were comparable (aflatoxins in the sample
lag slightly behind that of the pure standards due to the
presence of interfering substances in the sample), and there
was a similar color change when sprayed with sulphuric acid
the sample was considered positive. Additional
confirmation was carried out using the official method of the
AOAC, adopted from Przybylski (1975), in which aflatoxin B\textsubscript{1}
is converted into its hemiacetal (B\textsubscript{2a}) by the catalytic
action of trifluoroacetic acid (TFA).

The method of Van Egmond et al. (1978) was chosen to
derivatize aflatoxins B\textsubscript{1} and M\textsubscript{1} after separation by two-
dimensional TLC. The locations of the suspected aflatoxin
and standard spots on the plate were carefully marked with a
pencil. Each spot was treated with 1 - 2 \( \mu \)L TFA using a
microsyringe and the plate placed in a dark cupboard for 3-5
minutes, at room temperature. The TLC plate, on a pre-heated
glass plate (20 x 20 cm), was then put into the oven set at 75\(^\circ\)C.
Covering the treated spots with another heated glass plate the
TLC plate, between the two glass plates, was heated for 5
minutes. It was then allowed to cool for 1 minute, on a cold
surface. Development was carried out in the first direction in
a solvent system of chloroform - methanol-acetic acid
(90 : 10 : 1, v/v/v). The above solvent system was chosen as it
has been reported (Van Egmond et al., 1978) and found to be the
better developing mixture for the derivatized aflatoxins on the
Merck TLC plates that were used in this study.
The concentration of aflatoxins in the sample was calculated using the formula:

\[
\text{ng/ml} = \frac{C \times V_s}{V_1 \times V_2 \times V_3}
\]

where \( C \) = conc. of AF std in \( \mu g/ml \)

\( V_s \) = vol of std. spot giving fluorescent intensity equal to that of sample in \( \mu l \)

\( V_1 \) = final vol of sample extract in vial in \( \mu l \)

\( V_2 \) = vol of sample extract applied in \( \mu l \)

\( V_3 \) = vol of sample represented by final extract in ml.

The limit of detection of aflatoxin B₁ on a TLC plate was 0.5ng when applied as a pure standard which decreased to 0.8ng when mixed with an extract due to the background interferences.

E. SERUM SAMPLES

a) Extraction of Aflatoxins

It was proposed to use the method of Nelson et al. (1980) which was used by Hendrickse et al. (1982) for the determination of aflatoxins in serum from children. The serum is partitioned with hexane to remove the lipids and then extracted with chloroform. However, problems occurred after the addition of hexane and therefore the method was modified accordingly. On mixing equal volumes of serum and hexane in a separatory funnel a gel formed quite rapidly and the bottom
layer was impossible to remove. In a few instances if the serum was removed almost immediately, it was possible to proceed to the extraction step. However, on addition of the chloroform the two expected layers (aqueous and organic) could not be separated as a gel formed once again. So it was decided to dilute the serum with distilled water and then partition with an increased volume of hexane. The mixing was then done very gently and the partitioned serum obtained with little difficulty.

Initially, 4ml distilled water was added to 2ml serum, in a 20ml separatory funnel. To this, 10ml hexane was added and the separatory funnel gently inverted three times. The upper hexane layer containing the serum lipids was discarded and the procedure repeated twice with further 10ml aliquots of hexane. A few drops of saturated solution of sodium chloride was added to the aqueous phase obtained, before the extraction with chloroform. The serum was extracted four times with 10ml aliquots of chloroform. For each extraction, 10ml chloroform was added to the aqueous phase and the separatory funnel inverted three times. A glass funnel containing 5g anhydrous sodium sulphate in a fluted 12cm Whatman No. 1 filter paper was held under the separatory funnel and a 100ml round-bottomed flask placed beneath. The layers were allowed to separate and the lower chloroform layer collected in the flask. After the four extractions, 10ml chloroform was used to wash the sodium
sulphate in the filter paper to ensure removal of any aflatoxin residue and finally 5ml acetone was also passed through. The chloroform extracts and wash (both chloroform and acetone) were pooled, 10ml acetone was added (to azeotrope any remaining water (Trucksess and Stoloff, 1979) and the remaining mixture evaporated to near dryness at 40°C, on a rotary evaporator. Three 2 ml aliquots of chloroform were used to wash the sides of the flask and this was transferred to a 10ml vial (teflon-lined screw cap), together with 1ml of acetone. The vials were stored at -20°C until required for analysis. The remaining aqueous layer was also stored at -20°C to be tested for the presence of aflatoxin conjugates.

b) **Analysis of Aflatoxins by**

**High Performance Liquid Chromatography (HPLC)**

From the literature, HPLC appeared to be the most sensitive method for measuring aflatoxin levels in serum. As an HPLC was not available, the analysis of serum was performed at the Department of Biochemistry, University of Zimbabwe. There was very little time to develop a method suitable for HPLC analysis using the available machine. It was therefore decided to follow the reversed phase HPLC method described by Dr. Lamplugh (1983; pers. comm., 1986) as it appeared to be extremely sensitive which was an important criteria considering the small volume of serum that was used in the extraction. However, conditions were quite different from those expected and the results obtained were not very
satisfactory. In spite of this, it was decided to carry out the procedure as best as possible under the circumstances.

In the method described by Dr. Lamplugh (1983), separation of the sample components was achieved on an octadecylsilane bonded to 5µ silica (ODS) column, 25cm x 5mm. A fluorescence detector with a 365nm excitation filter and a 418nm emission filter was used to detect the separated components. The solvent system chosen as the mobile phase was a methanol - water (50 : 50,v/v) mixture pumped through the column at a flow rate of 2 ml/min and pressure of 23.3 MPa. It was found difficult to stabilize the pump with the new mobile phase consisting of methanol - water (50 : 50,v/v) as air bubbles accumulated in the pump, resulting in greatly fluctuating pressures. The problem was solved by passing the solvent system several times through a sintered filter after preparation and this was allowed to settle overnight (or longer if possible) and then argon was hooked onto the degassing system of the machine. As all the standards that had been brought from Zambia had been dissolved in chloroform (with 0.7% ethanol) the standards were injected using chloroform as the injection solvent but this had to be changed as a chloroform peak occurred which obliterated some of the aflatoxin peaks. Instead, the solvent mixture of methanol - water - acetonitrile (25 : 25 : 50,v/v/v), used for HPLC analysis by Lamplugh (1983), was tried and as this did not interfere with the aflatoxin peaks it was used as the
injection solvent system for subsequent analysis of standards and sample extracts.

Several concentrations of aflatoxin standards $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and aflatoxicol, were analyzed and plots of peak heights against the various concentrations drawn. Quantification of the aflatoxins in the sample extracts was therefore determined by measurement of peak heights. The various aflatoxins in the mixture of standards were clearly separated though the recorded peaks were not very sharp. The order of elution was aflatoxin $M_1$, $G_2$, $G_1$, $B_2$, $B_1$, and aflatoxicol with aflatoxin $M_1$ eluted at 5.8 minutes and aflatoxicol at 11 minutes (Fig.2.5).

The samples and standards were analysed after being redissolved in the solvent system chosen: methanol - water - acetonitrile (25 : 25 : 50, v/v/v). The sample extracts were redissolved in 100µl of the above solvent, of which 20µl was injected through the septum of the manual injector. Appropriate volumes of the standards were also injected at regular intervals to ensure that the retention times of the various aflatoxins had not changed. Identification was done by comparing retention times of the standard and sample peaks. The presence of an aflatoxin was confirmed by spiking an aliquot of the sample extract with a comparable quantity of the toxin in question, before injection.

In order to obtain better resolution, 1 percent acetic acid was added to the mobile phase. The flow rate was maintained at 2.0mls/min and the UV detector set at a wavelength of 350nm with an absorbance of 0.01. Various flow rates, wavelengths and
COLUMN: \( \mu \) Bondapak C18
FLOWRATE: 2.0 ml/min.
CHARTSPEED: 5 mm/min
ABSORBANCE
AT 350 nm: 0.01

Fig. 2.5: HIGH PERFORMANCE LIQUID CHROMATOGRAM
OF A SAMPLE SPIKED WITH AFLATOXIN STANDARDS
absorbance levels were experimented with until the ones above were chosen, as in combination they gave the best resolution and most consistent retention times. The pressures remained variable but as long as the flow rate was maintained and the fluctuation was not too great, this was not considered all that important. It was suspected that a few air bubbles continued to enter the pump even after precautions were taken and that this was the cause for the variable pressures. At the end of the day the whole system was flushed with a 90 percent solution of methanol and this served to clear the column of any residual aflatoxin as well as clear the pump of any air bubbles caught in it.

c) Analysis of Aflatoxins by Thin Layer Chromatography (TLC)

On return to Zambia, all the samples were re-analyzed by TLC. The samples had been evaporated to dryness for the journey back to Zambia and so they were redissolved in chloroform (with 0.7 percent ethanol) and stored at -20°C until required for analysis. It was constantly sought to keep the sample extracts in solution as it has been reported that aflatoxins stored as dry films, in glass vials, for long periods are adsorbed to the surface (Rodricks, 1969).

Before analysis, the sample extracts were evaporated to dryness on a Dri-Block at 40°C, under a stream of nitrogen. The samples being analyzed were redissolved in the appropriate volumes of chloroform (subtracting the amount used for the HPLC
analysis) and 20 μl spotted on a 10 x 10 cm TLC plate (as described before). As serum samples are relatively clear of chromatographic interferences (compared to urine samples) it was found that development in one dimension was sufficient. Therefore, 9 samples could be spotted on the same TLC plate and in the middle a mixture of aflatoxin B₁, aflatoxin M₁ and aflatoxicol. The solvent system used was diethyl ether-methanol - water (92 : 6.5 : 1.5, v/v/v).

d) Analysis of Total Protein and Albumin Concentrations

The serum total protein and serum albumin of all the samples was determined automatically with a Kem - O - Mat 2. The quantitative determination of serum total protein is based on the biuret test (Gornall et al, 1949). A color reaction is the basis of the test in which cupric ions, in an alkaline solution, form a violet-colored complex with protein. The color produced is directly proportional to the protein concentration and the absorbance of this colored complex was determined by a spectrophotometer set at 540 nm. From each serum sample 30 μl was taken for determination of total protein.

Serum albumin was similarly determined on the Kem - O - Mat 2 with the test being based on the binding of bromocresol green to serum albumin, at a controlled pH range, to produce a colored complex. The intensity of color produced is directly proportional to the albumin concentration and the absorbance read at 635 nm. The sample volume used for this determination was 40 μl.
**Determination of Conjugated Aflatoxin Metabolites in Serum**

The aqueous fraction of the serum sample extract containing water-soluble aflatoxin conjugates was acid hydrolyzed to permit analysis of the aflatoxins in the free form, as described by Gregory and Manley (1982). The aqueous phase was diluted with an equal volume of 0.4 M HCL, mixed and incubated for 2 hours in a 90°C water bath. After cooling the mixture to ambient temperature, it was extracted twice in a separatory funnel with 20 ml aliquots of chloroform (with 0.7 percent ethanol) and then once with 20ml aliquot of chloroform-acetone (50:50,v/v). Pooling the extracts in a 100ml round-bottomed flask and it was evaporated to near dryness on a rotary evaporator, under vacuum, at 40°C.

Preparation of the silica column for the clean-up of the above extracts was carried out following modification of Gregory and Manley (1981) procedure. Silica gel (100 - 200 mesh) was substituted for the silicic acid (100 - 200 mesh) since the latter could not be obtained. Both of them are obtained through the acidification of sodium silicate with the result that they are difficult to differentiate (Rose, 1966). A small wad of cotton wool previously soaked in chloroform was placed at the bottom of the column and about 5ml chloroform poured into the column. Using a stirring rod any air bubbles in the cotton wool were removed and then about 1g anhydrous sodium sulphate was added into the column. A slurry of 2g silica gel (1 percent water), prepared in 10ml chloroform was then poured into the
column. When the gel had settled it was topped with 1g anhydrous sodium sulphate and the chloroform drained to the top of the sodium sulphate layer.

The dried sample extract was redissolved in 2ml chloroform and applied to the top of the column. Once again the column was drained to the top of the sodium sulphate layer. The flask was further rinsed twice with 2 ml aliquots of chloroform and the rinses drained into the column. Elution of the aflatoxins was performed with 15ml chloroform - methanol (95: 5) (Gregory and Manley, 1982) and then 15ml chloroform - methanol (95 : 10) passed through the column, by gravity flow, into a 50ml round-bottomed flask. The eluates were pooled and then evaporated to dryness on a rotary evaporator, under vacuum, at 40°C.

Three 2ml aliquots of chloroform - acetone (4 : 1) were used to redissolve the dried residues and this was transferred to a 10ml screw-topped vial. Under a stream of nitrogen gas, the contents of the vial was evaporated to dryness at 40°C. The residue was dissolved in 50μl chloroform, of which 20μl was spotted on a TLC plate.

A mixture of aflatoxins was spotted on the TLC plate along with the samples and the TLC plate developed in one dimension, in a solvent system of diethyl ether - methanol - water (92 : 6.5 : 1.5, v/v/v). The mixture consisted of aflatoxin B₁, aflatoxin M₁, aflatoxin P₁, and aflatoxicol which may be conjugated as glucuronides or sulphates (Gregory and Manley 1982). The TLC procedure was performed as described before.
CHAPTER THREE

RESULTS
I URINE SAMPLES

A. AFLATOXINS IN URINE SAMPLES FROM ST. FRANCIS' HOSPITAL, KATETE, MAY - JUNE 1984

A pilot study was first carried out to determine the efficacy of carrying out a study in Zambia on the association between aflatoxin and kwashiorkor, as reported by Hendrickse et al. (1982). Urine samples from 69 patients, under four years of age, at St. Francis' Hospital were analyzed, of which 23 were from kwashiorkor cases, 15 were from marasmic, 8 were from marasmic kwashiorkor cases and 23 were age-matched controls. The results are presented in Table 3.1.

Only in one patient was the presence of aflatoxin detected and confirmed. Aflatoxin M₁ was the only aflatoxin species detected in the sample and this was at a level of 0.48 ng/ml. The sample, was from a patient diagnosed as a kwashiorkor case. Aflatoxin excretion was not observed in any of the other categories.

An earlier study carried out in April 1984 at the same hospital had shown, out of 23 urine samples tested for aflatoxins, just one positive sample as well. Aflatoxin M₁ was observed at a level of 0.22 ng/ml from a patient with marasmic kwashiorkor.

It is interesting to note that in both studies the only aflatoxin detected was aflatoxin M₁ and that too only in
Table 3.1  URINARY AFLATOXINS IN SAMPLES COLLECTED FROM ST. FRANCIS' HOSPITAL, KATETE, MAY - JUNE 1984

<table>
<thead>
<tr>
<th></th>
<th>Marasmus</th>
<th>Marasmic Kwashiorkor</th>
<th>Kwashiorkor</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>15</td>
<td>8</td>
<td>23</td>
<td>23</td>
<td>69</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Percentage of positive samples</td>
<td>-</td>
<td>-</td>
<td>4.3%</td>
<td>-</td>
<td>1.4%</td>
</tr>
<tr>
<td>Aflatoxin level (ng/ml)</td>
<td>-</td>
<td>-</td>
<td>0.48</td>
<td>-</td>
<td>0.48</td>
</tr>
<tr>
<td>Aflatoxins detected</td>
<td></td>
<td></td>
<td>AFM₁</td>
<td></td>
<td>AFM₁</td>
</tr>
</tbody>
</table>

(* Abbreviations - AFM₁ : aflatoxin M₁)
a kwashiorkor case and a marasmic kwashiorkor case. These findings were different from those of Hendrickse (1985) who reported that aflatoxicol was most frequently detected in kwashiorkor and marasmic kwashiorkor urine samples and that aflatoxin M₁ was least often detected in kwashiorkor urine. However, there did appear to be a difference in the aflatoxin excretion patterns between the various nutritional categories and therefore a one year survey was begun to measure aflatoxins in serum and urine samples from children with PEM and appropriate controls.

B. AFLATOXINS IN URINE SAMPLES FROM ST. FRANCIS' HOSPITAL KATETE, APRIL - AUGUST 1985

A total of 80 urine samples, collected from St. Francis' Hospital by the pediatrician Dr. N. Hone, were analyzed. These samples were obtained from 13 kwashiorkor, 14 marasmic kwashiorkor, 8 marasmic and 45 control patients, under the age of 4 years. The results are tabulated according to their various nutritional categories in Table 3.2, and according to their sex in Table 3.3.

Aflatoxins were detected in 3 urine samples, of which 2 were from control cases and 1 was from a kwashiorkor case. Aflatoxin B₁ and aflatoxicol were the only species detected, though the presence of aflatoxins B₂, G₁, G₂ and M₁ was also looked for. The incidence of aflatoxin excretion was 3.75 per cent, within a range of 0.063 - 0.8 ng/ml and
### Table 3.2  ANALYSIS OF URINARY AFLATOXINS IN SAMPLES COLLECTED FROM ST. FRANCIS' HOSPITAL, KATETE, APRIL - AUGUST, 1985

<table>
<thead>
<tr>
<th></th>
<th>Marasmus</th>
<th>Marasmic Kwashiorkor</th>
<th>Kwashiorkor</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of samples</strong></td>
<td>8</td>
<td>14</td>
<td>13</td>
<td>45</td>
<td>80</td>
</tr>
<tr>
<td><strong>Number of positive samples</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>Percentage of positive samples</strong></td>
<td>-</td>
<td>-</td>
<td>7.7%</td>
<td>4.4%</td>
<td>3.75%</td>
</tr>
<tr>
<td><strong>Aflatoxin range (ng/ml)</strong></td>
<td>-</td>
<td>-</td>
<td>0.0625 -</td>
<td>0.313 -</td>
<td>0.0625 -</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0.125</td>
<td>0.80</td>
<td>0.80 (Mean = 0.33)</td>
</tr>
<tr>
<td><strong>Aflatoxins detected and the concentrations (ng/ml)</strong></td>
<td>(i)*AFL-0.125</td>
<td>(i)AFB&lt;sub&gt;1&lt;/sub&gt;-0.313</td>
<td>AFB&lt;sub&gt;1&lt;/sub&gt;-0.0625</td>
<td>(ii) AFL 0.8</td>
<td>AFB&lt;sub&gt;1&lt;/sub&gt; - 0.188</td>
</tr>
</tbody>
</table>

(* Abbreviations - AFL : aflatoxicol; AFB<sub>1</sub> : aflatoxin B₁ *)
<table>
<thead>
<tr>
<th></th>
<th>KATETE</th>
<th>LUSAKA</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Marasmus</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Marasmic kwashiorkor</td>
<td>4</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Kwashiorkor</td>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>37</td>
<td>18</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>3</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Percentage of positive samples</td>
<td>7%</td>
<td>11%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>
with a mean concentration of 0.33 ng/ml.

The results indicate a higher incidence of aflatoxins in controls and kwashiorkor urine samples than in marasmic or marasmic kwashiorkor urine samples. The incidence of aflatoxin excretion was higher in kwashiorkor than in the controls but the total urinary aflatoxin concentration was lower in the kwashiorkor group. However, this observation was non-significant statistically (p > 0.05). Another point of interest is with respect to the sex of the children whose urine samples were found to be positive, in that they were all male. Unfortunately, all these samples were obtained in the dry season and it was not possible to obtain samples for the entire year from this hospital.

C. AFLATOXINS IN URINE SAMPLES FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986

Urine samples from 34 patients were analyzed, of which 10 were kwashiorkor, 3 were marasmic kwashiorkor, 3 were marasmic and 18 were control patients. Difficulties were encountered in obtaining as many urine samples as were hoped for from children at UTH. The results are presented in Table 3.4, and tabulated according to sex in Table 3.3 and season of the year in Table 3.5.

Aflatoxins were detected in 4 patients, of whom 2 were control cases, 1 was a kwashiorkor case and 1 was a marasmic kwashiorkor case. The incidence of aflatoxin excretion was
<table>
<thead>
<tr>
<th></th>
<th>Marasmus</th>
<th>Marasmic Kwashiorkor</th>
<th>Kwashiorkor</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Percentage of positive samples</td>
<td>-</td>
<td>33%</td>
<td>10%</td>
<td>11.1%</td>
<td>11.8%</td>
</tr>
<tr>
<td>Aflatoxin range (ng/ml)</td>
<td>-</td>
<td>0.5</td>
<td>0.125</td>
<td>0.25-0.63</td>
<td>0.125-0.63 (Mean =0.40)</td>
</tr>
<tr>
<td>Aflatoxins detected and the concentrations</td>
<td>-</td>
<td>(i) AFL- 0.5</td>
<td>(i) AFL- 0.125</td>
<td>(i) AFL- 0.5</td>
<td>AFB₁- 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) AFB₁- 0.25</td>
<td></td>
<td></td>
<td>AFL - 0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4. ANALYSIS OF URINE SAMPLES COLLECTED FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986
Table 3.5  
CLASSIFICATION OF URINE SAMPLES FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986, ACCORDING TO SEASON OF THE YEAR IN WHICH COLLECTED

<table>
<thead>
<tr>
<th></th>
<th>Total no. of samples</th>
<th>No. of positive samples</th>
<th>Percentage of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainy season (October - March)</td>
<td>16</td>
<td>3</td>
<td>18.8%</td>
</tr>
<tr>
<td>Dry season (April - September)</td>
<td>18</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>Marasmus</td>
<td>Marasmic kwashiorkor</td>
<td>Kwashiorkor</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------</td>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>No of samples</td>
<td>11</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>No of positive</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of</td>
<td></td>
<td>5.9%</td>
<td>8.7%</td>
</tr>
<tr>
<td>positive samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxins detected</td>
<td>(i) AFL</td>
<td>(i) AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>(i) AFB&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>and the concentrations</td>
<td>- 0.5</td>
<td>-0.0625</td>
<td>-0.25</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td>AFL-0.125</td>
<td>AFL-0.63</td>
</tr>
<tr>
<td></td>
<td>(ii) AFL-0.125</td>
<td>(ii) AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>AFL-0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.313</td>
<td>(iii) AFL-0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iv) AFL-0.5</td>
<td>(iv) AFL-0.8</td>
</tr>
</tbody>
</table>
11.8 per cent, within a range of 0.125 - 0.63 ng/ml and with a mean concentration of 0.4 ng/ml. Aflatoxicol was the only species detected in the kwashiorkor and marasmic kwashiorkor samples. In the control samples aflatoxin B₁ as well as aflatoxicol were detected.

Fig. 3.1 shows a sample that was positive for aflatoxin B₁ and aflatoxicol. It was observed that in the first direction aflatoxin B₁ was more polar than aflatoxicol, while in the second direction the reverse was observed.

Fig. 3.1. A POSITIVE URINE SAMPLE
II SERUM SAMPLES

A. AFLATOXINS IN SERUM SAMPLES FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986

A total of 108 sera, collected from UTH, were analyzed. Of these samples, 55 were from kwashiorkor, 17 from marasmic kwashiorkor, 11 from marasmic and 25 from control cases. The age range of these children was 9 - 48 months. The samples were analyzed by HPLC, as well as by two-dimensional TLC. Unfortunately, the results from the HPLC analysis did not correspond with those of the TLC analysis.

a) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

The HPLC equipment that was available at the University of Zimbabwe did not have a fluorescent detector and therefore a UV detector was used. As a result the level of detection was not as low as had been expected. Fig. 3.2 shows a plot of the peak heights against the concentration of aflatoxin B₁. A peak height of at least 1 cm was considered necessary to be seen above the base line of the sample and this was at a concentration of 2.8 ng. Lower levels of detection were only possible in pure standards and even that did not fall below 2ng and this is much higher than the level of detection by TLC.
Fig. 3.2 Plot of Peak Heights against the Concentration of Aflatoxin $B_1$
All the samples were analyzed by HPLC. The presence of aflatoxins: $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and aflatoxicol were looked for in the sera, by comparison of peak positions with those of standards. Tentatively, 13 samples were considered to be positive, however the results could not be confirmed so finally they were not considered. (See Fig. 3.3)

b) THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS

The 108 samples analyzed by HPLC were subsequently analyzed by two-dimensional TLC. It was decided to only present the TLC results as they were considered to be more reliable as the levels of detection were much lower than that of HPLC. These results have been presented in Table 3.7 and tabulated according to sex in Table 3.8, and season of the year Table 3.9.

Aflatoxins were detected in 3 samples, of which 2 were from kwashiorkor patients and 1 from a control patient. The incidence of aflatoxin excretion was 3 per cent, within the range 1.7 - 2.1 ng/ml and with a mean concentration of 1.9 ng/ml. Aflatoxin $B_1$ and aflatoxicol were the only species detected in the three samples, though the sera were analyzed for the other aflatoxins as well. Aflatoxicol was only detected in one kwashiorkor sample. The difference in aflatoxin incidence between the groups was not significant ($p > 0.05$).

A seasonal difference was not observed in the incidence of aflatoxins in the serum samples nor was there a significant difference in the aflatoxin incidence between the sexes.
AFLATOXIN B₁

COLUMN: µ Bondapak C₁₈
FLOW RATE: 2.0 ml/min.
CHART SPEED: 5 mm/min.

ABSORBANCE
AT 350 nm : 0.01

Fig. 3.3: HIGH PERFORMANCE LIQUID CHROMATOGRAM OF A POSITIVE SAMPLE
Table 3.7  ANALYSIS OF SERUM SAMPLES COLLECTED FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA
MAY 1985 - MAY 1986

<table>
<thead>
<tr>
<th></th>
<th>Marasmus</th>
<th>Marasmic Kwashiorkor</th>
<th>Kwashiorkor</th>
<th>Control</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>11</td>
<td>17</td>
<td>55</td>
<td>25</td>
<td>108</td>
</tr>
<tr>
<td>No. of positive samples</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Percentage of positive samples</td>
<td>-</td>
<td>-</td>
<td>3.6%</td>
<td>4%</td>
<td>3%</td>
</tr>
<tr>
<td>Aflatoxin range (ng/ml)</td>
<td>-</td>
<td>-</td>
<td>1.7 - 1.8</td>
<td>2.1</td>
<td>1.7 - 2.1 (Mean = 1.9)</td>
</tr>
<tr>
<td>Aflatoxins detected and the concentrations (ng/ml)</td>
<td>(i) AFB-1.7</td>
<td>(i) AFB1- 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ii)AFL-1.8</td>
<td>(ii)AFL- 2.1</td>
<td>(i) AFB1- 2.1</td>
<td>AFL- 1.8</td>
<td></td>
</tr>
<tr>
<td>Classification</td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------</td>
<td>--------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marasmus</td>
<td>4</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marasmic Kwashiorkor</td>
<td>10</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kwashiorkor</td>
<td>28</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of positive samples</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of positive samples</td>
<td>4%</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.9  CLASSIFICATION OF SERUM SAMPLES COLLECTED FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986, WITH RESPECT TO THE SEASON IN WHICH ADMITTED

<table>
<thead>
<tr>
<th>Season</th>
<th>Total no. of Samples</th>
<th>No. of positive samples</th>
<th>Percentage of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainy season</td>
<td>82</td>
<td>2</td>
<td>2.3%</td>
</tr>
<tr>
<td>(October - March)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Season</td>
<td>26</td>
<td>1</td>
<td>3.8%</td>
</tr>
<tr>
<td>(April - September)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
B. **CONJUGATED AFLATOXIN METABOLITES**

In a previous study, conjugated aflatoxin metabolites were looked for in urine samples from adult patients showing liver pathology (Dil, 1986). However, glucuronide and sulphate conjugates of aflatoxin M₁, aflatoxin P₁, and aflatoxicol were not detected in any of the samples, even those previously shown to be positive for free aflatoxin metabolites. For the present study it was decided not to test for conjugated metabolites in urine samples, as the previous study had shown that they could not be that important a route for aflatoxin excretion, added to the fact that the volume of urine obtained in most cases was only just sufficient to test for free aflatoxin metabolites.

Consequently it was decided to test for conjugated aflatoxin metabolites in the aqueous layer fraction of the serum samples. The method used was to acid-hydrolyze the glucuronide and sulphate conjugates. Enzymatic hydrolysis was precluded by the high ionic strength in the aqueous phase of the serum samples.

Conjugates of aflatoxins M₁ and P₁ and aflatoxicol were looked for in serum samples from 20 patients. This included samples that had been suspected to contain free aflatoxin metabolites by HPLC and the samples which were confirmed by TLC. No conjugates were detected in any of these samples by the method that was performed.
C. SERUM TOTAL PROTEIN AND SERUM ALBUMIN

It has been shown that serum albumin concentrations provide the most predictive information in the assessment of malnutrition (Alleyne et al., 1977). Biochemical measurements were therefore considered necessary to complement and confirm the clinical diagnosis made on admission of the patient. The results have been presented in Table 3.10.

The mean serum albumin values in g/l (g/dl) was 34 (3.4) for controls, and 25.3 (2.53), 18.1 (1.81) and 16.3 (1.63) for marasmic, marasmic kwashiorkor and kwashiorkor groups, respectively. It can be seen that though there was a marked reduction in total protein levels in kwashiorkor patients, it did not fall below 50 per cent of the control values as was observed in serum albumin values. This indicates that hypoalbuminemia is a more marked feature of kwashiorkor than is just hypoproteinemia. Concentrations of below 25 g/l have been considered clearly pathological by Alleyne et al. (1977) as below this level many of the children in a study done in Uganda began to show signs of edema. Clearly this is also the case in Zambia as it was found that children showing edema and classified as kwashiorkor and marasmic kwashiorkor cases also had albumin levels below 25 g/l.
Table 3.10 - SERUM TOTAL PROTEIN AND SERUM ALBUMIN LEVELS IN SERUM SAMPLES COLLECTED FROM THE UNIVERSITY TEACHING HOSPITAL, LUSAKA, MAY 1985 - MAY 1986

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Marasmus</th>
<th>Marasmic Kwashiorkor</th>
<th>Kwashiorkor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum total protein in g/l (g/dl)</td>
<td>65 (6.5)</td>
<td>62 (6.2)</td>
<td>44.8 (4.48)</td>
<td>43.7 (4.37)</td>
</tr>
<tr>
<td>Range observed</td>
<td>61 - 68</td>
<td>53 - 67</td>
<td>40 - 51</td>
<td>34 - 62</td>
</tr>
<tr>
<td>Level as a percentage of control value</td>
<td>95%</td>
<td>68%</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>Mean Serum albumin in g/l (g/dl)</td>
<td>34 (3.4)</td>
<td>25.3 (2.53)</td>
<td>18.1 (1.81)</td>
<td>16.3 (1.63)</td>
</tr>
<tr>
<td>Range observed</td>
<td>33 - 37</td>
<td>23 - 27</td>
<td>10 - 28</td>
<td>8 - 25</td>
</tr>
<tr>
<td>Level as a percentage of control value</td>
<td>-</td>
<td>72%</td>
<td>53%</td>
<td>48%</td>
</tr>
</tbody>
</table>

Normal range of Serum total protein in Zambia: 62 - 85 g/l
Normal range of serum albumin in Zambia: 35 - 45 g/l
CHAPTER FOUR

DISCUSSION
The results obtained in this study clearly indicate that young children in Zambia are exposed to aflatoxin and that this may be assayed in their sera and urine. Aflatoxin incidence in urine samples from Lusaka was higher than in samples from Katete, though this difference was not significant \( p > 0.05 \). This is not in agreement with the results of Dil (1986) who observed a significantly higher incidence in samples from Katete. The samples obtained for this study in Lusaka were only 34, compared with 80 from Katete, whereas in the study by Dil (1986), 136 samples were obtained from Lusaka and only 26 from Katete. This may have very well weighted the results observed. The total aflatoxin incidence in urine samples from Katete and Lusaka are similar in the two studies, 6.1 and 5.6 per cent, respectively. Aflatoxins were detected in urines more frequently in the rainy season (18.8 per cent) than in the dry season (5 per cent), but this difference was not significant \( p > 0.05 \). The incidence of total aflatoxin excretion in urine was found to be similar in kwashiorkor, marasmic kwashiorkor and control samples. Aflatoxins were not detected in the marasmus groups but as the sample numbers were small this difference was not significant at \( p = 0.05 \).

Taking the urinary aflatoxin concentrations into account it was interesting to note that aflatoxin excretion was highest in controls (mean of 0.63 ng/ml) and lowest in kwashiorkor (mean of 0.16 ng/ml). The marasmic kwashiorkor group showed intermediate aflatoxin excretory levels (0.5 ng/ml). Aflatoxin \( \text{B}_1 \) was excreted in both controls and kwashiorkor but there was a difference as
the concentration of aflatoxin B₁ in kwashiorkor (0.0625 ng/ml) was lower than the mean aflatoxin B₁ concentration in the controls (0.28 ng/ml).

The mean aflatoxin concentration in positive sera from kwashiorkor patients was found to be similar (1.75 ng/ml) to that of the control (2.1 ng/ml). The presence of aflatoxicol was only detected in a kwashiorkor child and this was found to be statistically significant (p < 0.0001). Though aflatoxins were more frequently detected (6.1 per cent) in urine samples as compared to serum samples (3 per cent), the aflatoxin concentration levels were higher in serum.

From the above discussion on the results the principal differences between kwashiorkor and controls were: the presence of aflatoxicol detected only in one serum from kwashiorkor; the lower mean concentration of urinary aflatoxicol observed in kwashiorkor; and the lower concentration of mean urinary aflatoxin B₁ observed in kwashiorkor.

The level of aflatoxin contamination in commonly eaten foods during the dry season from the Katete area has been previously determined to be 3 per cent (Lovelace and Salter, 1979). Evidently the incidence of aflatoxin detected in the urine samples from Katete (3.8 per cent) reflects the levels of aflatoxin that the people around Katete are exposed to, including children. The incidence of aflatoxin excretion observed in the pilot study which was carried out in the dry season had shown a similar incidence. Dil (1986) had however, observed a 15.4 per cent incidence of aflatoxin excretion in urine samples from Katete. These samples were obtained
in April which is usually taken as being part of the dry season but is at the end of the rainy season. This might possibly explain the increased incidence of aflatoxins observed since the aflatoxin contamination of foods is higher in the rainy season (Njapau, H., pers. comm.). Earlier work in Zambia indicated that aflatoxin contamination was a problem of the rural areas rather than of urban areas. This it was thought was due to the practices of small-scale farming in the rural areas where most of the food is home-grown. Insufficient drying and inadequate storage practices would therefore increase the chances of the growth of *A.flavus*, as well as the production of aflatoxins. The area chosen for the rural study was the Eastern Province (Fig. 2.1) where groundnuts are predominantly grown. Dil (1986) showed in her study that an increased incidence of aflatoxin was indeed found in Katete. However, though the present study showed differently, it should be realized that these samples obtained from Katete were obtained in the dry season.

An interesting finding of this study was the non-detection of aflatoxin M₁ in the sera and urine from children with PEM, as well as the controls. In the pilot study of urine samples, aflatoxin M₁ was the only aflatoxin species detected. Lovelace et al. (1983) similarly reported only the presence of aflatoxin M₁ in the urine samples analyzed. The possibility that the method used was not sensitive enough seems unlikely since the method used was the same as in the pilot study. The highest concentration of aflatoxin M₁ in the pilot study, was detected in a kwashiorkor child which implied that there had been metabolism of aflatoxin B₁ to M₁, a normal detoxification product. Campbell and Hayes (1976) in their review
of aflatoxin M1 toxicity concluded that excreted aflatoxin M1 generally only accounted for a small fraction of an aflatoxin B1 dose in animal studies. However, in the Phillipines, Campbell et al. (1970) observed that aflatoxin M1 was the only aflatoxin excreted in the urine of humans known to have ingested aflatoxin B1. Similarly, Coulter et al. (1986a) report that the incidence of aflatoxin M1 excretion in urine was higher than that of aflatoxin B1 and aflatoxicol. The presence of aflatoxin M1 in serum was also observed in the same study.

The purpose of this study was to investigate the association of aflatoxin in the etiology and pathogenesis of kwashiorkor in Zambia. However, due to the small sample size, it is not possible to draw any meaningful conclusions. The findings of this study will therefore be discussed in the light of the work carried out in the Sudan, where the sample size was considerably larger (Coulter et al., 1986a).

Differences in the mean levels of aflatoxin excreted, with the excretion levels in kwashiorkor children lower than in the controls, suggests that there may be an impairment in kwashiorkor with relation to the excretion of aflatoxin. However, the incidence of aflatoxin was higher in kwashiorkor than in either marasmic kwashiorkor or controls. A similar observation was reported by Hendrickse et al. (1982). In comparison aflatoxins were detected at higher concentrations in the sera of kwashiorkor and controls than in the urine samples. The incidence of aflatoxins in serum was similar in kwashiorkor and controls. This may support the view of Hendrickse
and his co-workers that in kwashiorkor there is a diminished
capacity to transport and excrete the aflatoxins, leading to higher
serum levels and lower urine levels of aflatoxins.

The presence of conjugates in serum of humans has apparently
not been reported so far. Attempts to detect them in human urine
have failed (Merrill and Campbell, 1974a; Campbell and Hayes, 1976).
Conjugates were also looked for in urine samples in Zambia but they
were not detected either (Dil, 1986). The method by Gregory and
Manley (1982) was tried out on the aqueous layer phase from serum
samples. TLC analysis of the acid-hydrolyzed samples did not detect
the conjugates of aflatoxin M\textsubscript{1} and P\textsubscript{1}. As recovery experiments
could not be carried out, it was not possible to determine the
sensitivity of the method, as performed in this study. It is
possible that the levels present were below detectable levels by
TLC analysis. Proteins would be precipitated during the early
stages of the extraction method and therefore the method of Gregory
and Manley (1982) would not detect aflatoxins covalently bound to
macromolecules. It has been reported that aflatoxin forms a
permanent complex in the blood with albumin (Nassar et al., 1982)
suggesting that due to the persistent aflatoxin-albumin conjugate
in the blood the level of aflatoxins excreted is decreased. This
might account for the low excretion levels detected in the urine
of animals, and possibly man.

Aflatoxicol was only detected in the serum of one kwashiorkor
child and not in any of the other nutritional groups. Aflatoxicol
has lower toxicity than aflatoxin B\textsubscript{1} however, due to a reversible
reaction it can serve as an intracellular reservoir of aflatoxin B\textsubscript{1}
in the liver and erythrocytes (Patterson and Roberts, 1972; Chang et al., 1985) and thus thought to prolong cellular exposure to the toxic effects of aflatoxin B₁. It is believed that the bioactivation of aflatoxicol is probably through the conversion to aflatoxin B₁, rather than the other way around (Wong et al., 1979). Aflatoxicol was also detected in the urine samples from kwashiorkor, marasmic kwashiorkor and controls cases, with 3/4 in controls and 2/3 in kwashiorkor, a higher frequency and lower concentrations being observed in the kwashiorkor group. This finding indicates that excretion of aflatoxicol does occur in kwashiorkor but that it is probably impaired.

The presence of aflatoxicol in kwashiorkor serum may indicate that kwashiorkor children are more susceptible to the acute and toxic effects of aflatoxin B₁ since this correlation has been observed with regard to animals (Hsieh et al., 1977; Wong and Hsieh, 1978). Aflatoxicol appears to be highly lipophilic and consequently high levels of distribution have been observed (Wong et al., 1979). If aflatoxicol serves as a reservoir of aflatoxin B₁ and since in vivo conversion of aflatoxicol to aflatoxin B₁ has been reported in hepatocytes (Wong et al., 1979) as well as in vitro conversion by erythrocytes (Chang et al., 1985), then aflatoxicol may very well facilitate the intracellular migration of aflatoxin B₁ to the target sites. Since the presumed 'ultimate carcinogen', aflatoxin B₁-2,3-epoxide is highly reactive, and a protein carrier system to transport the epoxide has not been detected, it is possible that aflatoxicol may increase the intracellular availability of aflatoxin B₁. If this is the case then the bioactivation of aflatoxin B₁ at the target site may lead to the development of
a toxic lesion.

The hypothesis, initially proposed by Hendrickse et al. (1982) that aflatoxins may be causally related to the etiology of and pathogenesis of kwashiorkor is an attractive possibility. It had been previously suggested by Schoental (1974) that hepatotoxins might play a role in the development of a syndrome assumed to be kwashiorkor, on the basis of the history taken. In support of this hypothesis, it has been suggested that the clinical, metabolic and pathological changes observed in aflatoxin toxicity are similar to those found in kwashiorkor e.g. hypoalbuminemia, immunosuppression and fatty liver. As with any hypothesis there have been criticisms as well as support of the suggested explanation.

Long (1982) reported that he had observed symptoms similar to that of kwashiorkor in guinea pigs on an adequate protein diet. It was later shown that aflatoxin was present in the groundnut meal that was fed to the guinea pigs and it was suggested that it was the aflatoxin in the diet that had caused this syndrome. However, this appears to be the only report of a direct kwashiorkor-like syndrome developing with aflatoxin toxicity.

A key feature of kwashiorkor is edema and though this has been observed in cases of aflatoxin toxicity, its occurrence is rare. In the outbreak of hepatitis in India (Krishnamachari et al., 1975), edema of the lower extremities was observed in some people. Similarly, in Kenya Ngindu et al. (1982) reported that edema of the legs and, to a lesser extent, of the face and/or trunk were features of acute hepatitis caused by aflatoxin toxicity.
Hypoalbuminemia, a suggested cause for the observed edema in kwashiorkor, is a feature of aflatoxin toxicity (Tung et al., 1975). Aflatoxin causes inhibition of protein synthesis and thus synthesis of albumin in consequently impaired. Determination of serum albumin levels in this study clearly show increased hypoalbuminemia in the kwashiorkor group as compared to the marasmic group. Marasmic kwashiorkor falls into an intermediate group. In kwashiorkor and aflatoxin toxicity fatty infiltration and hepatomegaly are observed. However, in treated kwashiorkor there is full recovery of the liver and there is no evidence of liver disease and cirrhosis being long-term sequels of this (Cook and Hutt, 1969; Alleyne et al., 1977; Chintu pers. comm., 1987). In contrast necrotic lesions are observed in aflatoxin toxicity which are not found in kwashiorkor (Amla et al., 1971).

Similarities in the seasonal prevalence and world distribution of kwashiorkor and aflatoxin incidence were also brought forward to support the hypothesis. However, studies in Sudan have so far not shown a seasonal prevalence of aflatoxin in urine and serum samples (Coulter et al., 1986a). The absence of a seasonal prevalence of aflatoxins in food samples has been reported in Swaziland as well (Peers et al., 1976). In Zambia the incidence has been observed to be slightly higher in the rainy season than in the dry season, though this was not statistically significant. Earlier studies have however shown a significant increase in incidence of aflatoxin in the rainy season (Lovelace et al., 1983; Dil, 1986). A seasonal prevalence of PEM is clearly indicated in the two hospitals from which the samples for this study were obtained (Fig 1.9 and Fig.1.10)
The world distribution of PEM and aflatoxin incidence is quite similar with a preponderance in the tropics. The warm, humid climate of the tropics make it an ideal environment for fungal growth. On the other hand there is an increased availability of starchy foods and thus an insufficient meal may be made more filling with the addition of extra carbohydrate. However in the drier areas, the diet has a higher protein level since starchy foods are more difficult to obtain. Thus in the event of a shortage of food the diet is insufficient in both protein and carbohydrate. This is quite definitely an over simplification of the problem but it indicates that generalizations can be made. In Zambia, malnutrition in urban areas has been reported to be associated with the economic condition of the family (Khan and Gupta, 1977) and in the rural areas with the rainy season (Hone, N., pers. comm.). However, both factors result in the child receiving less protein, including groundnuts which are a good source of protein as well as a major source of aflatoxins. This suggests that a lack of protein is probably the most proximate cause of kwashiorkor.

An interest point was recently made in reference to the causal role of aflatoxins in kwashiorkor. Professor Chintu (Pers. comm., 1987) commented that it was surprising that a child who ate little food and consequently developed kwashiorkor could be said to have developed it because of aflatoxins ingested, while a child who was given sufficient food remained healthy. If the food was from a common supply, as is the case in the urban areas, then the chance of the healthy child ingesting aflatoxins is much higher. However,
perhaps the case should be considered of the occurrence of kwashiorkor in totally breast-fed infants (Trowell et al., 1954). Aflatoxin excretion has been reported in breastmilk samples from Zambia (Bayley, pers. comm.) and Sudan (Coulter et al., 1984) and therefore this could be implicated as a cause in these cases.

In view of the findings of this study it is tentatively suggested that there may be an association between aflatoxins and kwashiorkor in Zambia. Decreased MFO activity has been associated with protein deficiency (Preston et al., 1976) and this would support Professor Hendrickse's suggestion that as a result of an impaired ability to metabolize aflatoxins a vicious cycle would develop with aflatoxin levels in the serum increasing as the ability to metabolize these aflatoxins progressively declines. The presence of aflatoxicol in this study would thus be understandable since it does not require the MFO for its formation but the cytoplasmic reductases (Patterson and Roberts, 1972). Furthermore, interconversion of aflatoxin B₁ and aflatoxicol in the plasma might also be a source of aflatoxicol. Increased levels of aflatoxin B₁ and/or aflatoxicol in the serum could mean a higher probability of toxic lesions in the various target organs. This might explain the severe hepatic lesions occasionally observed in kwashiorkor cases, quite often fatal and possibly a part of the high mortality rates reported (Alleyne et al., 1977). Any substance that may damage the liver and cause a decrease in levels of albumin could quite easily shift a child towards the kwashiorkor end of the malnutrition spectrum. As Oettlé (1965) has stated 'correlation does not necessarily prove causation' and therefore the available data at best only demonstrate a possible
association between the levels of aflatoxin in serum and urine and the different categories of PEM.

Immunosuppression is a feature of aflatoxin toxicity and it has been suggested that this immunosuppressive effect of the aflatoxins may have a role in the susceptibility of an individual to the Acquired Immune Deficiency Syndrome (AIDS) (Hendrickse, pers. comm.). AIDS is caused by the infection of a host with the Human Immunodeficiency Virus (HIV). It is characterized by a slow or rapid depletion of T cells eventually leading to the total destruction of the host's immunity. This then becomes a case of frank AIDS and the host becomes susceptible to a variety of infections. Eventually the host succumbs to the consequences of these infections and death follows.

Animal experiments on the effects of aflatoxin on immune responses in general show that the levels required to diminish the various immune responses are quite high at levels of over 2 mg/kg. Aflatoxin consumption at these levels would show up as acute aflatoxicosis in humans if the levels in the animal experiments are extrapolated. However, work is still required to determine the effect that aflatoxins in small quantities over an extended period of time would have on the immune responses of humans. The immunosuppressive effects of aflatoxin could be one of a whole series of environmental hazards affecting the population, which could have a similar effect.

In conclusion, the results in this study may be taken to represent preliminary finds. An extended study, similar to the one
being carried out in the Sudan would be necessary to look closely at the effect of aflatoxin on PEM. A survey over a longer period would be required to study the relationship between rainfall levels, crop harvest, aflatoxin contamination and PEM incidence.
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