STUDIES OF THE CAUSES AND MODULATING FACTORS OF LIVER CANCER DEVELOPMENT IN HUMANS AND IN EXPERIMENTAL ANIMAL MODELS.

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF NAIROBI.
DECLARATION:

THIS THESIS IS MY ORIGINAL WORK AND HAS NOT BEEN PRESENTED FOR A DEGREE IN ANY OTHER UNIVERSITY.

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THIS THESIS HAS BEEN SUBMITTED FOR EXAMINATION WITH MY APPROVAL AS UNIVERSITY SUPERVISOR.

DR. HEZRON S. NYANDIEKA.
This study is dedicated to the many patients suffering from cancer. May the Almighty God help us find a solution to this problem.
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<tr>
<td>P H C</td>
<td>Primary Hepatocellular Carcinoma</td>
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<td>H C C</td>
<td>Hepatocellular Carcinoma</td>
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<td>L C C</td>
<td>Liver Cell Carcinoma</td>
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<td>E F D V</td>
<td>Encephalopathy and Fatty Degeneration of Viscera</td>
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<tr>
<td>H B V</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HB&lt;sub&gt;s&lt;/sub&gt;Ag</td>
<td>Hepatitis B surface Antigen</td>
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<td>HB&lt;sub&gt;c&lt;/sub&gt;Ab</td>
<td>Hepatitis B core Antibody</td>
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<td>PB</td>
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AFP 1 = Aflatoxin P₁
AFB-GSH 1 = Aflatoxin-Glutathione Conjugate
AFB-N-Gua 7 = 2,3-Dihydro-2-(N-Guanyl)-3-Hydroxy-Aflatoxin B₁
AFB-FAPYR = 2,3-Dihydro-2-(N-Formyl2,5,6 Triamino-4-OXO-N-Pyrimidyl)-3-hydroxy-Aflatoxin.

DDT = Dichloro-Diphenyl-Trichloro-Ethane
WHV = Woodchuck Hepatitis Virus
GAHV = Ground Squirrel Hepatitis Virus
ELISA = Enzyme-Linked Immunosorbent Assay
HPLC = High Performance Liquid Chromatography
NADPH = Nicotinamide Adenine Dinucleotide Phosphate reduced
IAHA = Immune Adherence Haemagglutination
This study sought to define the roles played by hepatitis B virus (HBV) infection and aflatoxin (AF) ingestion in the causation of primary hepatocellular carcinoma (PHC) in humans. Some factors that may modulate the carcinogenic effects of aflatoxin in liver were studied in experimental animal models.

The study was divided into two main parts.

1) In the first part, the roles played by hepatitis B virus infection and aflatoxin ingestion in the aetiology of liver cancer in humans were studied by combined laboratory and epidemiological approaches as follows:

(a) Testing for presence of hepatitis B surface antigen (HB₅Ag) and anti-HBC antibodies in the serum of the individuals living in 12 districts of this country to show the frequency of HBV infection in these populations.
(b) detection of AFB-macromolecular adducts, especially 2, 3-dihydro 2-(N-guanyl)-3-hydroxy-aflatoxin B$_1$ (AFB-Gua) in urine samples collected from individuals living in 12 districts as above, to show the rate of human exposure to aflatoxin contamination of the staple foods.

(c) Epidemiological data on liver cancer prevalence in the areas of study was compiled from hospital records and the Kenya Cancer Registry.

(II) In the second part of the study the following areas were investigated in experimental animal models:

(d) Whether lactating mother rats dosed with aflatoxin B secreted an AFB$_1$-metabolite 1-AFM in their milk in sufficient amount to induce hepatocellular carcinoma in the offsprings that had suckled their mothers.
How modulating factors such as:-

(e) tumour promoters
(f) Co-carcinogens and
(g) anti-carcinogens influenced aflatoxin induced carcinogenic process in the liver.

(a) **Hepatitis B Virus Infection**

The incidence of hepatitis B virus infection was determined by the serological testing for hepatitis B surface antigen and hepatitis B core antibody using radioimmunoassay technique. The average frequency of $\text{HB}_S\text{Ag}$ in the 12 districts analysed was 9.5% with a male to female ratio of 3:2. The frequency rate varied from one area to another. The average for hepatitis B core antibody in the same 12 districts was 56% with a sex ratio of 1:1 male to female. No correlation was established between HBV carrier rate and PHC probably due to underreporting of liver cancer cases in hospitals.

(b) **Aflatoxin-Macromolecular Adducts.**

Aflatoxin exposure was determined by detection of aflatoxin-guanine adduct in the urine of individuals who had consumed aflatoxin either
through their foods or drinks. Measurement of aflatoxin-guanine adduct was carried out using high performance liquid chromatography (HPLC) and fluorescence spectrophotometry.

The results revealed that human exposure to aflatoxin is widespread in this country. However, the exposure rate varied from district to district ranging from 6% to 32% of the people exposed.

No association was established between aflatoxin exposure and liver cancer prevalence. A long-term aflatoxin exposure detection method would be more useful in establishing a fuller picture of aflatoxin exposure in a population. Moreover, improvement in liver cancer reporting in hospitals would probably increase the relationship between aflatoxin and PHC.

Liver Cancer Prevalence, HBV and Aflatoxin

The prevalence of primary hepatocellular carcinoma ranged from 1-6 per 100,000 persons with a sex ratio of 2.4:1 male to female. The combined effects of hepatitis B virus infection and aflatoxin exposure were compared to the prevalence of PHC in areas studied.
There was no significant correlation between these parameters. This is one of the first few studies utilizing hepatitis B virus infection, aflatoxin exposure and liver cancer measurements on the same population groups at individual level.

(d) **Animal Experiments:**

When lactating mother rats were dosed with aflatoxin B$_1$ they secreted an AFB$_1$-metabolite AFM in their milk in sufficient amount that induced hepatocellular carcinoma in the offsprings that had suckled their mothers. Development of liver tumours was observed macroscopically and histologically. The results revealed that more male than female offsprings that had suckled their aflatoxin dosed mothers developed hepatocellular carcinoma. This study suggests that people drinking milk from cows fed on aflatoxin contaminated feeds are at risk from aflatoxin M effects.

(e) Tumour promotion was studied in rats using aflatoxin B$_1$ as an initiator and phenobarbitone as a tumour promoter. The results of this study were obtained by examination of the liver macroscopically and histologically. It was observed that tumour promoters resulted in higher yields of liver
The results of this experiment were inconclusive in that the cercariae failed to develop into the mature schistosomes.

(g) Tumour inhibition was studied in rats by feeding rats on food fortified with B-carotene, ascorbic acid, reduced glutathione, vitamin E, selenium and uric acid and later challenged with aflatoxin B1. Pieces of the livers from these rats were examined after 24 months macroscopically and histologically. The results demonstrated that B-carotene, ascorbic acid, reduced glutathione, vitamin E and selenium significantly inhibited or suppressed aflatoxin hepatocarcinogenesis.
CHAPTER I:

INTRODUCTION:

A. THE INCIDENCE AND AETIOLOGIES OF PRIMARY HEPATOCELLULAR CARCINOMA

Primary hepatocellular carcinoma (PHC) is one of the most commonly encountered cancers in the World, particularly in the developing countries. It is estimated that probably more than a quarter million people develop this type of cancer each year in the World (WHO technical report series, 1983). Currently, it is recognized that PHC has a frequency which varies remarkably in different populations, with less than 1 case per 100,000 population reported in the Western World compared with over 60 cases per 100,000 population reported in Africa (Smuckler et al, 1984). Mozambique has the highest known frequency rates in the World with crude rates per 100,000 of 98.2-150 for males and 27 for females (Prates and Torres, 1965). A sex difference is very evident with a marked male predominance.

Primary hepatocellular carcinoma has for many years been a major problem in this country. It is the fourth most common malignant tumour (Kenya Cancer Registry). This type of
malignancy of the liver is aggressive, therapy shows limited success and the prognosis is poor with death commonly occurring within months after diagnosis. Many patients are affected in the prime of their lives when they have heavy family responsibilities.

The pattern of uneven geographic distribution of PHC in different parts of the World combined with epidemiological studies have yielded valuable clues to its causation. As Kennaways (1944) once pointed out, the very high liver cancer rate among blacks in Africa is unlikely to be genetically determined since the frequency among blacks in North America is not appreciably different from that among their white counter-parts. It therefore seems highly probable that the remarkably high frequency of liver cancer in certain areas especially in Africa and Asia must be the result of environmental factors often of quite sharply localized geographical distribution (Popper, 1986).

Significant progress has been achieved in research on possible aetiological factors and mechanisms of their actions in liver cancer induction. This progress has been achieved as a result of the combined research effort involving epidemiologists and laboratory
scientists. Although a multifactorial aetiology is most likely, involving hepatitis B virus, aflatoxin, alcohol consumption etc, studies have narrowed down the search for causative factors to hepatitis B virus (Beasley, 1982) and aflatoxins (Linsell, 1979) to be the major candidates in the aetiology of human liver cancer. This has been documented by the fact that geographically, PHC frequency is high in the areas that are highly endemic with hepatitis B virus infection and also with high prevalence of aflatoxin contamination of the staple food. Alcohol also plays a role in the causation of PHC but to a much less extent as a primary cause (Trichopoucos, 1981).

From the foregoing observations, it appears that two plausible and perhaps interactive aetiological factors for induction of liver cancer in humans have been identified. This study is designed to shed further light on the roles played by hepatitis B virus and aflatoxin in the aetiology of liver cancer in this country and to examine some factors which are likely to influence the carcinogenic process of the two agents using animal models. A literature review of each of the two major suspected aetiological agents plus that of modulating factors follows.
B. HEPATITIS B VIRUS:

Although the existence of hepatitis has been recognised since antiquity, the first evidence for transmissible, blood-borne, long incubation hepatitis came from an out-break of hepatitis in shipyard workers in Bremen, Germany in 1885 (Feinstone et al., 1979). The disease occurred in individuals who had been vaccinated against smallpox several months previously with lymph obtained from the vehicles of other vaccinated humans. Other outbreaks of hepatitis were reported in the first half of the 20th Century. These included epidemics among patients attending diabetes clinics or venereal disease clinics, where inadequately sterilised needles and syringes were the apparent vehicles of transmission. Proof that serum hepatitis (type B hepatitis) was distinct from infectious hepatitis (type A hepatitis) came from a series of epidemiologic studies and experiments conducted in human volunteers during the 1940s (Feinstone et al., 1979).

Prior to the late 1960s, intensive efforts to isolate the hepatitis B virus (HBV) or to demonstrate an antigen-antibody reaction specific for this disease were unsuccessful.
Furthermore, attempts to transmit HBV to many species of animals and birds were also unsuccessful, and inoculation of human volunteers remained the only method of demonstrating the presence of HBV. The discovery of Australia antigen now termed hepatitis B surface antigen (HB$_s$Ag) by Blumberg in 1965 and its subsequent association with type B hepatitis provided the first specific antigenic marker of HBV infection and stimulated more interest in viral hepatitis research. The antigen was found in the blood of 30-60% of adults with acute viral hepatitis and also in approximately 0.1% of normal individuals in the United States and Western Europe and in 5-20% of apparently healthy individuals in parts of Asia, Africa and South America.

In 1968, Bayer and Coworkers, using electron microscopy demonstrated that HB$_s$Ag was physically associated with particles 20nm in diameter and filaments of similar diameter and variable length and it was postulated that HB$_s$Ag previously thought to be a serum protein polymorphism was the HBV (Blumberg, 1964). Dane and Coworkers (1970) described (42nm) virus like particles that were associated with HB$_s$Ag particles and shared the antigen specificity of these particles. He proposed that the larger, more complex, virus-like particles were in fact the infective HB virions.
In 1971 Almeida et al, described a second antigen-antibody system associated with HBV infection. This antigen was on the surface of the core-like structure inside the particle noted by Dane (1970) and could be released by treatment with detergent or lipid solvent. This was called hepatitis B core antigen (HB₉Ag). The immune responses to (HB₅Ag) and (HB₉Ag) by HBV-infected patients were quite distinct and provided additional evidence that the Dane particle was the HBV. The intrahepatic localization of HBV antigens was demonstrated by immunofluorescence (F.A.) techniques by Edgington and his colleagues (1971) and HB Ag was found in the cytoplasm of hepatocytes while HB Ag was localized to the nucleus.

A third HBV antigen was described in 1972 by Magnus and Espmark. This antigen complex, HBₑ Ag was associated with a soluble protein in the serum of some patients acutely or chronically infected with HBV. Hepatitis Bₑ antigen was subsequently shown to correlate with the infectivity of the HB₅Ag positive sera (Shikata et al., 1977). Using serological markers for diagnosis coupled with microscopic studies the activity as well as the structure of hepatitis B virus has been well investigated.
Hepatitis B virus Antigenic Structure.

It is now established that hepatitis B virus is a DNA virus that infects only human and certain non-human primates and causes a wide spectrum of acute and chronic liver disease. It is a spherical particle, 42nm in diameter containing an outer lipid coat (envelope) and a spherical inner core (nucleocapsid) with a diameter of 27nm. The outer lipid-containing envelope bears the hepatitis B surface antigen (HBsAg). Within the core is a double stranded circular DNA molecule of about 2x10^6 daltons which has a variable gap in one strand together with an endogenous DNA-dependent DNA polymerase activity that can fill in this gap in in-vitro reaction using a template, the double stranded circular DNA. Nearly 30% of the circular DNA molecule is single-stranded and it is the complementary strand of the single stranded DNA portion that is synthesized by the polymerase \textit{in vitro} (Ruiz-Opazo et al., 1982). The nucleocapsid also consists of a Protein Kinase, a core protein that carries the HBcAg determinant; a 15,000-dalton subunit of the core protein carries the HBeAg determinant. Fig.1 shows the physical and genetic structure of HBV-DNA.
Fig. 1

(a) Schematic representation of the hepatitis-B virus particle.

(b) The Physical and Genetic structure of HBV-DNA.
Causes of infection and Host Responses

The host responses to HBV have been broadly separated into those associated with transient (acute type) infections and those associated with persistent infections. Transient HBV infection occurs when an immunologically mature host undergoes primary exposure to the virus. Most cases of acute type hepatitis B, posttransfusion hepatitis, accidental hepatitis in medical institutions and infections through drug abuse are of this type. Except for a few cases of fulminant hepatitis, the prognosis in this type of infection is generally better and sequelae of chronic hepatitis, liver cirrhosis and hepatoma are rare. In this case the transient infection of both \( \text{HB}_5 \text{Ag} \) and \( \text{HB}_e \text{Ag} \) is followed by clinical manifestation of hepatitis, then antibody responses against \( \text{HB}_c \text{Ag} \), \( \text{HB}_e \text{Ag} \), and finally \( \text{HB}_5 \text{Ag} \) are induced resulting in elimination of the virus followed by recovery from hepatitis. Transient infection with development of anti-HBS, leads to protection against subsequent infection with HBV and reduced risk of chronic liver disease. Until recently it had been thought that the disappearance of anti-HBs meant that the virus had also been cleared from the liver. This may be the general case, but in some instances whole virus or viral DNA may be retained in the liver. HBV infections in adults are transient in 90% of cases (London, 1983).
Persistent HBV infection occurs following primary exposure of an immunologically immature or impaired host to HBsAg, and specific immunological unresponsiveness to HBsAg occurs. Thus, when the host is exposed to HBV, immune elimination of HBV from the host does not occur. This type of infection is characterised by persistent HB antigenemia and HBsAb response. HBV and HBV infected liver cells are not eliminated and HBV-DNA is incorporated into host cells after a long-term carrier state.

Persons are classified as chronic carriers of HBV on the basis of persistence of HBsAg in their sera for more than 6 months (London, 1983). Persistent infections are associated with an increased risk of developing chronic active hepatitis, cirrhosis and PHC. Infections of infants less than 6 months old are persistent in 90% of cases.

Figure 2 shows the responses of HBV infection.

Differential diagnosis of the above two types of HBV infection is extremely important. This can be accomplished by measuring antibody against HBC. HBcAb titers are generally high in persistent HBV infection and are of the IgG class which can be measured by the IAHA method. HBcAg in liver cells can be detected only in cases with higher HBcAb not in those with lower titers (Nishioka, 1985).
Fig. 2

Shows responses to HBV infection

(a) Serology of Acute Hepatitis B Infection
Infection
HBsAg
HBeAg
anti-HBc
anti-HBe

(b) Serology of Persistent Hepatitis B Infection (Chronic Carriers)
Infection
HBsAg
anti-HBc
HBeAg
Anti-HBe
HBV-Like viruses in Animals.

Although for many years HBV was considered to be a unique virus, similar viruses called hepadna viruses have recently been found in three different animal species. Woodchuck hepatitis virus (WHV) was discovered in 1978 in sera of Eastern Wood chuck (Martoma Monax) a member of the squirrel family by Summers et al (1978) after Snyder (1968) had observed that the most frequent cause of death in captive woodchucks in the Philadelphia Zoo was hepatocellular carcinoma accompanied by chronic hepatitis. Another member of this virus family ground squirrel hepatitis virus (GSHV), was found in the Beechey ground squirrel (Spermophilus beecheyi), another genus of the sciuridae family by Marion et al, in 1980. The observation of frequent hepatomas in a species of domestic ducks in the people's Republic of China led to the discovery of yet another member of this virus family now called duck hepatitis B Virus (DHBV) in sera from ducks in China by Summers and Mason (1982). A similar virus has been found in Pekin duck (Anas domesticus) in the United States of America (Robinson et al., 1983). Knowledge gained from the study of hepadna viruses has certainly made a tremendous contribution to our understanding about the integration, replication and protein synthesis of the hepatitis B virus in humans but the mechanism by which it may cause malignant transformation still remains unresolved.
Molecular Biological Techniques: The development of methods in molecular biology using recombinant DNA and molecular hybridization technology has increased sensitivity and precision in the analysis of DNA sequences in living cells. DNAs are cleaved at specific sites using restriction endonuclease enzymes. This is followed by separation of the resulting DNA fragments by gel electrophoresis. The separated DNA is then transferred to nitrocellulose filters or other solid media by the Southern blotting technique and hybridized with radio-labelled DNA probes (32p-labelled HBV-DNA). Using probes made from such cloned HBV-DNA has facilitated the analysis of genomic liver DNA for traces of viral DNA and for the presence of the virions in blood. Furthermore, by using these methods, more definite analysis of the state of viral DNA in HCC tissue has led to a better understanding of the relationship between hepatitis B virus and hepatocellular carcinoma. On the basis of this principle integrated HBV-DNA was demonstrated in cultured human PHC cell lines and human PHC tissues that were positive for HBs Ag. Further analysis showed that integrated HBV-DNA was present in liver tissue adjacent to
the tumour, as well as in liver tissue from chronic HBV carriers without PHC (Nishioka, 1985).

Evidence Relating HBV to PHC.

Within the last decade a very strong correlation has been established between human primary liver cancer and hepatitis B virus (HBV) infection. A substantial body of evidence has accumulated which consistently indicates the close association of chronic infection with hepatitis B virus and primary liver cancer (Sun and CHU, 1984). Evidence is now overwhelming that HBV causes hepatocellular carcinoma and appears to be responsible for at least 80% of cases worldwide (Beasley and Hwang, 1984). There is therefore, no wonder that HBV has been assumed to be the single most important factor in the aetiology of liver cancer since most patients with this neoplasm show a positive serology for HBV antigeraemia.

(a) Strong arguments in favour of a causal relationship between HBV and liver cancer have come from the observations that HBV carriers have approximately a 200-fold excess risk of developing liver cancer relative to non-carriers, and the life-time risk
of hepatocellular carcinoma in a chronic HBV carrier may be as high as 50% in males.

(b) Secondly, studies using molecular hybridization techniques gave consistent results that HBV sequence was found to be integrated into the genome of hepatoma cells in culture, in the specimens of hepatocellular carcinoma and also in the non-tumourous hepatic tissue of the hepatoma patients and long-term carriers (Sun and Chu, 1984).

(c) Thirdly, there is a close geographic correlation of incidence between hepatitis B virus and primary hepatocellular carcinoma in that endemic areas of high HBV carrier rates, have usually a parallel high incidence of PHC. Family clusters of HB Ag-positive hepatocellular carcinoma have been recognized in some areas. The family clusters of HB Ag-positive HCC are of particular interest because they suggest that maternal transmission may in and of itself increase the risk of HCC.

(d) The fourth evidence supporting HBV as a cause of PHC is the discovery of viruses closely related to HBV, designated as hepadna viruses, in animals like woodchuck, (Marmota monax), Beechey ground squirrel (Spermophilus beecheyi) and Pekin duck (Anus domesticus). These viruses show extensive
homology in DNA and antigens to HBV. Hepadna viruses have been associated with acute and chronic hepatitis in a healthy carrier state and hepatocellular carcinoma in Woodchuck, Beechey squirrels and in Chinese ducks (H. Popper, 1986). In all these species HBV has been found associated with integrated viral DNA.

Evi~ence Against HBV as a cause of PHC

Although prevailing evidence strongly implicates HBV to be the single most important aetiological factor in the induction of primary hepatocellular carcinoma certain other observations have raised points which are difficult to reconcile with.

(a) If chronic HBV infection were indeed a sufficient cause for the development of hepatocellular carcinoma, a close geographical association between the two diseases would be universally expected, whereas several regions of anomalously high or low incidence for HCC relative to the occurrence of HBV have been reported (Van Rensburg et al., 1985). Lack of correlation in geographical distribution between HCC and HBV infection has been reported in Mozambique, China, Greenland and Kenya among others.
At a local scale in Mozambique, higher case numbers of HCC were found in district localities in eastern coastal areas of Panda, Inhambane, Inharrime and Morrumbene, while lower case numbers were found inland south and westwards in areas in Guija, Limpopo, Magude and Bilane with crude rates varying almost 9-fold from the lower to the highest (Harrington et al., 1975). Variations in the level of aflatoxin could be the cause of the differences that have been observed between regions within Mozambique. Furthermore, high prevalence of HB Ag have been reported from Taiwan both in control populations and among HCC patients than have been found in Mozambique (Van Rensburg et al., 1985), yet the incidence of HCC is higher in Mozambique than in Taiwan.

Secondly the geographic distribution of liver cancer in the Peoples Republic of China appears to correlate more closely to the possible exposure to aflatoxins rather than to HBV infection in that in areas of high prevalence, the incidence rate of liver cancer may be six times more than in other areas, whereas the difference of HBV-carrier rate is only about 2.5 fold (Sun and Chu, 1984).
Furthermore, in Guangxi region in China, the mortality rate of PHC in HB$_S$Ag positive individuals was higher when the individuals lived in villages with high aflatoxin exposure (Henderson, 1985). In the same country, when ducks in areas where flocks were commonly infected with the HBV-like virus were fed on aflatoxin contaminated corn, 18% developed hepatomas whereas no hepatomas were found in the 40 ducks fed on non-contaminated corn (Harris and Sun, 1984).

(c) Thirdly, prevalence of HB$_S$Ag as high as anywhere in Africa or Asia have been reported from Greenland's Eskimos and yet there is no evidence for an elevated risk of HCC above the low level that is found in neighbouring European or North America territories (Melbye et al., 1984).

(d) Fourthly in Muranga District, Kenya, the incidence of HBV infection does not vary among people living in the low and high altitude areas whereas both the rate of food contaminated by aflatoxin and the incidence of HCC are higher in the low altitude areas (Peers and Linsell, 1973). If hepatitis B virus played the dominant role in the aetiology of HCC in this area, transmission and infection should probably be more common in the low altitude areas where the incidence of HCC was greater (Bagshawe et al., 1975).
A case control study of HCC patients all of whom were born and reared in rural areas but half of them had moved in to an urban setting was conducted by Kew et al., (1983). These workers concluded that HBV status which was similar in both groups could not account for the differences in the incidence and age at onset of HCC in rural and urban populations.

The hepatitis B virus itself does not have oncogenic activity. In other words, no experimental studies have so far demonstrated a transforming potential for HBV, even in cases where intact HBV genes have been integrated into cellular DNA and the viral gene products were expressed (Garner et al., 1985).

It is therefore probable that infection with HBV alone cannot be responsible for the induction of liver cancer; other factors must be involved thus implying a multifactorial origin of human liver cancer. In fact a number of other studies have demonstrated the importance of other environmental factors such as aflatoxins.
Aflatoxins were first discovered as a consequence of an outbreak in 1960 of a disease that resulted in the deaths of many thousands of young turkeys, ducks and pheasants in Eastern and Southern England (Goldblatt, 1969). This disease, known as turkey X disease, was characterized by lethargy and loss of appetite resulting in death within a week. The birds exhibited extensive hepatic haemorrhagic necrosis.

A similar extensive outbreak of the disease occurred almost simultaneously in ducklings in Kenya and Uganda and later pigs and cows were also affected (Allcroft, 1969). The source of the toxin that caused the disease in animals in England was ultimately traced to a shipment of Brazilian peanut meal, used in mixing feed for various species of animals. The meal was referred to as Rossetti meal after the name of the ship that brought it to England. The peanut meal proved to be highly toxic for many species of animals and from it the common fungus Aspergillus flavus was isolated. It was also
later isolated as a secondary fungal metabolite of Aspergillus parasiticus. The A.\textit{flavus} fungus produced toxins that were subsequently designated "aflatoxins" from the contraction of "A.\textit{flavus} toxin" (Groopman et al., 1986).

Subsequent to the observations in England and Africa, liver tumours in trout were observed in brood stock fish from hatcheries throughout the United States. These tumours in fish were eventually traced to rations that contained either aflatoxin - contaminated peanut meals or cottonseed meal (Newberne, 1984). Aflatoxicosis may well have remained for a long time essentially a veterinary problem had it not been for the interest of the World Health Organization in providing peanut meal and peanut butter as a protein supplement to undernourished children (Stoloff, 1977). In the intervening years since the early reports became available, it has been revealed that virtually every domestic and laboratory animal species is sensitive to either the toxic or the carcinogenic effects of aflatoxins.
(i) Acute Aflatoxicosis

Acute Aflatoxicosis in humans associated with aflatoxin consumption have been reported. In Taiwan, the poisoning of 26 persons in two rural villages was linked to the consumption of aflatoxin-contaminated food. Those poisoned developed vomiting, abdominal pain, palpable livers and oedema of the legs (Newberne, 1984). Other cases of acute aflatoxicosis have been reported from Uganda, India and Thailand. There is strong evidence that a disease of children in Thailand, with symptoms identical to those of Reye's Syndrome, was associated with aflatoxicosis. The disease was characterized by vomiting, convulsions, coma and death with cerebral oedema and fatty involvement of the liver, kidney and heart (Groopman et al., 1986).

Better documentation of an outbreak of aflatoxin poisoning from the ingestion of heavily contaminated maize was reported from India in
which 100 fatalities were recorded (Van Rensburg, 1977). The illness occurred only in households where humans and dogs were consuming contaminated maize and ceased when the suspect crop of maize had exhausted.

Here in Kenya, an outbreak of aflatoxin poisoning was reported in Makueni division of Machakos district by Ngindu et al., in 1982. These workers reported 20 cases of aflatoxicosis occurring mostly in family clusters, twelve of which were fatal. Further investigations revealed that these families had been consuming maize meal which was heavily contaminated by aflatoxin. There was similarity in the Indian outbreak in that death in dogs preceeded human illness and the unweaned infants were not affected by the poison. Aflatoxin was demonstrated in the liver biopsies and blood from the affected individuals.

The recent study of Hendrickse et al., (1982) to establish the relationship between aflatoxin and kwashiorkor in Sudanese children is of tremendous relevance to us in this country. This study provided evidence that children in Sudan were exposed to aflatoxins in their diets and the
kwashiorkor group was at the greater risk. They postulated that either the children with kwashiorkor had a greater exposure to aflatoxins or that their ability to transport and excrete aflatoxins was impaired by the metabolic derangements associated with kwashiorkor.

(ii) **Carcinogenicity:**

Aflatoxins have been shown to be potent carcinogens in many species of animals, including rodents, nonhuman primates and fish. The liver has been the organ principally affected, where the toxin induced a high frequency of hepatocellular carcinomas and lower frequencies of other tumour types. Under appropriate circumstances, dependent on such variable as animal species and strain, dose, route of administration and dietary factors, significant frequencies of tumours have been induced at sites other than the liver.

Most information on aflatoxin B carcinogenicity has been obtained from studies in rats, which are highly susceptible
to the toxin. In recent years, however, there has been increasing information on the carcinogenic responses of the rainbow trout (which is more sensitive than the rat) and the monkey (a more appropriate model for human risk estimation). Such experiments have often examined dose-response characteristics and the influences of such parameters as route of administration, size and frequency of dose and the sex, age and strain of the test animals. Effects of various modifying factors on carcinogenic responses have been evaluated, including diet, hormonal status, liver injury, microsomal enzyme activity relationships of aflatoxin congeners, structural analogs and metabolites as inducers of liver tumours.

$\text{AFB}_1$ has been shown to induce liver tumours in two species of lower primates fed a diet containing 2 mg $\text{AFB}_1$ /Kg, the tree shrew (Tupaia glis) and the marmoset (Saguinus Oedipomidas) (Groopman et al 1986). Rhesus monkeys have also been demonstrated to be susceptible to $\text{AFB}_1$ carcinogenicity.
(iii) **Mutagenicity**

Aflatoxins have been shown to be mutagenic by observations of chromosomal damage and determinations of altered genetic expression in a variety of procaryotic and eucaryotic systems in addition to mitotic damage. Experimental evidence for DNA damage following exposure to AFB₁ has been shown with different experimental designs. Using alkaline and neutral sucrose density gradient techniques, single and double strand DNA breaks were demonstrated in Hela cells incubated with 32 μg AFB/ml for 24 hours (Umeda et al., 1980). Phage production in lysogenic bacteria was potentiated by AFB (Busby and Wogan, 1979). The mutagenic activity of AFB₁ in a wide variety of organisms including unicellular algae and higher plants might reflect the capacity of the organism to metabolize AFB and the propensity of AFB to bind non covalently with DNA. The incidence of AFB₁-induced metaphase
chromatid breaks in normal and Xeroderma pigmentosum human skin fibroblasts was increased 12-and 127-fold respectively, when a rat liver microsomal activation system was employed (Busby and Wogan, 1979). Ong (1971) examined AFB for mutagenicity in the ad-3 test system of Nersospora crassa vegetative cells and found a 24-fold increase in mutation frequency of AFG versus a 177-fold increase for AFB at a toxin concentration of 40 ug/ml. AFG was somewhat less mutagenic in the Ames assay relative to AFB. The mutagenicity of many of the known AFB metabolites has been examined in the Ames assay and aflatoxicol (AFL) was highly mutagenic being 23-31% as potent as AFB. This may reflect the ability of aflatoxicol to be reduced to AFB by the enzymes present in the rat liver postmitochondrial supernatant (Busby and Wogan, 1979).

(iv) **Teratogenicity:**

The hamster has been reported to be susceptible to the teratogenic effects of AFB₁ (Elis and Dipaolo, 1967). These
workers reported severe foetal malformations such as encephaly, neural tube disorganization and pronounced growth retardation when pregnant mothers were dosed up with 4mg AFB/kg on day 1 of pregnancy. Birds and fish appeared to be more susceptible to the teratogenic effects of AFB. Malformed fore- and hind limbs were a major teratogenic feature in chicks when eggs were treated with 0.2 to 0.6 ug AFB (Busby and Wogan, 1984).

Chemistry and Metabolism:

Aflatoxins have been described as compounds which are highly substituted coumarins containing a fused dihydrofurofuran moiety which are produced as secondary metabolites by specific strains of Aspergillus flavus and Aspergillus parasiticus. The toxic principles were extracted and isolated from A. flavus and were designated as B, B, G, and G. Aflatoxins B and B were so designated because of their strong blue fluorescence under ultraviolet light whereas aflatoxins G1 and G2 fluoresced greenish - yellow. The B toxins were characterized by the fusion
of a cyclopentenone ring to the lactone ring of the coumarin structure but the G toxins contained an additional fused lactone ring. AFB₁ and to a lesser extent, AFG₁ were responsible for the biological potency of aflatoxin-contaminated meals. These two toxins possessed an unsaturated bond at the 2,3 position on the terminal furan ring. The essentially inactive AFB₂ and AFG₂ were saturated at this position.

Aflatoxins have been shown to be soluble in methanol, chloroform and other organic solvents but are only sparingly soluble in water. The toxins strongly absorb ultraviolet light (362 nm), with extinction coefficients in methanol or ethanol varying from 17,000 for AFG₂ to 24,000 for AFB₂. Fluorescence emission occurs at 425nm for AFB₁ and AFB₂ and at 450nm for AFG₁ and AFG₂. Although aflatoxins are quite stable in foods or feeds they are rapidly deactivated by extremes of PH less than 3 or more than 10, oxidizing agents or exposure to ultraviolet light in the presence of oxygen (Groopman et al., 1986).

Aflatoxins are metabolized by the microsomal mixed function oxygenase system, a complex
organization of cytochrome-coupled, \( \text{O}_2 \) and NADPH-dependent enzymes localized mainly on the endoplasmic reticulum of liver cells but also present in kidney, lungs, skin and other organs. These enzymes catalyze the oxidative metabolism of AFB, resulting in the formation of various hydroxylated derivatives, as well as an unstable, highly reactive epoxide metabolite. Detoxification of AFB is accomplished by enzymatic conjugation of the hydroxylated metabolites with sulphate or glucuronide esters that are excreted in urine or bile. Another route for removal of AFB from the organism involves the enzyme catalyzed reaction of the epoxide metabolite with glutathione and its subsequent excretion in the bile.

Interest in cancer chemotherapy using antioxidants has recently produced evidence that suggests that the protective effects of these compounds arise from enhanced carcinogen inactivation through selective induction of enzymatic detoxification pathways. Many chemoprotective agents against cancer act by their ability to induce glutathione S-transferase activities and the,
induction of this family of glutathione conjugating isozymes is felt to be an effective mechanism for ameliorating aflatoxin toxicity and carcinogenicity (Kensler et al. 1985). Figure 3 shows the various metabolic transformations of AFB

During the course of AFB metabolism the reactive electrophilic epoxide can react with various nucleophilic centres in cellular macromolecules such as DNA, RNA and protein to form covalently bound adducts. The consequences of this activation reaction are opposite to those of detoxification and potentially pose a biological hazard to the cell or organism and constitute a putative mechanism by which many compounds, including AFB exert toxic, carcinogenic and genotoxic effects (Groopman et al. 1986). AFB forms covalently linked adducts with guanine in DNA after oxidative metabolism to a highly reactive 2,3-epoxide. The major reaction product formed between the activated species and DNA has been identified as 2,3-dihydro - 2 - (N-guanyl)-3-hydroxy-aflatoxin B$_1$ (AFB - N - Gua). AFB -N -Gua modification in DNA is unstable and can be (a) removed from DNA either enzymatically and/or
FIG. 3

METABOLIC TRANSFORMATIONS OF AFLATOXIN B₁

[Diagram showing metabolic transformations of aflatoxin B₁, including reactions and structures labeled with chemical formulas and names.]
FIG. 4

THE STRUCTURES OF MAJOR AFB, MACROMOLECULAR ADDUCTS

-structures-diagram-
spontaneously leaving an apurinic site, or (b) the imidazole ring of the guanine moiety can open and the putative 2,3-dihydro-2-(N-formyl 2,5,6-trimino-4-oxo N-pyrimidyl)-3-hydroxy-aflatoxin B\textsubscript{1} (AFB\textsubscript{1} FAPyr) is produced. This putative derivative has been found to be a non-repairable persistent adduct in DNA of rat liver in \textit{Vivo} and in human lung cells in culture after acute or chronic administration of AFB\textsubscript{1}. This kind of interaction between DNA and Aflatoxin resulting in DNA modification is considered an important step in carcinogenesis. This analysis of AFB\textsubscript{1} - DNA adducts provides the chemical basis for producing monoclonal antibodies that recognize these DNA adducts so that the antibodies may be employed as highly specific probes for quantifying the occurrence of adducts as well as other products of AFB\textsubscript{1} metabolism in biological samples. Figure 4 shows how AFB\textsubscript{1} is metabolized into macromolecular - binding species.

(vi) \textbf{Methodologies of Aflatoxin Analysis:}

Common methods of aflatoxin analysis include either thin-layer chromatography or HPLC of extracted samples with confirmation of identity by physicochemical methods such as fluorescence photon counting or mass
spectrometry with selective ion monitoring. These methods, however, are time consuming and costly besides requiring technical skill. So they have not been widely applied to measurements of aflatoxin metabolites in human body fluids. The development of immunoassays for detection of AFB and its metabolites appears promising. A number of reports have appeared on the generation of antibodies against AFB, AFM$_1$, AFB$_{2a}$ and AFB-DNA and others (IARC Internal Technical Report No. 84/003/1984). Some of these antibodies have been produced in rabbits while others are monoclonal in origin. In the last few years Groopman and Coworkers. (1986), have produced various monoclonal antibodies that recognize aflatoxins using antigens ranging from aflatoxin-modified DNA to aflatoxin-adducted proteins. These antibodies are being used in conjunction with such chemical analytic techniques as noninvasive screening methodologies to monitor human exposure to these environmentally occurring mycotoxins. Chemical analytic and radiometric procedures have been combined with a monoclonal antibody-affinity chromatography column, both to purify the aflatoxin adducts and metabolites from urine and to confirm their identity (Groopman et al, 1986).
Polyclonal antibodies appear to be particularly suitable for measurements of exposure since various aflatoxin-derived materials can be assayed; thus giving a more representative picture of the exposure. In contrast, several monoclonal antibodies might be advantageous in determining at an individual level the pattern of aflatoxin metabolites present in the urine (Garner et al., 1985). Miniaffinity columns coupled with polyclonal or monoclonal antibodies of high avidity against aflatoxins have been very effectively used to immunoconcentrate minute quantities of mycotoxins in food extract, alcoholic beverages and urine (Sun and Chu, 1984) followed by assay using enzyme immunoassay.

ELISA and RIA methods have been used to assay aflatoxins also (Groopman et al., 1986). RIA requires radiolabelled substrates, access to scintillation counters in addition to technically skilled personnel. ELISA on the other hand does not require radioactivity, expensive analytical equipment, or highly trained technical personnel yet the procedure is as sensitive as, if not more sensitive than RIA. ELISA methods should therefore be the best choice for the developing countries.
HUMAN EXPOSURE TO AFLATOXIN:

Aflatoxins are found all over the world; although they are more frequently detected as contaminants of grains, peanuts, cotton seed oil, cassava, milk and milk products and in other commodities in the human food supply in the tropical and sub-tropical developing countries where a hot, humid climate coupled with inadequate storage and food processing facilities are conducive to fungal growth. In certain areas of these countries, social and economic conditions require that the population must eat whatever foods are available in order to survive, irrespective of the quality of the food. Aflatoxin B₁ is usually found in the highest concentrations and is the most potent toxin of the group. Aflatoxin M which was first isolated from the milk of lactating animals fed on aflatoxin contaminated feeds is the other significant toxin (Busby and Wogan 1984). Contaminated foods and drinks are the main sources of human exposure to aflatoxins.
They get into humans by:-

(a) Direct ingestion of aflatoxins in contaminated foods of plant origin such as groundnuts, oilseeds including cotton seeds, cassava and maize.

(b) Ingestion of aflatoxins carried over from feeds into animal products appearing as aflatoxin \( M_1 \). It has been observed that the amount of aflatoxin \( M_1 \) secreted in milk is proportional to the amount of ingested aflatoxin \( B_1 \) (Applebaum et al., 1982). When lactating cattle, sheep or goats are given feed contaminated with aflatoxin \( B_1 \) they will secrete aflatoxin \( M_1 \) in their milk.

A report appearing in the Environmental Health Criteria II, (1979), pointed out that in the cow there was a linear relationship between the amount of aflatoxin \( B \) ingested daily and the level of aflatoxin \( M_1 \) in the milk. It indicated that about 1.5% of aflatoxin \( B_1 \) was secreted as the metabolite \( M_1 \) in the milk plus smaller quantities of unmetabolized aflatoxin \( B_1 \).
One of the main sources of human exposure to aflatoxins is by ingestion of aflatoxins carried over from feeds into milk products including cheese and powdered milk, where they appear mainly as M₁. This study, using rats, sought to demonstrate whether the milk from lactating mothers dosed with aflatoxin B₁ can induce liver cancer in their suckling offsprings. This will imply that milk from lactating cows fed aflatoxin contaminated feeds poses health hazards to humans consuming it.

(vii) Evidence Relating Aflatoxin to PHC:

Aflatoxin B₁ has been given particular attention as a real or potential aetiological agent for human hepatocellular carcinoma because of its widespread distribution in staple food products in areas where HCC is prevalent and because of convincing evidence from epidemiological observations and laboratory studies that it is important in the causation of HCC in man. Several epidemiological studies have been designed to obtain inform-
ation on the relationship of estimated dietary intake of aflatoxin to the incidence of human hepatocellular carcinoma in different parts of the world.

(a) Information obtained from Uganda, Philippines, Swaziland, Kenya, Thailand and Mozambique showed a positive association between high intakes of aflatoxin and high incidence rates of liver cancer in man (Groopman et al., 1986). The association was most apparent in adult men for which large numbers of cases were involved, consequently yielding more precise estimates of disease incidence of a putative causal relationship between aflatoxin ingestion and liver cancer in humans. Although this evidence does not constitute proof that aflatoxin is the cause of human liver cancer, these data together with the extensive animal data on aflatoxin carcinogenicity, are sufficient to indicate that exposure to the carcinogen is associated with
elevated risk of this form of cancer and therefore warrants continued investigations into effective means for monitoring and control of aflatoxin occurrence as food contaminants.

(b) Further supportive work for aflatoxin as an important causal agent for human hepatocellular carcinoma comes from the fact that aflatoxin has been shown to be present in human tumour tissue by both spectrophotometric and chromatographic methods (Shanka et al., 1971, Stora et al., 1981).

(c) Human exposure to aflatoxin has been shown by excretion of AFB-metabolites in human urine (Campbell et al., 1970, Martin et al., 1984, Wu, 1984) and has been detected in human blood samples (Tsuboi et al., 1984).

Urine samples collected in Muranga district Kenya, showed detectable levels of 2,3-dihydro-2-(N-guanyl) hydroxy-AFB (AFB-Gua), indicating that the consumed aflatoxin was not only metabolized but that the activated species also reacted with cellular nucleic
acid (Autrup et al., 1983). Furthermore, people who had been exposed to AFB from dietary sources were identified for a pilot study in Beijing, China. Urine samples from individuals who had been exposed to the highest dose (87.5mg) the previous day, were run on an antibody affinity column and then analyzed by analytic HPLC demonstrating the presence of the major AFB-DNA adduct, $\text{AFB}_1^\text{N-Gua}$ at levels representing between 7 and 10 ng of the adduct. These data indicate that monoclonal antibody columns, coupled with HPLC can be used to quantify aflatoxin DNA adducts in human urine samples obtained from environmentally exposed people in less than one hour (Groopman et al., 1986).

d) Aflatoxin is carcinogenic in a wide variety of animal species including rodents and nonhuman primates (Busby and Wogan, 1984). Furthermore, aflatoxin has been shown to be a potent transforming agent in cell transformation assays.

e) A recent report of an excess of cancer deaths among workers at a Dutch oil-pressing facility in which exposure to aflatoxin
primarily occurred via the respiratory route (Hayes et al., 1984) adds further credence to the evidence that aflatoxin is a human carcinogen.

(D) MODULATING FACTORS IN LIVER CARCINOGENESIS:

Most naturally occurring cancers including liver cancer result from a complex interaction between endogenous (host) factors and exogenous (environmental) factors thus giving rise to a multifactorial origin. In addition, the carcinogenic process proceeds via several discrete steps and over a time span that occupies a considerable fraction of the lifespan of the individual, thus giving rise to a multistep (multistage) process. There is considerable evidence that successive steps (stages) in carcinogenic process may involve qualitatively different events, and that, they can be enhanced or inhibited by quite different types of environmental and host factors (Weinstein et al., 1980). This should provide an exploitable basis for strategies of liver cancer prevention that emphasize protection of the host. Some of the environmental factors that play roles in the carcinogenic process are:-
The pioneering and elegant studies of Isaac Berenblum and his colleagues (1941) led to the definition of two distinct processes during carcinogenesis experiments on mouse skin, initiation and promotion. These studies, as well as subsequent related studies by others, represent a major landmark in experimental carcinogenesis research. A third step (stage) known as progression has since been added to the two stage processes (Weinstein et al., 1984).

A major tribute to the studies by Berenblum and other investigators (1941) of the two stage mouse skin carcinogenesis is that their findings have served as a paradigm for more recent studies on the multistep aspects of carcinogenesis in several other tissues and species. Evidence that hepatocellular carcinoma, bladder cancer, colon cancer and breast cancer also proceed via processes analogous to initiation and promotion is well established (Weinstein et al., 1980).

(a) Initiators (Carcinogens)
(b) Co-Carcinogens
(c) Tumour Promoters
(d) Inhibitors
The three stages of carcinogenic process fit well in the concept of Foulds (1975) who described carcinogenesis as "a process of sequential neoplastic development extending over a long period of time which in man, might amount to several decades and be manifested by a wide variety of lesions that might emerge contemporaneously or consecutively at various times and places."

**Initiation of Liver Cancer.**

Initiation is defined as a change in the target cells, which induces an essential irreversible genetic alteration and increases the number of cells at risk as well as the probability for malignant transformation significantly. A solitary carcinogen can be defined as a chemical physical or biological agent that causes/initiates cancer.

In liver, like in many other organs, initiation results from a limited exposure to a carcinogen, either a high dose given once or several small doses given for a short duration. This initiated cell will only develop into clinically recognizable tumour if the tissue is exposed continually to the carcinogen or to a repeated exposure of a tumour promoter. In the absence of further
treatment initiated cells may not develop into clinically recognizable tumours, but the cellular changes are heritable though not grossly recognizable.

Cellular metabolism of the carcinogen plays an important role in initiation as most of the chemical carcinogens are inactive per se and require metabolic activation effects. The carcinogens are metabolized by cellular enzymes, particularly the cytochrome p-450 mixed function oxidase, to reactive intermediates which then form covalent bond-adducts with cellular constituents such as DNA, RNA and proteins (Miller, 1970). In many cases the process of metabolic activation involves multiple enzymatic steps leading from the procarcinogenic parent compound to a proximate carcinogenic intermediate which is further metabolized to the ultimate carcinogenic species.

The activation of a procarcinogenic form is accompanied by several competing pathways resulting in non-carcinogenic metabolites, so that the amount of the ultimate carcinogen represents only a small fraction of the total metabolites. Hence cellular metabolism is important for
deactivation as well. In the absence of metabolic activation, procarcinogens are neither mutagenic nor carcinogenic. The ultimate carcinogenic form binds to DNA to form adducts. The metabolism of several carcinogens and the identification of carcinogen-DNA adducts has been demonstrated in several animal systems (Lin et al., 1977) and in humans (Autrup et al., 1983).

Furthermore, a positive correlation between the total level of DNA-modification, mutation frequency and tumour formation has also been demonstrated (Ashurst et al., 1983).

The immediate and irreversible nature of initiation and the demonstration that most ultimate carcinogens are mutagens, have strongly supported a mutational mechanism for initiation. However, mammalian cells treated with carcinogens under identical conditions have a higher neoplastic transformation than single gene mutation frequency, suggesting that carcinogenesis involves one of multiple oncogenic sites; damage to the genome at sites less likely to be repaired, e.g. tandem repeats or genetic damage other than point mutations (Yuspa, 1984). Faber and Cameron (1980) described
initiation as consisting of a mutation-like event or a heritable rare event, in a small segment of the exposed target cell population.

**Co-Carcinogenesis:**

A cocarcinogen is a compound that augments tumour induction when administered concurrently with the carcinogen or the tumour promoter.

The mechanism of most cocarcinogens is presently unknown. However, since the genotoxic activity of carcinogens may depend on the ratio of metabolic activation/deactivation, a cocarcinogen could affect these competing metabolic pathways towards an unfavourable direction, resulting in the formation of a higher amount of the ultimate carcinogen. Cocarcinogens may also act by activation of oncogenic Viruses or by inhibition of DNA repair. Cocarcinogens stimulate cell proliferation and therefore make the organs more sensitive to carcinogen exposure. Some examples of cocarcinogens in the human environment are asbestos, alcohol, some parasitic infections and hormones.
As pointed out above, tumour promoters can be defined as compounds that have very weak or no carcinogenic activity when applied alone but that markedly enhance tumour yield when applied repeatedly following a low or suboptimal dose of a carcinogen (initiator). Tumour promotion involves those cellular events which lead to the phenotypic expression of initiation by the selective multiplication of the initiated cell population. The net effect of tumour promoters is the clonal expansion of the initiated cells. Tumour promotion, therefore, acts to induce an identifiable neoplasm by forcing expression of structurally altered DNA within the initiated cell. Most studies in multistage liver carcinogenesis and tests of chemical compounds for their ability to promote hepatic tumours have been conducted with rats. Several studies for tumour promoters have used the original protocol of Peraino et al, (1971). Weaning rats were fed 2-acetyl-aminofluorene for a few weeks as initiator, followed some weeks later by chronic feeding with the tumour promoter. Phenobarbitone has been extensively used as a model tumour promoter in rat liver.
Tumour promoters enhance tumour development only after initiation even if a long interval separates the two treatments. Promotion in the absence of or prior to initiation does not result in tumour formation. In contrast to the irreversible nature of the action of initiators, the action of promoters is reversible. Also in contrast to initiators of carcinogenesis, promoters do not require metabolic activation. The difference with respect to reversibility may be due to the fact that initiators bind covalently to cellular DNA and produce heritable changes, while the action of tumour promoters appears to be epigenetic and is not mutagenic (Weinstein et al., 1977). The cellular targets for the action of tumour promoters are not known with certainty, but recent studies in cell culture systems have provided important clues. The effects seen in cell culture can be classified into three categories:

(a) Mimicry of transformation. Tumour promoters induce several properties in normal cells that mimic those often seen in transformed cells.

(b) Modulation of differentiation. Tumour promoters act as potent modifiers of terminal differentiation.
Membrane effects. The cell surface membrane is said to be the initial and major target of tumour promoters. One of the earliest effects of tumour promoters is a change in the membrane phospholipid metabolism.

Progression:

Progression is defined as the stage of neoplastic development characterized by visible karyotypic alterations leading to malignancy. Tumour progression is often thought of in terms of a series of successive mutations and selections eventually resulting in the clonal outgrowth of a fully malignant tumour. Although the crucial event in carcinogenic process is the initial change which somehow frees the normal cell from growth control and sets the whole train of events in motion, it should be realized that subsequent changes conferring increasing cellular autonomy e.g. the capacity to invade and metastasize are those which make cancer so life-threatening a disease
Inhibition of Liver Carcinogenesis:

Posed against the impact of environmental exposures to carcinogenic agents is the presence of agents that inhibit these carcinogens. Most of the inhibitors presently investigated in experimental system are synthetic compounds, however, some are constituents of normal products including vegetables consumed by humans. The inhibitors identified so far display a great diversity of chemical structures making it likely that only a limited knowledge of the total spectrum of compounds having this property is available. Inhibitors present in the environment must be having an important impact on the response of humans to carcinogenic agents. Geographic differences in the frequency of cancer of particular organs as well as variations in frequency over a period of time have generally been attributed to changes in the magnitude of exposure to carcinogenic agents. However, it is possible that in some instances the observed differences may as well be due to alterations in the level of protection against carcinogenic agents.
Inhibitors act by either scavenging on the reactive carcinogenic species or by altering the activity of the microsomal mixed function oxidase. Since many initiators require metabolic activation usually via oxidative pathways, antioxidants and agents which alter microsomal metabolism can reduce or prevent neoplastic transformation by inhibiting the formation of ultimate carcinogens and thus prevent the critical reactive species that are capable of forming covalent bonds with cellular macromolecules or by accelerating their detoxification. The induction of alternate metabolic pathways leading to non-carcinogenic metabolites or the trapping of reactive species by molecules acting as scavengers may also be involved in the anticarcinogenic action of these agents. Inhibitors of tumour promotion also act by preventing promoter-induced cellular proliferation and promoter-induced alterations in epithelial differentiation. During neoplastic progression, anti-carcinogens may act directly on the transformed cell altering its biological potential or its response to promoting agents, and/or enhancing the host defence system against these cells. Included in this group of inhibitors
are retinoids and protease inhibitors. Other inhibitors, which are structurally related to specific carcinogens act by competitively inhibiting the effect of these carcinogenic compounds.
(E) OBJECTIVES:

1. The first objective of this study is to define the roles played by aflatoxin and hepatitis B virus in the aetiology of liver cancer in humans. The objective will be studied by a combined laboratory and epidemiological approach as follows:

a) Detection of AFB$_1$ - Macromolecular adducts especially 2,3-dihydro 24N-guanyl)-3-hydroxy-aflatoxin B (AFB-Gua) in urine samples collected from individuals living in areas with varying rates of aflatoxin contamination of the staple foods.

b) Testing for the presence of hepatitis B surface antigen (HBsAg) and anti-HBV core in the serum of the above individuals to show the incidence rate of HBV infection in these populations.

(c) Epidemiological data on liver cancer prevalence in the area of study will be compiled from hospital records and the Kenya Cancer Registry.
2. The second objective will be to study whether lactating rat mothers dosed with aflatoxin B₁ can pass some of the carcinogen or its metabolites to their offsprings through their milk during suckling.

3. The third objective is to study in experimental animal models how modulating factors such as promoters, co-carcinogens and inhibitors may influence aflatoxin-induced liver carcinogenic process.

   This objective will be achieved by:

   a) Studying the mechanism of tumour promotion in rat liver carcinogenesis using phenobarbitone.

   b) Studying the effects of schistosomiasis on rat liver carcinogenesis.

   c) Studying the effects of anti-carcinogens such as B-carotene, ascorbic acid, reduced glutathione, Vitamin E (tocopherol), selenium and uric acid on carcinogenesis.
EXPERIMENTAL.
CHAPTER 2:

AFLATOXIN-GUANINE ADDUCT, HEPATITIS B VIRUS INFECTION AND PRIMARY HEPATOCELLULAR CARCINOMA

HUMAN STUDY

INTRODUCTION:

This study has sought to establish which of the two major aetiological agents - hepatitis B virus or aflatoxin is more important singly or whether the two agents act synergistically in the causation of primary hepatocellular carcinoma. The first objective is to define the roles played by aflatoxin B₁ and hepatitis B virus in the aetiology of human liver cancer. Once the roles of these two agents are clearly defined, it will be possible to design effective prevention programs against these agents and thus reduce or prevent the risk of liver cancer. Although epidemiological studies have strongly favoured hepatitis B virus infection as the major risk factor in the induction of liver cancer and intervention strategies based on vaccination against its infection are already underway in some countries, there is evidence that aflatoxin may be playing a more significant role than HBV in the induction of this
disease in other areas, particular in this country. The above objectives were studied by a combined laboratory and epidemiological approach as follows:

(a) By detection of AFB$_1$ - macromolecular adducts, especially 2,3-dihydro-2, 3-dihydro-2-(N-guanyl)-3-hydroxy-aflatoxin $B_1$ (AFB-Gua) in urine samples collected from individuals living in areas with varying rates of aflatoxin contamination of the staple foods.

(b) By testing for the presence of hepatitis B surface antigen (HB$_S$ Ag) and anti-HBV core antibody in the serum of the above individuals to show the frequency rates of HBV infection in those populations.

(c) Epidemiological data on liver cancer prevalence in the areas of study was compiled from hospital records and the Kenya Cancer Registry.
MATERIALS AND METHODS

MATERIALS.

Methanol AR, ethanol AR, acetonitrile AR, Hydrochloric acid AR, Methylated spirit, sodium hydroxide AR, were purchased from British Drug House (BDH). Synthetic AFB-Gua, Ausria 11-125 and Corab kits (Abbot Laboratories, North Chicago 111, U.S.A.), C-18 sep-pak cartridges, C-18 bondapak column, ultrasil-si column and cartridge rack (Waters Associates, Waltham, MA, U.S.A.) were kindly donated to me by Dr. Herman Autrup of the Fibiger Institute, Copenhagen, Denmark. Aloka auto well gamma system ARC-500, High performance liquid chromatography (Perkin Elmer), Fluorescence spectrometer (Perkin Elmer MPF 44B), vacuum pump, centrifuge, plastic bottles, cooling boxes with ice bags, pasteur pipettes, cotton wool, universal bottles, 10 ml or 20 ml syringes, 21 G.hypodermic needles. Ausria kits included bottles of 100 beads each of antibody to hepatitis B surface antigen (Guinea pig), vials of antibody to hepatitis B surface antigen 11-125 (human),vials of negative control,vials of positive control and
cover slips, plastic gloves, precision pipettes with disposable tips to deliver 200µl, single bead dispenser, water bath, aspiration tips for washing coated beads. Corab kits included hepatitis B core antigen (human), coated beads vials (1 ml each) of antibody to hepatitis B core antigen 125 (human), preservative 1.0% sodium oxide, vials (3 ml) of negative control.

METHODS:

(a) Collection of Urine and Blood Samples:

Urine samples (over 50 ml) were collected into 125 ml sterile plastic bottles from patients attending the outpatient clinics at various hospitals and health centres. The bottles were tightly capped, labelled and kept in ice cooled boxes. Information from each patient regarding his/her name, address, sex, age, place of birth and place of residence was recorded. Venous blood (over 10 ml) from each patient was taken from a peripheral vein and put in a universal bottle. The urine samples were then transported to the Department of Surgery in cooling boxes where they were stored frozen at -30°C until they were processed. Blood samples were transported at 4°C and centrifuged.
to separate serum on arrival. The serum was kept frozen until analysed.

The following hospitals and health centres were used for the project: Kenyatta National Hospital, Nairobi and Kiambu; Muranga District Hospital, Murang'a; Machakos General Hospital and Makueni Hospital in Machakos District; Kericho District Hospital, Kericho; Homa Bay District Hospital in South Nyanza; Sio Port Health Centre in Busia; Kitale District Hospital in Trans Nzoia; Kapenguria District Hospital in West Pokot and Lodwar District Hospital in Turkana.

Clearance to collect biological materials in various districts had been granted from the President's Office.

(b) **Processing of Urine Samples.**

The frozen urine samples were thawed and adjusted to pH 5 using IN HCl. They were deproteinized by the addition of 2.3 volumes of isopropyl alcohol and stored at 4°C overnight. Precipitated material was removed by centrifugation of the samples at 1500xg for 10 minutes. The supernatant (25 ml) was subjected to initial clean up on C-18 sep-pak cartridge using a sep-pak cartridge rack connected
to a vacuum pump, at a flow rate not exceeding 2 ml/min. The sep-pak cartridges were washed with 10 ml of 7% methanol followed by 5 ml of 7% acetonitrile and wrapped in parafilm, labelled and kept at -30 until analysis.

(c) Analysis of Urine Samples.

During analysis the C-18 sep-pak cartridges were washed with 5 ml of 10% ethanol and 5 ml of 7% acetonitrile, followed by elution with 10 ml of 80% methanol. The eluate was concentrated by evaporation to 0.5 ml using a speedivac evaporator and analyzed for AFB-Gua by a two step HPLC procedure.

Samples were injected manually through a rheodyne column and the absorption of eluates was monitored at 365 nm. In the first step, isocratic elution using a C-18 Bondapak column and 18% ethanol, 10 nM ammonium formate, pH 5.1 at a flow rate of 1 ml/min was used. Authentic AFB-Gua eluted at 22 min and fractions eluting at 20-25 min were collected. Several other substances from the urine samples also eluted during this time interval. In the second step, isocratic elution of an ultrasil column with 4.5% acetonitrile
at a flow rate of 1 ml/min yielded the AFB-Gua at
5.5 min. 3H-AFB-Gua samples obtained by hydrolysis of
AFB-DNA adducts, were run in parallel with the test
samples to ensure that the procedure was reproducible.
Figure 5 shows the HPLC profile (UV, 365 nm) of
urine sample positive for AFB-Gua on Ultrasil-si
column.

Verification of the chemical identity of AFB-Gua
isolated from the urine samples was carried out
by scanning the 5.5 min eluate using fluorescence
spectrophotometer with synchronous luminescence
and photon counting. Scanning with a fixed wavelength
difference of 34 nm and a 5 nm bandwidth from 250
nm to 600 nm yielded a characteristic spectrum with
a single peak at 415 nm. A sample was considered
positive for AFB-Gua if the 365 nm absorption peaks
were obtained in both HPLC systems and if the characteristic
synchronous fluorescence spectrum was obtained with
the fraction collected from the second HPLC run.
Figure 6 shows a synchronous fluorescence emission
spectrum for AFB-Gua.

(d) **Analysis of Blood Samples:**

Blood samples were centrifuged at 1500 x g for 15
min for separation of sera. The sera were analysed
HPLC profile (UV, 365 nm) of a urine sample positive for AFB-Gua on Ultrasil-SI column, eluted with 4.5% acetonitrile.
Synchronous fluorescence emission spectrum for chemically synthesized AFB-Gua (A) and a positive test sample (B). Same sample as in fig. 5.
for HBV serological markers, i.e. for hepatitis B surface antigen (HBsAg) and anti-Core (anti-HBC) using standard radioimmunoassay (RIA) procedures. Ausria 11-125 and Corab kits were used for these essays according to the general procedures described by the manufacturer as detailed below.

1. **Ausria 11-125 Test Procedure (HBsAg)**

Seven negative and three positive controls were assayed with each run of samples.

i) The waterbath temperature was adjusted to 45°C.

ii) Reaction tray wells for each specimen or control were identified and entered on the data sheets.

iii) 200 ul of the negative control and positive control and 200 ul of the specimens were dispensed into assigned wells.

iv) One bead for each specimen or control to be tested was dispensed into each well on the reaction tray.

v) A cover sealer was applied to each tray. The tray was tapped to release any air bubbles.
It was ensured that the beads were completely covered with the liquid.

vi) The trays were then incubated in 45°C waterbath for two hours.

vii) After the incubation period the cover sealers were removed and discarded. Beads and wells were washed three times using a pipette attached to a vacuum source.

viii) 200 ul 125₁ - Anti - HBS was pipetted into each reaction well.

ix) A new cover sealer was applied to each tray, and the tray tapped to remove air bubbles.

x) The trays were again incubated in 45°C water bath for one hour.

xi) Beads and wells were washed three times again.

xii) Beads from reaction wells were transferred to assay tubes. Inverted rack of oriented assay tubes was aligned over walls, then tray and tubes were inverted together so that beads fell into corresponding tubes.
Radio activity of each tube was determined. Controls and samples were counted together on an Aloka auto well gamma system ARC-500.

The presence or absence of HBsAg was determined by relating net count per minute (CPM) i.e. (gross CPM minus background CPM) of the sample to the cut-off value. The cut-off value is the net CPM of the negative control mean (NCX) times the factor 2.1. For the run to be valid, the mean value for the positive controls (PCX) specimens should be at least 20 times the (NCX).

2. **Test Procedure for the detection on Anti-HBC(CORAB)**

Five negative and five positive controls were assayed with each run of samples.

i) All reagents were brought to 20°C to 30°C, mixed gently before beginning the assay.

ii) The position of each specimen or control was marked in the reaction tray for proper identification.
iii) 100 ul antibody to hepatitis B core antigen 125 I (Human) was pipetted into each designated well.

iv) 100 ul of each specimen or control was pipetted into its assigned well, using a separate pipette tip for each sample.

v) Each tray was tapped to mix the reagents, being careful not to splash or cross contaminate the reaction mixtures.

vi) One antigen coated bead was carefully added to each well.

vii) The reaction trays were covered with the sealers. Trays were then gently tapped to ensure that each bead was covered with the reaction mixture, care being taken to avoid splashing liquid onto the cover.

viii) The trays were incubated on a level surface at room temperature for 18 to 22 hours.
xi) At the end of the incubation period the cover sealers were removed and discarded. The contents of the wells were aspirated into a collection bottle for radioactive waste. The beads were washed three times with distilled water.

x) Beads were transferred from reaction wells to properly identified assay tubes. Inverted rack of tubes was aligned over the reaction tray, the tubes were pressed tightly over wells, then tray and tubes were inverted together so that beads fell into corresponding tubes.

xi) Assay tubes were placed in an Aloka auto well gamma system ARC-500 and the net count rate per one minute determined.

The presence or absence of anti-HBC was determined by comparing the net count rate per minute (CPM) of the samples to a cut-off value. The cut-off value was calculated from the negative and positive control net count rates, calculated from their respective
mean. The ratio between the negative and the positive mean count rates was calculated as a measure of the validity of the test. The mean value for the negative control samples was at least 5 times the positive control mean.

(e) Liver Cancer Incidence Records.

A search was made through patients records in the liver clinic and cancer registry for reported cases of hepatocellular carcinoma covering a period from 1975 to 1982. 5,000 cases were reviewed and the positive cases analysed. The diagnosis of hepatocellular carcinoma was confirmed on the basis of:

(a) Presence of alphafoetal protein and
(b) Histological examination.

Confirmed cases were analysed with respect to sex, age, tribe, area of residence (district) and year of diagnosis. The 1979 Kenya population census was used for the calculation of the frequencies of hepatocellular carcinoma in the districts.
(f) Statistical Analysis.

The individual roles of aflatoxin, hepatitis B virus in the induction of PHC were analysed using Spearman rank correlation analysis to demonstrate if any association between the two agents and primary hepato-cellular carcinoma in the twelve districts existed.

The animal datas were analysed using chi-square.
(a) **Hepatitis B Virus Markers:**

2000 blood samples were taken from persons attending out-patient clinics in hospitals and health centres in twelve different districts of this country. The samples were analysed for hepatitis B surface antigen to find out the carrier rate of HBV in these areas. Nearly all the samples were also analysed for hepatitis B core antibody to establish the previous exposure to hepatitis B virus. Of all the samples tested 9.5% were positive for HBₐg marker while 56% were positive for the core antibody marker. There was a male to female sex ratio of 3:2 for hepatitis B surface antigen carrier rate and a sex ratio of 1:1 for hepatitis core anti-body.

Analysis of the distribution of the hepatitis B surface antigen carrier rate in various age groups showed that at the early age of 10-19 years the prevalence of this marker was 9%. This rate remained nearly the same upto the age group of 20-29 years when it started rising gradually, reaching a peak of 16% at the age group of 40-49 years. It then started falling off gradually so
that by the age of 70 years and over, it had dropped
to a low level of 3%.

Hepatitis core antibody had a prevalence of 45% in the
early age group of 10-19 years. It gradually rose
to 50% at the age groups of 20-29 and 30-39 years.
Thereafter there was a sharp rise in the prevalence
which reached a peak of 70% at the age group of
40-49 years. The prevalence gradually dropped
to 60% and then remained steady at this level through
the later years. Figure 7 shows the prevalence
of hepatitis B surface antigen and hepatitis B
core antibody at various age groups.

A district by district breakdown involving twelve
districts showed that Turkana had the highest carrier
rate of hepatitis B surface antigen with 30.7%.
Next to Turkana was Meru which had a carrier rate
of 14.2% of this marker. Busia district came third
with a carrier rate of 13.5%. Kericho district
had the lowest carrier rate of hepatitis B surface
antigen with only 4.7%. Next to Kericho in the
lower scale of HB Ag carrier rate was Nairobi with
6.6%. West Pokot came third in the district with
low carrier rate of 7.0%. A table showing the distribution
and sex ratio of hepatitis B surface antigen is
shown in Table 1.
FIG. 7

PREVALENCE OF HBsAg AND ANTI-CORE AT VARIOUS AGE GROUPS.

KEY

TOTAL

FEMALE

MALE

ANTI-CORE

SURFACE

RATE OF HBsAg AND ANTI-CORE IN % AGE

AGE GROUPS IN YEARS
**TABLE I**

THE DISTRIBUTION AND SEX RATIO OF HBsAg IN 12 DISTRICTS

<table>
<thead>
<tr>
<th>DISTRICT</th>
<th>% TOTAL +VE</th>
<th>% MALES +VE</th>
<th>% FEMALES +VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MACHAKOS MAKUENI</td>
<td>(220) 11.8%</td>
<td>(95) 14.9%</td>
<td>(125) 10.4%</td>
</tr>
<tr>
<td>MURANG'A</td>
<td>(42) 7.1%</td>
<td>(24) 4.2%</td>
<td>(18) 11%</td>
</tr>
<tr>
<td>KIAMBU</td>
<td>(61) 8.1%</td>
<td>(31) 9.7%</td>
<td>(30) 6.7%</td>
</tr>
<tr>
<td>NAIROBI</td>
<td>(92) 6.6%</td>
<td>(73) 5.5%</td>
<td>(19) 11.1%</td>
</tr>
<tr>
<td>MERU</td>
<td>(48) 14.2%</td>
<td>(27) 11.1%</td>
<td>(21) 20%</td>
</tr>
<tr>
<td>KERICHO</td>
<td>(192) 4.7%</td>
<td>(102) 3.9%</td>
<td>(90) 5.6%</td>
</tr>
<tr>
<td>SOUTH NYANZA</td>
<td>(175) 11.3%</td>
<td>(85) 13.2%</td>
<td>(90) 9.4%</td>
</tr>
<tr>
<td>BUSIA</td>
<td>(200) 13.5%</td>
<td>(49) 24.5%</td>
<td>(151) 8.6%</td>
</tr>
<tr>
<td>TRANS-NZOIA</td>
<td>(129) 7.8%</td>
<td>(65) 12.3%</td>
<td>(64) 2.6%</td>
</tr>
<tr>
<td>WEST POKOT</td>
<td>(59) 7.0%</td>
<td>(30) 12.9%</td>
<td>(29) 0%</td>
</tr>
<tr>
<td>TURKANA</td>
<td>(138) 30.7%</td>
<td>(82) 31.3%</td>
<td>(56) 30.1%</td>
</tr>
<tr>
<td>NYERI</td>
<td>(28) 10%</td>
<td>(16) 8.6%</td>
<td>(12) 11.4%</td>
</tr>
</tbody>
</table>

The numbers in brackets indicate the number of cases analysed.
When a district by district analysis was carried out on hepatitis B-core antibody Turkana again came out with a high rate of this marker. 96% of Turkanas were found to be positive, implying that this percentage of Turkanas had a previous exposure to hepatitis B virus. Next to Turkana was Muranga district with 64.3% positive cases of hepatitis anti-core. Busia district came third with 63.5% positive cases. On the lower end of the scale for this marker was Nyeri district with a rate of 40% positive cases of anti-core and Kericho district which had 40.6% positive cases. Second placed from the bottom of the scale were Machakos/Makueni and Trans-Nzoia both of which had 45% positive cases of anti-core.

2.68% of the tested individuals were positive for both AFB₁ exposure and HB s Ag presence while 17.32% of the tested persons were positive for AFB₁ exposure and HB Ab.

(b) **Aflatoxin-Guanine Adducts (AFB-Gua)**

Over 2000 urine samples were collected from persons attending out-patient clinics in hospitals and health centres in 12 different districts of this country.
The urine samples were analysed for the presence of aflatoxin-guanine adduct using high performance liquid chromatography and fluorescence spectrophotometry. The method used to determine the amount of AFB-Gua adduct in the positive samples was a semi-quantitative one and the lowest level of detectibility for the adduct by this method was 0.3 p. moles/25 ml urine. This adduct accounts for more than 80% of the adducts in the liver two hours after administration of AFB\textsubscript{1} to rats (Busby and Wogan 1984). The adduct is quite unstable and may not be accurately detected after 48 hours following exposure to aflatoxin.

Out of the 1800 samples analysed 20.4% were found to be positive for the presence of AFB-Gua adduct. This indicates that a fairly high percentage of people in this country are exposed to aflatoxin contaminated food products. The detection of AFB-gua adduct in the urine of positive individuals was an indication that these individuals had consumed aflatoxin.

Out of the 866 samples from males analysed 22.9% were positive and of the 934 samples from females analysed 17.1% were positive. This gave an overall male to female ratio of almost 3:2.
A break down on district by district analysis showed that Busia district had the highest rate of exposure to aflatoxin as measured by AFB-Gua adduct excretion. Out of the 203 samples analysed from that district 32.34% had a detectable level of aflatoxin-guanine adduct. The sex ratio of aflatoxin guanine adduct excretors was approximately 1:1 male to female respectively. Meru district came next to Busia with the second highest rate of aflatoxin-guanine adduct positive cases of 30.77% and with a male: female ratio of 2:1 respectively. Turkana district came third with 29.41% positive cases and with a male:female ratio of approximately 2:1.

The district with the lowest level of excretors of aflatoxin-guanine adduct was Nairobi which had 6.22% positive cases, followed by Machakos/Makueni which had 8.71% positive cases. The results from Machakos/Makueni were surprising since an outbreak of aflatoxin poisoning had been reported in the inhabitants of these areas by Ngindu et al, in (1982). The low AFB-Gua adduct positive rate showing in this area could be explained by the fact that samples were collected during the dry season during which time aflatoxin contamination of the staple food was low. The other districts such as Kiambu, Murang'a Nyeri, Kericho, South Nyanza, Trans Nzoia and West
The distribution and sex comparison of AFB-GUA adducts excretion in 12 districts of Kenya

<table>
<thead>
<tr>
<th>District</th>
<th>% Total +VE</th>
<th>% Males +VE</th>
<th>% Female +VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Machakos/Makueni</td>
<td>(218) 8.71%</td>
<td>(87) 11.49%</td>
<td>(181) 6.87%</td>
</tr>
<tr>
<td>Murang'a</td>
<td>(128) 15.63%</td>
<td>(64) 20.31%</td>
<td>(64) 10.94%</td>
</tr>
<tr>
<td>Kiambu</td>
<td>(91) 15.63%</td>
<td>(31) 22.58%</td>
<td>(40) 5%</td>
</tr>
<tr>
<td>Nairobi</td>
<td>(93) 6.22%</td>
<td>(73) 6.85%</td>
<td>(20) 4.76%</td>
</tr>
<tr>
<td>Meru</td>
<td>(39) 30.77%</td>
<td>(24) 37.5%</td>
<td>(15) 20%</td>
</tr>
<tr>
<td>Kericho</td>
<td>(77) 22.08%</td>
<td>(46) 26.0%</td>
<td>(31) 19.35%</td>
</tr>
<tr>
<td>South Nyanza</td>
<td>(192) 23.96%</td>
<td>(93) 26.09%</td>
<td>(99) 22.22%</td>
</tr>
<tr>
<td>Busia</td>
<td>(201) 32.34%</td>
<td>(50) 32%</td>
<td>(151) 53.11%</td>
</tr>
<tr>
<td>Trans Nzoia</td>
<td>(165) 23.64%</td>
<td>(82) 24.38%</td>
<td>(83) 22.89%</td>
</tr>
<tr>
<td>West Pokot (Kapenguria)</td>
<td>(65) 24.62%</td>
<td>(35) 22.86%</td>
<td>(30) 26.67%</td>
</tr>
<tr>
<td>Turkana</td>
<td>(68) 29.41%</td>
<td>(38) 36.84%</td>
<td>(30) 16.67%</td>
</tr>
<tr>
<td>Nyeri</td>
<td>(46) 15.38%</td>
<td>(21) 14.29%</td>
<td>(25) 16.67%</td>
</tr>
</tbody>
</table>

The numbers in brackets indicate the number of cases analysed.
FIG. 8

THE DISTRIBUTION OF AFB-GUA EXCRETORS AT VARYING AGE GROUPS IN ALL THE 12 DISTRICTS COMBINED.

KEY

X X X MALES

○ ○ ○ FEMALES

% AGE OF AFB-GUA EXCRETORS

0-19  20-29  30-39  40-49  50-59  60-69  70 & OVER

AGE IN YEARS
Pokot had AFB-Gua positive cases ranging from 12.68% to 24.6%. Table II shows the distribution and sex comparison of AFB-Gua adduct excretion in 12 districts of this country.

Analysis of AFB-Gua adduct excretors at specific age groups revealed that there was a high rate (over 25%) of positive cases right from the early age of 10-19 years. The percentage of AFB-Gua excretors remained high between the age groups of 20-29 years, to 40-49 years and started falling off at 50 years. In the age groups of over 50 years, whereas the percentage of female excretors continued falling off till 70 years, that of males dropped only a little and then started to rise, continued rising till the age of 70 years. Fig.1 shows the distribution of AFB-Gua adducts excretors at varying age groups.

(c) Prevalence of Primary Hepatocellular Carcinoma in various Districts of Kenya.

5,000 cases were reviewed from patients records in liver clinic, cancer registry and the district disease control records to identify positive cases of hepatocellular carcinoma covering
from 1975-1982 in 27 districts. The diagnosis of hepatocellular carcinoma was confirmed on the basis of histological report and serology of alphafoetal protein. The 1979 Kenya population census was used for the computation of the prevalence of hepatocellular carcinoma in various districts. The prevalence of PHC ranged from 1 to 6 cases per 100,000 persons per year. The district which reported the highest prevalence of PHC was Nyeri with 6.78 cases per 100,000 persons per year. This was followed by Elgeyo Markwet with 5.37, Kitui with 5.17, West Pokot with 4.41 and Turkana with 4.21 cases per 100,000 persons per year. Nairobi with 0.604 cases, Nakuru with 0.57, Baringo and Siaya with 0.49 cases each per 100,000 persons had the lowest prevalence of PHC. A histogram showing the prevalence of PHC in 27 districts is shown in fig.9. There was a male to female sex ratio of 2.4:1; although the ratio varied widely from one district to another. In most districts the male/female ratio was greater than 1.

Table III shows the annual prevalence of PHC and sex comparisons in 27 different districts.

When the relationship between specific age groups and the combined national annual average PHC cases
<table>
<thead>
<tr>
<th>DISTRICT</th>
<th>MALES</th>
<th>FEMALES</th>
<th>TOTAL</th>
<th>PREVALENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NYERI</td>
<td>21</td>
<td>12</td>
<td>33</td>
<td>6.783</td>
</tr>
<tr>
<td>ELGEYO MARKWET</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>5.374</td>
</tr>
<tr>
<td>KITUI</td>
<td>17</td>
<td>7</td>
<td>24</td>
<td>5.169</td>
</tr>
<tr>
<td>WEST POKOT</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>4.412</td>
</tr>
<tr>
<td>TURKANA</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>4.205</td>
</tr>
<tr>
<td>MOMBASÃ</td>
<td>13</td>
<td>1</td>
<td>14</td>
<td>4.104</td>
</tr>
<tr>
<td>EMBU</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>3.040</td>
</tr>
<tr>
<td>KIAMBU</td>
<td>11</td>
<td>9</td>
<td>20</td>
<td>2.914</td>
</tr>
<tr>
<td>TAITA</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>2.710</td>
</tr>
<tr>
<td>KAJIADO</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2.684</td>
</tr>
<tr>
<td>MURG'A</td>
<td>10</td>
<td>7</td>
<td>17</td>
<td>2.622</td>
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<td>KISUMU</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>2.488</td>
</tr>
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<td>0</td>
<td>5</td>
<td>2.143</td>
</tr>
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<td>MACHAKOS</td>
<td>16</td>
<td>2</td>
<td>18</td>
<td>1.760</td>
</tr>
<tr>
<td>KILIFI</td>
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<td>3</td>
<td>7</td>
<td>1.624</td>
</tr>
<tr>
<td>KAKAMEGA</td>
<td>7</td>
<td>9</td>
<td>16</td>
<td>1.552</td>
</tr>
<tr>
<td>KISII</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>1.380</td>
</tr>
<tr>
<td>BUSIA</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1.343</td>
</tr>
<tr>
<td>TRANS NZOIA</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1.156</td>
</tr>
<tr>
<td>SOUTH NYANZA</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td>1.100</td>
</tr>
<tr>
<td>NANDI</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0.998</td>
</tr>
<tr>
<td>KERICHO</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>0.947</td>
</tr>
<tr>
<td>NAKURU</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0.574</td>
</tr>
<tr>
<td>BARINGO</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.491</td>
</tr>
<tr>
<td>SIAYA</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0.491</td>
</tr>
<tr>
<td>NAIROBI</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>0.491</td>
</tr>
</tbody>
</table>
was examined, it was observed that PHC cases at the early age of 0-19 years was quite low—showing only 28 cases per year. This figure rose sharply reaching a peak of 110 cases per year at the age group of 30-39 years. This peak was maintained although with a slight drop, up to the age group of 50-59 years. Thereafter there was a sharp drop in the number of cases, so that by the age of 70 years and over, the number had dropped to almost 28 cases per year. A distinct contrast was observed between the male and female frequencies. The male PHC frequency started off with a low number of 20 cases in early age group and rose sharply to a peak of 90 cases at the age group of 30-39 years. It then slightly dropped to 74 cases at the age group of 50-59 years and thereafter dropped sharply in the late ages of 60-69 years. At the age of 70 and over years, the frequency had dropped to a low of 17 cases per year. The female frequency rose gradually from the low frequency of 6 cases per year in the early age and reached a peak of 29 cases per year at the age group of 40-49 years. It then remained steady at this peak through the age group of 50-59 years up to 60-69 years. Thereafter it dropped rather gently to 10 cases per year at 70 and over years.
FIG. 10
DISTRIBUTION OF HCC CASES ACCORDING TO AGE AND SEX GROUPS

KEY.

- TOTAL HCC FREQUENCY
- HCC FREQUENCY FOR MALES
- HCC FREQUENCY FOR FEMALES

AGE GROUPS IN YEARS.
FIG. 11

ANNUAL VARIATION IN HCC INCIDENCE IN KENYA (1975-1982).

KEY:
- TOTAL ANNUAL FREQUENCY
- TOTAL ANNUAL FREQUENCY FOR MALES
- TOTAL ANNUAL FREQUENCY FOR FEMALES

YEAR:

HCC FREQUENCY:
10 20 30 40 50 60 70 80 90 100 110
The ratio of male to female cases varied from 3.3:1 at the age group of 0-19 years and increased to 5:1 at the age group of 30-39 years then decreased to a ratio of 1.5:1 at the age group of 60-69 years. The graph showing the frequency of PHC at various age groups is shown in fig.10.

Annual variation in the frequency of PHC was analysed between 1975 and 1982. This analysis revealed that there was a marked fluctuation in the frequency of PHC from year to year. In 1975, 60 cases of PHC were reported. The number rose to 94 cases in 1976. The frequency then sharply dropped to 54 cases in 1977, and remained at this level through 1978. After that year the frequency of PHC started again rising sharply reaching 83 cases in 1979. The frequency continued rising and reached a peak of 93 cases in 1980 and then slightly dropped to 90 cases in 1982. The graph showing the annual variation in the frequency of PHC is shown in fig.11.

(d) Correlation between AFB–Gua, HBsAg, HBsAb and PHC

The rates of aflatoxin B1 guanine adduct excretion, hepatitis B surface antigen and hepatitis B core
### Table IV

Comparison between the prevalence of PHC, HBsAg and AFB-Gua in 12 districts of Kenya

<table>
<thead>
<tr>
<th>District</th>
<th>PHC Prevalence per 100,000 Persons</th>
<th>Frequency of HBsAg</th>
<th>Rate of AFB-GUA Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Nyeri</td>
<td>6.783</td>
<td>(28) 10% 40%</td>
<td>(46) 15.38%</td>
</tr>
<tr>
<td>2 West Pokot</td>
<td>4.412</td>
<td>(59) 7.0% 47.4%</td>
<td>(65) 24.62%</td>
</tr>
<tr>
<td>3 Turkana</td>
<td>4.205</td>
<td>(138) 30.7% 96%</td>
<td>(68) 29.4%</td>
</tr>
<tr>
<td>4 Kiambu</td>
<td>2.914</td>
<td>(61) 8.1% 52.5%</td>
<td>(71) 12.68%</td>
</tr>
<tr>
<td>5 Meru</td>
<td>2.650</td>
<td>(48) 14.2% 50%</td>
<td>(39) 30.77%</td>
</tr>
<tr>
<td>6 Murang'a</td>
<td>2.622</td>
<td>(42) 7.1% 64.3%</td>
<td>(128) 15.63%</td>
</tr>
<tr>
<td>7 Machakos</td>
<td>1.760</td>
<td>(220) 11.8% 45%</td>
<td>(218) 8.71%</td>
</tr>
<tr>
<td>8 Busia</td>
<td>1.343</td>
<td>(200) 13.5% 63%</td>
<td>(201) 32.34%</td>
</tr>
<tr>
<td>9 Trans Nzoia</td>
<td>1.156</td>
<td>(129) 7.8% 45%</td>
<td>(165) 23.64%</td>
</tr>
<tr>
<td>10 South Nyanza</td>
<td>1.100</td>
<td>(175) 11.3% 62%</td>
<td>(192) 23.96%</td>
</tr>
<tr>
<td>11 Kericho</td>
<td>0.947</td>
<td>(192) 4.7% 40.6%</td>
<td>(97) 22.08%</td>
</tr>
<tr>
<td>12 Nairobi</td>
<td>0.604</td>
<td>(92) 6.6% 59.8%</td>
<td>(93) 6.22%</td>
</tr>
</tbody>
</table>

The numbers in brackets indicate the number of cases analysed.
antibody were compared to the prevalence of PHC in 12 districts of this country as shown in table IV. A Spearman rank correlation analysis of the rate of aflatoxin exposure and PHC prevalence in the 12 districts gave no statistically significant correlation between the two parameters, \( r=0.054 \) (\( t=0.171, \text{P}>0.1 \)). The same type of analysis did not show any statistically significant association between HB\(_S\) Ag and PHC, \( r=0.231 \) (\( t=0.710, \text{P}>0.1 \)). There was also no significant association between HB\(_C\) Ab and PHC, \( r=0.115 \) (\( t=0.366, \text{P}>0.1 \)). Neither was there any statistically significant correlation between the combined effects of HBV and aflatoxin exposure when compared to the prevalence of PHC in this study, \( r=123 \) (\( t=0.392, \text{P}>0.1 \)).
DISCUSSION:

(a) **Hepatitis B virus infection:**

The seroepidemiology of hepatitis B virus infection was carried out in more or less the same individuals in whom AFB-Gua excretion was studied. The frequency of HBV infection was determined by the serological testing for HBsAg and anti-HBC antibody using radioimmunoassay technique. The average frequency of HBsAg in the 12 districts analysed was 9.5% with a male to female ratio of 3:2. This frequency is rather low when compared with the results of Bowry and Shah (1983) who reported a carrier rate of 14% for HBsAg in volunteer blood donors in this country. Zumla and Voller (1982) reported a carrier rate of 14% of the healthy controls in Zambia, while rates of 18.7% in Swaziland and 14% in Senegal were reported (Peers et al, 1987). The carrier rate reported in this study is fairly low compared to the carrier rate in most sub-Saharan African Countries. This is because the subjects covered in this study had a wider age-range than those in the other studies.

The average rate for hepatitis B core antibody in the same district was 16%, which is much higher than the...
This means that over one half of the persons living in the 12 districts analysed have at one time or the other had HBV infection. Generally, most infections with hepatitis B virus are followed by an apparent complete recovery with the development of a virus specific antibody (K.Koike et al.,1983) However, a significant proportion of infections may produce chronic hepatitis of various types, liver cirrhosis and hepatocellular carcinoma. Many epidemiological studies have shown a close association between chronic active hepatitis infections and primary liver cancer (Decker and Kuhns 1986).

The frequency of HBsAg in different specific age groups showed that HBsAg appeared at an early age group in a few cases. A significant rise in the incidence rate of HBsAg started showing from the age of around 20 years. This suggests that vertical transmission from mothers to offsprings accounted for only a small proportion of the infection in the affected population of this country. This consequently means that vaccinating babies from infected mothers soon after birth will only take care of a small part of the problem involved.
The main route of transmission of HBV in this country is parenteral, involving human exposure to infected materials such as blood and other body fluids. Blood transfusion, injection or taking blood using contaminated syringes and needles and handling of contaminated blood and other body fluids are the main sources of infection. Health workers are therefore, at high risk to HBV infection in this country. Tatooing and other skin abrasives also play a role in the transmission of HBV. However, the role played by insects such as mosquitoes in the transmission of HBV infection is not clear.

The district by district analysis of the frequency of HB\textsubscript{S}Ag and HB\textsubscript{C}Ab indicated that Turkana had the highest rates of both of these two HBV markers. In fact the frequencies (30.7%) for HB\textsubscript{S}Ag and 96% for HB\textsubscript{C}Ab of these two markers were high up beyond those of the other districts. Over all, no geographical correlation between HB\textsubscript{S}Ag carrier rate and PHC was observed in the 12 districts. If hepatitis B virus infection of itself, played a major role in the aetiology of primary hepatocellular carcinoma, then an association between the two diseases would have been demonstrated. West Pokot had a relatively
low frequency of HBs Ag and yet high PHC prevalence. Similarly, Nyeri, Kiambu and Murang'a had low frequencies of HB Ag and yet they had high PHC prevalences relative to the other districts. On the other hand, South Nyanza had a fairly high incidence rate of HBs Ag and yet the prevalence of PHC was relatively low.

If chronic HBV infection were indeed a sufficient cause for the development of PHC, a close geographical association between the two diseases would be expected, whereas anomalously high or low prevalence for PHC relative to the incidence rates of HBV have been observed in the above mentioned districts. These observations need to be viewed with caution because of the problem of PHC under-reporting in various hospitals. Nevertheless, these results somehow discount or down play the significance of the role played by HBV as a cause of PHC singly and call for a serious consideration of other factors that may be involved.

(b) Aflatoxin-guanine adduct excretions

This study showed that 20% of people in this country are exposed to aflatoxin contamination. Correlation between doses of AFB and cancer rates in different animal species is generally poor. However, when carcinogen-DNA binding in target organs is measured, good correlation with cancer rates
has been observed for several carcinogens including AFB (Neumann, 1984). Total AFB-DNA binding level has been shown to strongly correlate with carcinogenic response. Therefore, determination of the binding of AFB to DNA in humans as well as measurements of urinary excretion of AFB-Gua should give a better basis of a non-invasive method for monitoring aflatoxin exposure.

The fact that AFB-Gua adduct was detected in urine excreted by some individuals was an indication that those persons had consumed aflatoxin either through some foodstuffs or some drinks. Not only had these persons consumed aflatoxin, but that the aflatoxin so consumed had interacted with DNA or RNA to form AFB-DNA adducts which were hydrolysed and released as AFB-guanine adducts.

The parent aflatoxin compound is not carcinogenic per se. It is the activated species which exerts toxic, mutagenic and carcinogenic effect. Aflatoxin is metabolized by the microsomal mixed function oxygenase system. This system incorporates a complex organization of cytochrome P-450-coupled, O$_2^-$ and NADPH dependent enzymes localized mainly on the endoplasmic reticulum
of livers and also present in kidneys, lungs, skin and other organs. The enzymes catalyse the oxidative metabolism of aflatoxin which results in either the formation of various hydroxylated derivatives or in an unstable highly reactive epoxide metabolite. The suspected carcinogenic lesion is formed by the interaction of the activated electrophilic species, $\text{AFB}_1\text{2,3-oxide}$ with the 7th position of guanine in DNA (Essignmann et al; 1977). $\text{AFB}_1\text{2,3-oxide}$ interacts with DNA to form 2,3-dihydro-2-(N-guanyl)-3-hydroxy aflation B (AFB-N-Gua) as the major DNA adduct both under in vitro conditions and also in the liver of rats injected with $\text{AFB}_1$ (Bennet et al.; 1981). This adduct accounted for more than 80% of the adducts in the liver 2 hours after administration of $\text{AFB}_1$ to rats. An identical adduct profile has been observed when cultured human tissues including foetal liver explants were incubated with $\text{AFB}_1$ (Autrup et al.; 1983). $\text{AFB}_1$ substitution at the N position of guanine in DNA induces a strongly positive charge in the imidazole ring while the adduct is present in DNA. This in turn renders the N-glycosidic bond linking the adduct to DNA labile to hydrolysis resulting in the release
of the complete $\text{AFB}_1 - \text{N-Gua}$ moiety, and leaving an apurinic site in the DNA strand—a possible mutagenic lesion. Apurinic sites have been shown to lead to TA-GC transversions in eucaryocytes. This particular change in base composition has been found in several activated oncogenes isolated from human tumour cell lines (Dragsted et al., 1986). A second reaction, occurring most readily under mildly alkaline conditions, resulted from OH-attack on the C-8 of guanine. This attack caused the opening of the imidazole ring, producing the putative persistent ring-opened DNA-bound formamido-pyrimidine derivative, $\text{AFB}_1 - \text{FAPyr}$. Formation of this derivative has important implications with respect to its possible functional significance, since it results in the stabilization of the deoxyglycosidic bond, reducing the facility with which depurination takes place and stabilizing the adduct in DNA. (Dragsted et al., 1986).

$\text{AFB-Gua}$ adduct is very unstable and is lost rapidly by way of excretion through the urine and by its partial conversion into the persistent $\text{AFB}_1 - \text{N-FAPyr}$ adduct.
The major DNA adduct in rat liver 2 hours after i.p. injection of 0.3 mg of AFB$_1$ /kg was AFB$_1$-N-Gua but at 24 hours most of this adduct had disappeared with some of it being converted to the now more prevalent and persistent AFB$_1$-N-FAPyr. Essentially only AFB$_1$-N-FAPyr was detectable 48 hours after injection (Busby and Wogan, 1984).

It is well established that modification of cellular DNA by AFB has the potential for producing profound and deleterious consequences to the organism in terms of toxicity, mutagenicity and carcinogenicity. The degree of the damage to DNA in turn depends on the total amount of DNA adducts formed since a positive correlation has been demonstrated between total adduct levels in the DNA and the known toxic, mutagenic and carcinogenic effects (Busby and Wogan, 1984). The specific types of adduct present will also affect the biological effect in that persistence and possible continued accumulation of AFB$_1$-N-FAPyr in liver DNA during carcinogenic treatment might be the primary molecular event of tumour initiation (Croy and Wogan, 1981). The time frame in which the adduct persists in DNA is also critical in that if within that time the cell goes into mitotic division then the damage will be more
effective. Also the particular areas of the genome affected will be critical as far as DNA modification is concerned.

Each organism has its own mechanism of DNA repair processes by which it tries to restore the damaged DNA strand to its original undamaged state. This then means that the ability of an organism to resist damage to its DNA will depend on the efficiency and fidelity of its DNA-repair enzymes. Deficiency of these enzymes will have far reaching effects on the maintenance of DNA in its proper form.

AFB-Gua has been detected in the urine collected from rats treated with AFB and from humans living in an area with a high exposure to dietary AFB (Astrup et al., 1987). Detection of AFB-Gua is proof that genotoxic damage has occurred after exposure to AFB. In rats, approximately 1% of the administered AFB dose was excreted as AFB-Gua within 24 hours. Assuming the same rate of excretion in humans, the daily minimum rate of AFB exposure in the individuals studied was 360 ng. At the lowest level of detectability, the exposure corresponded to 0.3 AFB-modification/10^6 nucleotides in liver DNA. This level of AFB exposure
compares very well with that found in Gambia but measured by a different approach (I.A.R.C. Internal Technical Report No. 84/003).

The staple diet prepared from contaminated maize, millet, cassava, groundnuts is the main source of AFB-contamination, although alcoholic beverages brewed from aflatoxin contaminated grain may also be an important source of aflatoxin. Another source may be milk and milk products from dairy cows feeding on aflatoxin contaminated feeds and forage.

There was a sex ratio of 3:2 male to female in AFB-Gua excretion. The higher exposure rate of AFB in males has been attributed to consumption of AFB contaminated alcoholic drinks since more males drink it more than females. A hormonal effect might also be playing a role for this sex ratio difference. Hormonal effects have been clearly demonstrated in rats and other animal species (Busby and Wogan, 1984). The higher exposure rate of AFB in males than females, may also account for the higher incidence of primary hepatocellular carcinoma in males than in females that was observed in this study.
The results obtained from each district, showed that aflatoxin contamination in the staple food is quite widespread in this country although the percentage of AFB-Gua excretors varied from district to district.

In an earlier study, Dragsted et al. (1986) had reported a seasonal variation in AFB-Gua excretion in some districts of this country. This seasonal variation may account for the annual variation of PHC that was observed in this study. No seasonal or annual variation has been reported for hepatitis B virus infection.

The results of AFB-Gua adduct excretion at varying age groups revealed that aflatoxin is consumed by the population of this country from a fairly young age. By the age group of 10-19 years, excretion of AFB-Gua adduct was already high. This may account for the early age at which PHC appears in this country. Persons at an early age are more susceptible to genetic and carcinogenic effects since at this age their cells
are rapidly undergoing mitotic division during the period of their rapid growth. Also clearly seen from this study was the fact that AFB-Gua adduct excretion was observed throughout the range of the various age groups.

(c) Primary Hepatocellular Carcinoma

Analysis of the results obtained from the district disease control registry and the cancer registry gave an annual prevalence of 1 to 6 cases per 100,000. This compares well with the prevalence of 3-12.5/10 reported by Peers and Linsell (1973) for Murang'a district. Whereas Nyeri, West Pokot, Turkana, had relatively high prevalences, South Nyanza, Kericho and Nairobi had low prevalences of PHC.

The sex ratio of 2.4:1 male to female ratio reported in this study is fairly close to that of 2:1 reported by Peers et al., (1976). The sex ratio varied considerably from one district to another, indicating that probably it is not just the hormonal effect that accounted for the differences in the sex ratio but other factors may be involved in different areas. Apart from cultural practices being involved in various ethnic groups,
genetic factors could also be involved as has been demonstrated in debrisoquine metabolism (Astrup personal communication).

When the age-specific case numbers for PHC were analysed in the 12 districts combined, it was shown that the number of cases of PHC were fairly few (28 cases per year) below the age of 20 years. The numbers increased sharply and by the age group of 30-39 years, it had reached a high peak of 110 cases per year. It remained high up to the age group of 50-59 years and then sharply declined. This observation indicates that induction of PHC occurs in early age group, especially in males. It is interesting to recall that aflatoxin exposure as measured by AFB-Gua excretion was quite high even in early age.

The situation in females was somehow different in that besides the number of PHC cases being low compared to that of males in the early age group, the increase in the female cases was very gradual and reached a peak at the age of 40-49 years and remained at that level up to the age group of 60-69 years. It then declined. The high case number of PHC in middle aged male persons agrees with the observation
of Beasley and Hwang (1984) that PHC was seen in younger age in areas of high prevalence than in areas where it was rare. The pattern of distribution of PHC in various age groups in females, on the other hand, tended to agree with what has been reported in Europe and U.S.A. where most cancer cases occurred in the late age of over 50 years (Szumuness, 1978).

The increasing sex ratio of male to female from 3.3:1 at the age group of 0-19 years to 5:1 at the age group of 30-39 years, signifies some important differential factors in relation to PHC induction as the two sexes develop through those age groups. After the age group of 30-39 years the ratio between the two sexes decreased until it reached 1.5:1 at the old age of 60-69 years. Whatever factors are involved in bringing about the different trends in the two sexes during that development stage (30-39 years) whether social or biological, can be beneficially exploited to reduce the prevalence of PHC in our society.

When the annual variation in the number of PHC cases was analysed, there was significant fluctuation from year to year between 1975 and 1982. This yearly variation
in the number of PHC cases tended to agree with the earlier report of seasonal variation in aflatoxin ingestion (Dragsted et al., 1986).

(d) Correlation Between AFB-Gua, HBV and PHC

The fact that the joint effects of aflatoxin ingestion and HBV infection showed no statistically significant correlation with the prevalence of PHC in the twelve districts in which this study was conducted should not be construed to mean that the two agents play no significant role in the aetiology of liver cancer in humans. Considering the evidence that has accumulated about HBV and aflatoxin, these two agents have been implicated to be serious candidates in the aetiology of liver cancer either singly or synergistically (Groopman et al., 1987, Sun and Chu 1984, Peers et al., 1987 and Van Rensburg et al., 1985).

A very strong correlation has been established between human primary liver cancer and chronic HBV infection. Several lines of evidence have converged to indicate that human primary liver cancer and chronic HBV infection are causally related although no association exists between PHC and Previous HBV infection (Groopman et al., 1986). The numerous case control studies
that indicated a causative role for HBV infection in the development of PHC have been supported by evidence that suggests that chronic active infection with HBV may be a necessary initial factor in the development of all cases of PHC. A prospective study in Taiwan by Beasley et al. (1981) indicated a relative risk of over 200 with all but one case occurring among persons who were carriers of HBsAg prior to development of PHC (Van Rensburg et al., 1985). Furthermore HBV-DNA has been found incorporated into the host genome of PHC patients including those who had no markers of HBV infection (Brechot et al., 1982).

However, as Groopman et al. (1986) pointed out, the complete elucidation of the pathogenesis of human PHC must take into account the fact that chronic HBV infection is not the sole aetiological factor in PHC since some cases are not related to HBV.

Secondly, chronic HBV infection is by itself not sufficient to cause PHC since only a fraction of the individuals infected ever go on to develop PHC. Furthermore, although geographical and regional relationships between PHC and HBV in South East Asia, China, India and sub-Saharan Africa have been established, some exceptions to the
association have emerged. If chronic HBV infections were indeed a sufficient cause for the development of PHC, a close geographical association between the two diseases would be expected, whereas several regions of anomalously high or low incidence for PHC relative to the occurrence of HBV infection have been reported. For instance, PHC is uncommon and yet HBV infection is prevalent in Greenland's Eskimos (Melbye et al., 1984). Bagshawe et al. (1975) found no association in the prevalence of Hb Ag and PHC in areas where an association had been demonstrated between PHC prevalence and aflatoxin contamination of the staple food (Peers and Linsell, 1973). In Guangxi region in China, the mortality rate of PHC in HB Ag positive individuals was higher when the individuals lived in villages with high aflatoxin exposure (Henderson, 1985). A case control study of PHC patients all of whom were born and reared in rural areas but half of them had moved to an urban setting was conducted by Kew et al. (1983). A higher incidence in PHC and a lower age of its onset in rural than in the urban populations was
observed. It was concluded that HBV status which was similar in both groups could not account for the differences in the frequency and age at onset of PHC in rural and urban populations. Thus, other factors including aflatoxin exposure are possibly important determinants in the development of PHC.

A positive association has been found to exist between aflatoxin ingestion and liver cancer frequency in Kenya, Swaziland, Thailand and Mozambique (Peers et al. 1987), Busby and Wogan, 1984, Van Rensburg et al., 1985). However, these studies were a measure of AFB in the diet and not at individual level. High intakes of aflatoxin were consistently associated with high frequencies of liver cancer. The association was most apparent in connection with the frequencies for adult males. These data provide strong circumstantial evidence of a putative causal relationship between aflatoxin ingestion and liver cancer frequency in humans. Although this evidence does not constitute proof that aflatoxins are the cause of human liver cancer, these data together with the extensive animal data on aflatoxin carcinogenicity are sufficient to associate exposure to the carcinogen with elevated risk of this form of cancer.

Further to the above observation, the findings of
Adekuule et al. (1983) that a part from aflatoxins, plant medicinal preparations containing active compounds; imperatorin, dictamine, marmersin and heraclenin may be responsible for some human cancers in Nigeria and other parts of Africa, must be viewed seriously. The use of aqueous extracts of the leaves, bark, wood and roots of various plants has been adopted by native doctors (herbalists) as a method of health care in many African countries. Most of these extracts have been found to contain the coumarin nuclei with 2,3-furan double bonds complemented to the coumarin as in aflatoxins. This observation has far reaching implications for, besides the examples given above, unanalysed plant preparations are widely used in various parts of Africa within traditional methods of healing. Some of these plant preparations might be tumour promoters although others might carry inhibitory effects.

Areas of high, prevalence of HBV in Africa and Asia also coincide with areas of elevated aflatoxin contamination of the staple human food supply. This implies that two plausible and perhaps interactive, aetiological factors for induction of liver cancer in humans have been identified. Thus hepatitis
B virus and aflatoxin appear to act synergistically in the pathogenesis of PHC. Integration of HBV-DNA into cellular genome of PHC tissues and the in vivo formation of adducts between AFB and nucleic acids led Vyas et al (1986) to suggest that hepatocytes with integrated HBV-DNA preferentially accumulated AFB; the AFB-nucleic acid adducts formed may then initiate cell transformation by modifying the expression of critical host genes, especially since both AFB and HBV are considered to be immunosuppressive agents.

Why then was there no positive correlation between aflatoxin ingestion or HBV infection and PHC prevalence? This may be due to the fact that the cause and development of PHC involves more complex interactions involving several aetiological factors and going through several stage processes. In other words, the cause and development of PHC are also dependent on other modifying factors both exogenous such as promoters and inhibitors and endogenous factors such as hormones, genetic influence etc. Thus suggesting that the cause of PHC is multifactorial and its process is multistage.
Modifying factors may influence the metabolism of aflatoxin so that the ultimate reactive species is not formed. For instance, the excretion of AFB-Gua adducts depends not only on the aflatoxin exposure level but also on the individual's ability to metabolise aflatoxin to its ultimate carcinogen. Thus following in vitro incubation of human bronchial or colonic explants with AFB, Autrup et al. (1979) found a 100-fold variation among individuals in the formation of adducts between AFB and DNA.

Underreporting of PHC cases seems to have significantly contributed to the lack of correlation between HBV or aflatoxin to PHC in this study. It is evident that only a small fraction of patients with PHC were reported from some of the districts covered in the study. It is likely that many patients with PHC might have died without going to hospital and so were never reported. It is well known that in this country attendance at hospital falls markedly with distance from the patient's home. Equally significant is the number of patients with PHC who might have
attended their nearest district hospitals or health centres but were never reported in the hospital records or cancer registry.

The method used for assaying aflatoxin guanine adduct excretion only measures short-term exposure to aflatoxin. This might have contributed to the lack of correlation between aflatoxin and PHC by excluding the long-term exposure cases. Measurement of long-term as well as short-term aflatoxin exposure would be preferred when one becomes available. Human exposure to hepatitis B virus can be reliably measured retrospectively via immunological methods so a positive association between HBV markers and PHC was expected if HBV infection indeed played a major role in the cause of PHC in this country. On the other hand, the relationship between aflatoxin exposure and the risk of liver cancer has not been adequately evaluated at an individual level in human studies. This was so because aflatoxin exposure was very difficult to accurately measure at an individual level. Tests for aflatoxin metabolites or DNA adducts are useful in measuring recent exposure within the past few hours or days,
but as yet cannot be used to evaluate long-term exposure. There is therefore an urgent need to develop a reliable method for the detection of long-term exposure to aflatoxin at individual level. The procedure used in this study has an advantage that in addition to proving recent aflatoxin exposure, it also proves modification of genetic materials in the individuals involved. The present study is one of the first of its kind to utilize measurements of both aflatoxin and hepatitis B virus exposure at individual level made on the same population groups.

Taking a broad view, aetiological agents for PHC seem to differ from place to place in the world according to environmental, genetic, ethnic, hormonal, socioeconomic or human ecological backgrounds. Whereas, in some areas of the world hepatitis B virus may appear to be significant, in other parts aflatoxin seems to be more important for inducing PHC. In either of the two cases, it should be remembered that carcinogenesis of PHC involves a combination of activities of several possible cofactors in its aetiology
some acting as initiators and/or promoters. In his country, aflatoxin seems to be playing a major initiating role with the other agents such as HBV probably acting as co-carcinogens. The fact that aflatoxins seem to play a stronger role in the aetiology of liver cancer in this country should not polarize research efforts into the camp of chemical carcinogenesis at the expense of viral carcinogenesis. Rather, greater progress will be made only if the view is taken that liver cancer probably results from complex interactions between the virus (HBV) and chemicals, and that the final pathways by which the two classes of agents produce cell transformation are probably similar.

Despite the fact that there was no significant correlation between aflatoxin and hepatitis B virus exposure with the prevalence of PHC in this study every effort should be made to control aflatoxin contamination by using improved food storage system and by using healthy seed for crops. HBV infection can be controlled by better hygiene and vaccination of high risk cases.
CHAPTER 3:

EFFECTS ON OFFSPRING RATS SUCKLING MILK FROM AFLATOXIN B₁-TREATED MOTHERS

INTRODUCTION

The hepatotoxicity and carcinogenicity of AFB₁ has been thoroughly documented (Wogan 1973, Campbell and Hayes, 1976, Busby and Wogan 1984). However, relatively little is known about the carcinogenic potency of AFM₁; a major hydroxylated metabolite which is produced by animals exposed to AFB₁. AFM₁ was the first aflatoxin metabolite to be identified by Allcroft and Carnaghan (1963) and has been found in many human foods such as milk from cows fed on AFB₁-contaminated feeds. Exposure to AFM₁ can occur in two ways, either as a metabolite formed after AFB₁ exposure or indirectly through the ingestion of food products from animals that have metabolized AFB₁ to AFM₁.

One of the main sources of human exposure to aflatoxins is by ingestion of aflatoxins carried over from feeds into milk and milk products including cheese and powdered
milk, where they appear mainly as aflatoxin M₁. This study using rats sought to establish whether the milk from lactating mothers dosed with aflatoxin B₁ can induce hepatocellular carcinoma in the offsprings. This will imply that milk from dairy cows fed aflatoxin B₁-contaminated feeds poses health hazards to humans.

MATERIALS AND METHODS

MATERIALS

Chloroform, ether, Xylene, mayor's haematoxylin, eosin and dimethyl sulphoxide were purchased from Sigma Chemical Co. Aflatoxin B₁ was purchased from Makor Chemicals Ltd. Jerusalem, Israel. Microtome, incubator, plastic rat cages, paraffin wax, slides, cover slips and stomach tube.

ANIMALS

Female albino wistar rats bred in our animal house were used. They were housed in plastic cages and fed on mice pellets from Unga Feeds Ltd. They received water ad libitum.
METHODS:

Ten pregnant mother rats were housed singly in different cages. They were fed on mice pellets and water ad libitum. Five days after giving birth each mother rat received 250 ug/kg body weight of aflatoxin B1. A second dose of similar concentration was given seven days later. The third dose of similar concentration was given another seven days later. The babies suckled their mothers freely from the time of their birth. At the age of 30 days, the offsprings were weaned from their mothers, sexed and kept in groups of five rats per cage. The offsprings of three mother rats which did not receive aflatoxin B1 were used as controls. The offsprings were observed for a period of 26 months. The surviving offsprings were sacrificed, dissected and their livers examined grossly for any changes. The liver biopsies were taken, fixed in 10% formalin and later processed and stained for histological examination.
RESULTS

A total of 79 offsprings were born of the mothers which were administered with 250ug/ml dose per animal of aflatoxin B once a week for 3 weeks. 1 26 offsprings were born of the control mothers. Of the offsprings from the test mothers 43 were males while 36 were females. Seven offsprings (2 males and 5 females) died within the first eighteen months all with normal livers. No offspring born of the control mothers developed liver cancer. Furthermore, none of the AFB1-treated mothers rats developed liver cancer.

The first rat with liver tumour was identified 22 months after exposure to aflatoxin B1. By that time 41 male offsprings and 31 female offsprings were surviving. From the 22nd month onwards, rats showing signs of weakness or sickness were sacrificed and their livers were examined grossly and histologically. The experiment was terminated after 26 months and all the surviving rats were sacrificed. Their livers were examined grossly and histologically.

Out of the 72 total offsprings that survived for over 22 months 18 of them developed liver tumours and 14 had liver dysplasia. This clearly demonstrated that aflatoxin dosed lactating mother rats excited
through their milk, sufficient amount of aflatoxin that could induce the development of liver tumours in the offsprings suckling the milk.

Of the 41 male offsprings 16 (39%) had normal liver, 14 (34%) had liver tumours while 11 (26.83%) had liver dysplasia. In contrast to the male offsprings 24 (77.42%) of the 31 female offsprings had normal livers, 4 (12.90%) had liver tumours while 3 (9.68%) had liver dysplasia. This showed that male offsprings were approximately 3 times more susceptible to developing liver tumours than their sister female offsprings. The results further demonstrated that whereas only 39.02% of the male offsprings resisted the development of liver tumour, 77.42% of the female offsprings resisted the development of liver tumour within that period. 26.83% of the male offsprings had liver dysplasia whereas only 9.68% of the female offspring showed liver dysplasia. Table V shows the results of the effect on offsprings suckling aflatoxin $B_1$ -treated mothers. Fig.12 shows the gross appearance of normal liver and liver tumour. Fig.13 shows the histological appearance of the normal liver and liver tumour from same specimens as of Fig.12.
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<td>LIVER DYSPLASIA</td>
<td>11</td>
<td>26.83%</td>
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a) Gross appearance of rat liver of offsprings from control mothers

b) Gross appearance of liver tumour of offsprings from AFB$_1$-treated mother
NORMAL: X40 section of normal liver tissue

TUMOUR: X40 Liver showing presence of a moderately differentiated hepatocellular carcinoma
When lactating cattle, sheep or goats are given feeds contaminated with aflatoxin B, they will secrete aflatoxin M in their milk (Environ. Health Criteria, 1979). In the cow there is a linear relationship between the amount of aflatoxin B ingested daily and the level of aflatoxin M in the milk, indicating that about 1.5% of aflatoxin B is secreted as the metabolic M (Environ. Health Criteria, 1979). Smaller quantities of unmetabolized aflatoxin B have been found in cow's and sheep's milk. In this investigation 34.15% of the male rat offsprings and 12.90% of the female rat offsprings which had suckled aflatoxin B dosed mothers developed liver tumours indicating that aflatoxin M has a high carcinogenic potency in rat liver. AFM has been reported to be about 2-10% as potent a carcinogen as AFB (Cullen et al, 1987). The male rat offsprings were nearly three times as sensitive as the female rat offsprings thus confirming the well established fact that male rats are more susceptible to aflatoxin carcinogenicity than the female rats. I cannot rule out the possibility that unmetabolized AFB might also be secreted in the milk. However, only a small quantity of unmetabolized AFB might have been secreted.
Infant animals are reportedly significantly more susceptible to the carcinogenicity of AFM than adults (Busby and Wogan, 1984). This implies that human infants or babies may be more susceptible to AFM than adults and since babies are the ones who drink more milk, this complicates the situation in humans. A study in Sudan provided evidence that children in that country are exposed to aflatoxins in their diet. The data suggested that children with kwashiorkor had a greater exposure to aflatoxins or that their ability to metabolize and excrete aflatoxins was impaired by the metabolic rearrangement associated with kwashiorkor (Hendrickse et al., 1982).

The fact that liver cancer has been observed in early age groups in this country, follows from the probability that some of our population becomes exposed to aflatoxin at the infant age through drinking aflatoxin contaminated milk. Adults may also become exposed to aflatoxin by drinking AFM contaminated milk and dairy products. This has been reported in the United States and Denmark where human exposure to AFM occurs by ingestion of milk and other dairy products (Cullen et al., 1987). The dairy cattle in turn ingest aflatoxin through eating aflatoxin contaminated feeds such as corn, forage grasses
and silage. In this country, there is a tendency of some farmers to give partly rotten dried maize, unfit for human consumption to their cattle including dairy ones. Some of this rotten maize may be highly contaminated with aflatoxin B which may find its way to humans through the cows milk and other dairy products as aflatoxin M. Such presence of AFM in milk and other dairy products destined for human consumption raises grave concern regarding the health risk associated with their consumption. This calls for an urgent measure to curb or control this kind of human exposure to aflatoxin risks.
CHAPTER 4.

TUMOUR PROMOTION STUDY

INTRODUCTION:

The induction of liver cancer in man is thought to be the final result of complex interactions between initiators and factors that favour tumour development (promoters). This is exemplified by the multistage process and the multifactorial action. Tumour promoters play an important role in the oncogenic process in experimental animals and several attempts to translate the findings on tumour promotion to the problems of human liver cancer are being made. The presence of a tumour promoter substantially increases the overall cancer risk and/or shortens the latency period of tumour development. In this project, therefore, the effects of a tumour promoter have been studied in rat liver carcinogenesis using phenobarbitone.
MATERIALS AND METHODS

MATERIALS:

Chloroform, ether, xylene, mayor's haematoxylin, eosin, phenobarbital, and dimethyl sulphoxide were purchased from sigma chemical co. Aflatoxin B₁ was purchased from Makor Chemicals Ltd., Jerusalem, Israel; Microtome, centrifuge, incubator, plastic rat cages, paraffin wax, slides, cover slips and stomach tubes (improvised from a 14g needle with a blunt end and 2ml syringe).

ANIMALS:

Male albino wistar rats bred in our animal house were used. They were housed in plastic cages and fed on mice pellets from Unga Feeds Ltd and received water ad libitum. Aflatoxin analysis carried out on the mice pellets revealed no contamination.

METHODS:

78 male albino wistar rats aged 6 to 8 weeks and weighing 120 to 130 grams were started on an experiment
designed to test for the effects of phenobarbitone as a liver tumour promoter with aflatoxin B₁ serving as an initiator. The rats housed in plastic cages were fed on mice pellets checked for AFB and received water ad libitum. Phenobarbitone was given dissolved in water while aflatoxin B₁ dissolved in dimethyl sulphoxide was given using a stomach tube.

The rats were divided into 13 groups of six rats in each group and treated as follows:–

**Group 1:**

The six rats in this group received no initiator and no promoter. They served as controls for spontaneous tumours.

**Group II:**

The six rats in this group received a single high dose (500 µg/kg body weight) of aflatoxin B₁. No promoter was administered.

**Group III:**

The six rats in this group received a single dose (50mg/kg body weight) of phenobarbitone each. No initiator was given.
Group IV: The six rats in this group received a single low dose (100 ug/kg body weight) of aflatoxin B. No promoter was given.

Group V: The six rats here received a single low dose (10mg/kg body weight) of phenobarbitone each. No initiator was given.

Group VI: Rats in this group, each received low doses (100 ug/kg body weight) of aflatoxin B each, three days a week for a total period of one month.

Group VII: Rats in this group, each received low doses of phenobarbitone (10mg/kg body weight) three days a week for a total period of one month.

Group VIII: Rats in this group each received a single low dose, 100 ug/kg body weight of aflatoxin B then followed by low doses of phenobarbitone (10mg/kg body weight) three times.
a week for a total period of one month.

**Group IX:** The six rats each received low doses 10mg/body weight) phenobarbitone three times a week for a total period of one month, then followed by a single low dose (100 μg/kg body weight) of aflatoxin B₁.

**Group X:** The six rats here each received a single low dose (100 μg/kg body weight) of aflatoxin B₁ followed by a few (only three times in one week) low doses of phenobarbitone.

**Group XI:** The six rats in this group each received a single low dose of aflatoxin B₁, then followed by low doses of phenobarbitone at wide intervals (one a week) for a period of one month.

**Group XII:** The six rats in this group each received a single dose (100μg/body weight) of aflatoxin B₁, rested for one month and then received low doses (10mg/body weight) of phenobarbitone three times a week for another one month.
GROUP XIII: The six rats here each received only dimethyl sulfoxide (solvent of aflatoxin B₁) to serve as another control.

All the rats were observed for a period of 24 months. They were then sacrificed using ether to suffocate them. Each was opened up and the livers were examined grossly before liver biopsies were taken, fixed in 10% formalin and processed for histological examination.

Processing Sections for Histological Examination

After trimming the fixed liver biopsies, they were put on automatic tissue processor. The specimens were passed through increasing concentration of isopropyl alcohol as follows:-

70%, 80%, 95% isopropyl alcohol, each step taking 1½ hours. They were then passed through absolute alcohol twice, each taking again 1½ hours and cleared by chloroform for 3½ hours then for 2½ hours. The specimens were passed through paraffin wax twice each step lasting 1½ hours and then they were embedded in paraffin wax to form blocks. The embedded sections
in blocks were sectioned at 5 microns using a microtome.

The sections on slides were incubated in the oven at 60°C overnight. The sections were stained with haematoxylin and eosin staining technique.

**Haematoxylin and Eosin Staining Technique**

i) The sections on slides were passed through two sets of xylene each taking 2 minutes.

ii) They were dipped in isopropyl alcohol or absolute alcohol several times.

iii) Then washed in running tap water for 5 minutes.

iv) The sections were stained by Meyer's haematoxylin for 10 minutes.

v) They were rinsed in tap water and differentiated in 1% acid alcohol.

vi) They were again rinsed in tap water.

vii) They were then blued in scots tap water for 30 seconds.

viii) They were then rinsed once more by tap water.
ix) They were counterstained in 1% aqueous eosin for 5 minutes and then rinsed in tap water.

x) The sections were dehydrated in three changes of isopropyl alcohol and cleared in three changes of xylene.

xi) They were finally mounted with cover slips in DPX. They were then microscopically examined.

RESULTS:

The six rats in the first group, which served as controls, received no initiator and no promoter. One rat died in the initial stages of the experiment leaving five rats. The rats were sacrificed after 24 months and on dissecting them they looked grossly normal. Histological examination did not show any structural changes of the normal morphology.

The second group of six rats received a high dose of aflatoxin \( B_1 \) (500 \( \mu \)g/kg body weight). Two rats died in the early stage (first week) of the experiment due probably to the toxic effects of \( AFB_1 \). The remaining four were sacrificed after 24 months. In three rats, the livers
showed necrosis while the liver in one rat looked normal. Histological examination of the sections taken from the rats with necrotic livers showed widespread areas of moderate to severe dysplasia in two while hepatocellular carcinoma was noted in one section from the other rat.

The rats in the third group each received a high dose (50 mg/kg body weight) of phenobarbitone as a promoter. Two rats died in the course of the experiment (after 5 months). The remaining four rats were sacrificed after 24 months. All the liver specimens were found to be grossly normal and the histological examination confirmed them to be normal.

The rats in the fourth group each received a low dose (100 µg/body weight) of aflatoxin B₁. One rat died early in the course of the experiment. The five remaining rats were sacrificed after 24 months. Macroscopic examination showed that the livers from four rats were normal and one looked necrotic. Histologically the tissues from the four rat livers were found to be normal. The tissues from the 5th rat showed mild dysplasia of parenchymal cells.
The rats in the 5th group each received a single low dose (10mg/kg body weight) of phenobarbitone. One rat died in the early stages of the experiment leaving five. When the five surviving rats were sacrificed after 24 months their livers were found to be macroscopically normal. Histological examination of these liver specimens confirmed them to be normal.

The rats in the 6th group received repeatedly low doses (100 μg/kg body weight) of aflatoxin B₁ three days in a week for a period of one month. No promoter was given to them. Two rats died in the course of the experiment. The four which survived were sacrificed at the end of 24 months. Macroscopic examination of the livers showed necrosis. Histologically two sections showed nodules of hepatocytes with moderate to severe dysplasia while two other sections showed well differentiated hepatocellular carcinoma.

Rats in the 7th group received a low dose (10mg/kg body weight) of phenobarbitone three times a week for one month. Two rats died in the course of
the experiment. Those which survived were sacrificed after 24 hours and their livers were macroscopically found to be normal. Histologically all the sections were found to be essentially normal.

The rats in the 8th group received a single low dose (100 µg/kg body weight) of aflatoxin B1 followed by low repeated doses (10 mg/kg body weight) of phenobarbitone three times a week for a period of one month. All the six rats survived and were sacrificed after 24 months. On opening them, all the six rats had clear tumours. Histological examination confirmed the tumours in all the rats to be hepatocellular carcinoma.

The rats in the 9th group each received low doses (10 mg/kg body weight) of phenobarbitone three times a week for a period of one month. This was followed by a single low dose (100 µg/kg body weight) of aflatoxin B1. The rats were sacrificed at the end of 24 months and their livers were macroscopically found to be normal. Histological examination showed all the sections to have mild dysplasia only.
Group 10 rats each received a single low dose (100 μg/kg body weight) of aflatoxin B₁ followed by a low dose (10mg/kg body weight) of phenobarbitone on Wednesday, Friday and Monday for only one week. One rat died in the course of the experiment. The surviving five were sacrificed at the end of 24 months and their livers were grossly normal. Histological examination showed the sections to have mild dysplasia.

Group 11 rats each received a single low dose (100 μg/kg body weight) of aflatoxin B₁ followed by repeated low doses (10mg/kg body weight) of phenobarbitone at widely spaced intervals (once a week) for a period of one month. All the six rats survived and were sacrificed after 24 months. Livers from five rats were grossly normal while the sixth rat showed necrotic lesions. Histological examination revealed moderate to severe hepatocyte dysplasia while a section from one rat showed nodules of hepatoma.
Group 12 rats each received a single low dose (100μg/kg body weight) of aflatoxin B₁. They rested for a period of one month and then were re-started on low doses (10mg/kg body weight) of phenobarbitone three times a week for another period of one month. Two of the rats died in the course of the experiment. Of the four rats that survived, two had necrotic liver lesions and two had normal liver, when they were sacrificed after 24 months. The histology of these livers showed moderate to severe dysplasia in two rats with two other rats showing hepatoma.

Group 13 rats received dimethylsulphoxide (the solvent of aflatoxin B₁), three times a week for one month. All the six rats survived and were sacrificed at the end of 24 months. Their livers were found to be normal both grossly and histologically.
SCHEMATIC DIAGRAM OF TUMOUR PROMOTION IN RAT LIVER CARCINOGENESIS

DURATION OF EXPERIMENT

RESULT

1. NO INITIATOR & NO PROMOTER → NO HCC
2. AFB₁, HIGH DOSE 500 µg/rat → HCC
3. PB HIGH DOSE 50 mg/rat → NO HCC
4. AFB₁ LOW DOSE 100 µg/rat → NO HCC
5. PB LOW DOSE 10 mg/rat → NO HCC
6. M W F M W F M W F M W F → HCC
7. M W F M W F M W F M W F → NO HCC
8. M W F M W F M W F M W F → HCC
9. M W F M W F M W F M W F → NO HCC
10. M W F M → NO HCC
11. M F F F F F → HCC
12. M W F M W F M W F M W F M W F → HCC
13. CONTROL → NO HCC

KE-1

X = AFB₁
Δ = PB
× = AFB₁, High dose
M = Monday
W = Wednesday
F = Friday
(a) Shows the gross appearance of rat liver from normal control group.

(b) Shows the gross appearance of liver tumour from AFB-treated followed by phenobarbitone
(a) Normal: X40 Section of normal liver tissue
(b) Tumour: X40 liver section showing the presence of a moderately differentiated hepatocellular carcinoma.
DISCUSSION:

When a single high dose (500 μg/kg body weight) of aflatoxin B₁ was given to the rats, tumours developed. However, when a single high dose (50mg/kg body weight) of phenobarbitone was given to the rats no tumours developed. This shows that a high dose of a carcinogen initiates as well as promotes tumour development. Phenobarbitone on the other hand is well known for its promoting potency but since it has no initiating ability, it produced no tumours. When a single low dose (subcarcinogenic dose) of a carcinogen (100 μg/kg body weight) of AFB was administered to the rats, no tumours of the liver were observed at the end of the experiment. The subcarcinogenic dose did cause initiation of tumour lesions but the lesions remained latent since the dose was too small to evoke their promotion. When
the subcarcinogenic dose was given repeatedly (three times a week) for a month, liver tumours developed. In this case, after the initial dose initiated liver tumour lesions, the subsequent doses produced a promoting effect on the initiated lesions resulting in tumours. This is in agreement with Hecker's (1984) finding in which he observed that the single subcarcinogenic dose of a solitary carcinogen may produce in the cells of target tissues latent irreversible lesions. If these initiated cells are followed by exposure to repeated identical doses of the same carcinogen, the lesions induced by the single doses may add up to the manifestations of cancer within the life span of the host. It is now widely accepted that solitary carcinogens are the most critical yet not the only class of causative agents in the aetiology of cancer. Initiation of normal cells of target tissues is postulated to be the key biological event in the process of carcinogenesis (Hecker, 1984). It is essentially irreversible and involves the covalent binding to DNA. In the case
of aflatoxin, the activated electrophilic species, AFB\textsubscript{-2}, 3-oxide interacts with the DNA of the target tissue to form AFB-DNA adducts. Hydrolysis of this adduct results in the release of AFB-Gua adduct leaving an apurinic site in the DNA strand – a possible mutagenic lesion. A second reaction occurring under mildly alkaline and also under physiological, conditions, causes the opening of the imidazole ring, producing the putative persistent ring-opened DNA-bound formamido-dopyrimidine derivative the $\text{AFB}_1\text{- FAP yr}$ (Chetsanga and Frenette, 1983, Croy and Wogan, 1981).

The events observed in this protocol closely mimic the kind of situation that is likely happening in the human environment where small doses of the same type of a prevalent carcinogen are being consumed repeatedly in contaminated foods and drinks. In the case of aflatoxin B the doses consumed are probably too small to produce any toxic effects so that those affected may never be aware of when they consumed it. The effects will only manifest as liver cancer many years later in the host's lifespan.

In the group of rats where a promoting agent, phenobarbitone was given in small repeated doses for
a month, no tumours were observed in their livers after 24 months. This is in agreement with the hypothesis that promoters are not expected to produce tumours on their own even after chronic exposure of the target cells (Hecker, 1984). It was only when a single sub-carcinogenic dose of a solitary carcinogen preceded the non-carcinogenic protocol of the tumour promoter that tumours developed. When a small single dose of a carcinogen was given and then followed by small repeated doses of a promoter for a month, tumours were observed in the livers of all the rats. This protocol proved to be the most efficient way of producing liver cancer in rats experimentally. Looking at these results from the human situation, this seems to be the commonest way by which humans develop liver cancer. It is conceivable that some people become exposed to a single small dose of a solitary carcinogen and then repeatedly keep consuming small doses of promoting agents in their foods as food additives or drugs. In some areas, the vegetables that are eaten at meal times may themselves contain potent promoters.
Numerous carcinogens have been detected as exogenous carcinogenic risk factors in animals and of these several have also been identified as carcinogenic risk factors in the generation of human cancers by the international Agency for Research on cancer (IARC Tomatis et al., 1978). Exposure of the host, even to a single, subcarcinogenic dose of a solitary carcinogen may produce in the cells of target tissues latent, irreversible lesions. If later in the lifespan of the animal or human, the initiated lesions are exposed to repeated doses of a promoter then cancer will manifest. Promoters therefore, amplify carcinogenesis, although they are non-carcinogenic entities per se. This multi-factorial protocol verifies the model of co-carcinogenesis in the form of a combination of a solitary and a co-carcinogen/promoter. Initiation/promotion is the most sensitive protocol in the tests for liver tumour promotion (Autrup and Las Dragsted, 1987) and represents one of the commonest ways by which humans develop cancer.

The recognition that many human cancers are caused by many factors and occur in a multi-stage process complicates the task of extrapolation of results from animal models to humans. The above made observations coupled with
the results of this experiment, imply that those concerned with carcinogenic risk assessment in the human environment must address themselves to the presence of promoting or co-carcinogenic agents in that environment. This factor had hitherto been either underplayed or completely ignored because promoting/co-carcinogenic factors are difficult to assess.

When a promoting agent was given repeatedly in small doses for a month and then followed by a single dose of a carcinogen no tumours developed. This means that a promoting agent has to follow a carcinogenic effect (initiation) for tumours to develop but not vice-versa. When a single small dose of a carcinogen was given followed by few (only 3 times) repeated small doses of a promoting agent, no tumours developed. This means that withdrawal of a promoting agent after only a few applications, will have no promoting effect on the initiated cells. Hence these cells will not progress to tumour expression, but will remain latent for a long time. Yamasaki and Weinstein (1983) reported that when the application of a promoter stopped, some of the papillomas that had already appeared on mouse skin regressed.
In one interesting protocol, rats were given a single small dose of a carcinogen and then were left to rest for one month. After the rest period the rats received repeated small doses of a promoting agent for another month. When the experiment was terminated after 24 months, tumours were observed in the livers of these rats. This means that initiated cells retain the memory of the action of an initiator that can be recalled to progress to tumours even after a long rest period. In this case, the subsequent exposure to tumour promoters is what will determine whether or not tumours will develop.

The results of this experiment support the findings of other researchers who have observed that the cause and development of most malignant tumours was multifactorial and multistage (Weinstein, 1982). The process involves the interaction of target cells with multiple factors both exogenous and endogenous. This complex aspect of carcinogenesis must therefore be taken into account when analysing the carcinogenic risk factors of environmental agents to humans.

It is now evident that tumour promoters and certain other modifying agents act on the cells through
mechanisms different from those of carcinogens. Whereas a carcinogenic initiator covalently binds to DNA and is mutagenic, this is not the case with tumour promoters. The action of tumour promoters is mainly on the cell surface. Tumour promoters have been reported to alter the membrane structure and function of the cell surface. They have been reported to inhibit cell-cell communication as well as inhibiting cell differentiation (Yamasaki et al., 1983).

It is probably appropriate to end this section by quoting Hecker's (1984) words: "It appears important to point out to the public that in both principal classes of defined carcinogenic risk factors—solitary and cocarcinogens—the most active entities known, the aflatoxins produced by moulds, as well as the diterpenes ester-type promoters produced by Euphorbiaceae and Thymelaeaceae species; are of plant origin". Therefore the presence in the human environment of natural carcinogenic risk factors certainly cannot be neglected.
CHAPTER 5:

EFFECT OF SCHISTOSOMIASIS INFECTION ON AFLATOXIN B₁ - TREATED RATS:

INTRODUCTION.

Biological factors, in particular parasites have been identified as significant carcinogenic risk factors. Schistosomiasis has long been associated with cancer. The association has been most prevalent between \textit{Schistosoma haematobium} and bladder cancer (Nomura, 1979). Considering the substantial amount of supportive evidence for the co-carcinogenic effects of schistosomes in bladder cancer, this study attempted to establish whether there is also any co-carcinogenic effect of \textit{Schistosoma haematobium} and aflatoxin B in liver cancer.

MATERIALS AND METHODS.

MATERIALS:

Dimethyl sulfoxide, xylene, Meyer's haematoxylin, Chloroform, ether, eosin were purchased from
Sigma Chemical Co. Aflatoxin B was purchased from Makor 1 Chemicals Ltd. Jerusalem, Israel. Microtome, incubator, plastic rat cages, paraffin wax, slides, cover slips and stomach tube.

**ANIMALS:**

Female albino wistar rats bred in our animal house were used. They were housed in plastic cages and fed on mice pellets from Unga Feeds Ltd., and received water ad libitum.

**METHODS:**

**Preparation of Cercaria**

i) 3 - 5 life infected snails of biomphalaria or bulinus species were collected and put in each tube of size 3" x 1" half-filled with rain water.

ii) The snails were exposed to strong sunlight or electric bulb light for 30 to 60 minutes. The snails started shading cercariae.

iii) A drop of the water carrying cercariae was put on a microscope slide using a pasteur pipette and the number of
iv) The number of drops contained in 1 ml of the water were counted.

vi) 250,000 to 400,000 cercariae were then injected intradermally into each rat.

Treatment of the Rats:

45 female rats aged 7 - 8 weeks and weighing 140-150 grams were divided into three groups, each group consisting 15 rats. The rats were housed in groups of five rats per cage.

Group 1: Rats received between 250,000 to 400,000 cercariae of Schistosoma haematobium by intradermal injection.

Group II: Rats received both cercariae (250,000-400,000) of Schistosoma haematobium and 500 µg/1 ml /kg body weight of aflatoxin B₁.
Group III: Rats received only 500 μg/1 ml/kg body weight of aflatoxin B<sub>1</sub>. The rats were observed for a period of 26 months. They were then sacrificed using ether. Their livers were grossly examined and pieces taken from them fixed in 10% formalin and sent for histological examination.

RESULTS:

Of the rats that received *Schistosoma haematobium* alone, 4 rats died in the early stages of experiment. Out of the 11 rats that survived for 26 months, livers from 3 rats showed some lesions and the rest had normal livers macroscopically. Histological examination showed that livers from two rats had dysplasia while livers from 9 rats were normal. No tumours were seen in any rat from this group.
In the group of rats that received aflatoxin B<sub>1</sub> alone, 4 rats died in the early stage of the experiment. Of the rats that survived for the 26 months, 5 rats macroscopically showed lesions, 2 had liver tumours while 4 had normal livers. Histological examination confirmed 2 livers from 2 rats to have tumours, three showed dysplasia while 6 had normal livers.

In the group of rats that received combined aflatoxin B<sub>1</sub> and Schistosoma haematobium, 7 rats died in the early part of the experiment. Out of those which survived the entire experimental period, livers from 2 rats showed lesions macroscopically, two had tumours while the rest were normal. Histological examination confirmed liver tumours in two rats, two other rats had liver dysplasia while four had normal livers. Table VI shows the effect of Schistosomiasis on AFB<sub>1</sub> hepatocarcinogenicity. A check on whether the cercariae injected in the rats survived and developed into the adult schistosomes
TABLE VI

EFFECT OF SCHISTOSOMIASIS ON AFB, HEPATOCARCINOGENICITY

<table>
<thead>
<tr>
<th></th>
<th>INITIAL NO. OF RATS</th>
<th>DEATHS</th>
<th>TUMOURS</th>
<th>DYSPLASIA</th>
<th>NORMAL LIVER</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCHISTOSOME ALONE</td>
<td>15</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>AFLATOXIN ALONE</td>
<td>15</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>SCHISTOSOME + AFLATOXIN</td>
<td>15</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
revealed no presence of the Schistosomes. It appears that most of, if not all the cercariae died in the early stages of the experiment.

**DISCUSSION**

Multiple mechanisms interrelate in the induction and development of liver cancer in humans. A large number of biological factors have been implicated in predisposing individuals to later development of liver cancer. These include viral infections as well as parasitic infestations (Gentile, 1985). Although bilharziasis has been implicated in bladder cancer, its exact role has not been fully elucidated. Schistosoma Mansoni has been associated with hepatocellular and bile duct carcinoma while Schistosoma japonicum has been linked with hepatoma (Nakashima et al., 1975). However, most of the available evidence involves Schistosoma haematobium which has been associated mainly with bladder cancer and other cancers to a lesser extent.
The results of this experiment did not show any co-carcinogenic effect between aflatoxin and schistosomiasis on rat liver. The reason for this failure to demonstrate co-carcinogenesis between these two agents was because the cercariae failed to survive and develop into adult Schistosomes in the rats. At the time of sacrificing the rats, the bladders and livers were examined for evidence of live schistosomes but none were seen. It is likely that no schistosomiasis effect was therefore exerted on aflatoxin carcinogenesis in these rats. Furthermore very few rats developed liver tumours. This can be explained by the fact that female rats which are normally resistant to aflatoxin carcinogenic effect were used. Only two rats from AFB alone group and three from the combined AFB - schistosome group developed liver tumours. Since there was no difference in carcinogenic effect in the two
groups, it can be concluded that the tumours which developed from both groups were due to aflatoxin B with no additive effect from schistosomiasis. If there was any additive effect then more rats with liver tumours would have been seen in the combined AFB - schistosome group than in the AFB - alone or schistosome alone group. Moreover tumours would have appeared in the rats of the combined AFB - schistosome group earlier than in the other two groups.

Schistosomiasis is said to lead to reduced immune surveillance and alteration of the host's metabolism of carcinogens (Camus et al., 1981). Schistosome infested individuals have elevated levels of enzymes that cleave the parent carcinogenic compounds to their ultimate reactive species (Gentile, 1985). It has further been suggested that schistosomiasis provides the proliferative
stimulus necessary to accelerate cancer growth from latent tumour foci produced by carcinogenic exposure (Hicks, 1980).

On the whole, the results of this experiment are inconclusive about the co-carcinogenic effect of schistosomiasis on aflatoxin hepatocarcinogenesis in rats. However, since schistosomiasis is endemic in some areas of this country where aflatoxin exposure to humans is prevalent, more work should be carried out to establish whether there is any significant interrelationship between aflatoxin, schistosomiasis and liver cancer.
CHAPTER 6.

INHIBITION OF AFLATOXIN CARCINOGENESIS.

INTRODUCTION:

A variety of chemicals have been shown to protect animals against neoplastic, mutagenic and toxic effects of carcinogens. A striking feature of the chemoprotectors is that they belong to totally unrelated chemical classes such as retinoids, tocopherols, flavones, indoles and selenium compounds. Some of these protective substances alter the metabolic fate of carcinogens by metabolizing enzymes (Talalay et al., 1985). Induction of enzymes that detoxify electrophilic metabolites of carcinogens may interrupt the neoplastic process.

The ultimate carcinogenic form of aflatoxin is 2,3-oxide, which binds predominantly to the 7N atom of guanine in DNA, as well as to RNA and protein. Apart from binding to macromolecules, AFB-2,3-Oxide can undergo hydrolysis to aflatoxin 2,3-dihydrodiol and conjugation with glutathione to form AFB-SG (Kensler et al., 1986). This glutathione
conjugate is a major biliary metabolite that is formed through the catalytic action of a family of isozymes, the glutathione s-transferases, and is considered to be a detoxication product (Kensler et al., 1986). Antioxidants substantially inhibit the formation of aflatoxin-DNA adducts in target tissues in the rat. A major determinant for these protective actions has been reported to be the elevation of glutathione s-transferase activities, facilitating the detoxication and elimination of the 23-oxide (Kensler et al., 1987).

In this study the chemoprotective effects against aflatoxin tumourigenicity in rats fed β-carotene, ascorbic acid, reduced glutathione, vitamin E (tocopherol), selenium and uric acid were investigated.

**MATERIALS AND METHODS**

**MATERIALS:**

Chloroform, ether, xylene, meyer’s haematoxylin, eosin, selenium and uric acid were purchased from British Drug House (BDH). β-carotene, vitamin E (tocopherol) reduced glutathione and dimethyl sulfoxide were purchased from Sigma Chemical Co. Aflatoxin B₁ was purchased from Makor Chemicals
ltd., Jerusalem, israel. Microtome, incubator, plastic rat cages, paraffin wax, slides, cover slips and stomach tubes.

**ANIMALS:**

Male albino wistar rats bred in our animal house were used. They were housed in plastic cages and fed on mice pellets from Unga Feeds Ltd.; and received water *ad libitum*. Aflatoxin analysis carried out on the mice pellets revealed no contamination.

**METHODS:**

42 male albino wistar rats aged 7 to 8 weeks and weighing 120 to 130 grams were started on an experiment designed to test for the inhibitory effects of the following compounds in aflatoxin $B_1$ carcinogenicity in rat liver. The rats were divided in seven groups and housed in plastic cages with each cage housing six rats.

**Group 1** The rats in this group were started on crushed mice pellets and received water *ad libitum* for seven days.
Group II: Rats in this group were started on crushed mice pellets mixed with 1mg/kg body weight of β-carotene for seven days. They received water ad libitum.

Group III: Rats in this group were started on crushed mice pellets mixed with 10mg/kg body weight of ascorbic acid for seven days. Water was given ad libitum.

Group IV: Rats were started on crushed mice pellets mixed with 1mg/kg body weight of reduced glutathione for seven days. Water was given ad libitum.

Group V: Rats were started on crushed mice pellets mixed with 1mg/kg body weight of vitamin E (tocopherol) for seven days. Water was given ad libitum.

Group VI: Rats received crushed mice pellets mixed with 1mg/kg body weight of selenium for seven days. They received water ad libitum.
GROUP VII  Rats received crushed mice pellets mixed with 1 mg/kg body weight of uric acid for seven days. Water was given at libitum.

On the eighth day each rat was given 500 μg/kg body weight of aflatoxin B₁ using stomach tube. All the rats were left to continue on their respective food regimens for a further three weeks and thereafter resumed feeding on mice pellets only and received water ad libitum. They were opened up and their livers were grossly examined for any changes. After that, liver biopsies were taken from them and fixed in 10% formalin. They were then sent for processing, staining and histological examination.

RESULTS:

The first group of rats received no anti-carcinogen in their food but on the 8th day each received one dose of 500 μg/kg body weight of aflatoxin B₁
This group of rats was used as a positive control group. One rat died a few days after receiving aflatoxin. Two rats were noticed to be weak and were sacrificed after 18 months. Macroscopically, both rats had massive liver tumours and pieces of the tumours were taken, fixed in formalin and examined histologically. The remaining three rats were sacrificed after 22 months and two were found to have liver tumours while one had normal liver macroscopically. All together four rats were confirmed to have hepatocellular carcinoma while one had severe dysplasia. Aflatoxin \( B_1 \) therefore, produced hepatocellular carcinoma in 80% of the rats in this group and 20% showing dysplasia. No normal liver was observed in any rat in this group.

The six rats of group two were fed on mice pellets fortified with \( \beta \)-carotene for seven days prior to treatment with one dose of aflatoxin \( B_1 \) 500 \( \mu \)g/kg body weight. The animals continued to feed on the fortified food for a further period of three weeks. All the six rats in this group survived the entire period of the
experiment. When they were sacrificed after 22 months, 4 rats had liver lesions macroscopically while the other two had normal livers. Histology showed moderate dysplasia in four rats while the other two had normal livers. No rats from this group developed liver tumour. β-carotene therefore produced 100% inhibition of aflatoxin B₁ tumourigenicity in the livers of these rats in the 22 months period of experiment.

The six rats of group three were fed on mice pellets fortified with ascorbic acid for seven days. They were each challenged by 500 μg/kg body weight of aflatoxin B₁ and left to continue on the fortified food for three more weeks. One rat died during the first week of aflatoxin administration. One more rat died at month 20 and when it was dissected its liver was macroscopically found to be normal. A piece of liver was taken for histological examination. A third rat was sacrificed in the 21st month after it had been noticed to be weak and sick. Its liver had massive liver tumour from which pieces were taken and sent for histological
examination. The surviving three rats were sacrificed after 22 months. One of them had small liver lesions, while the other two had normal livers. Histological examination showed that one rat had developed liver tumour, one had dysplasia while three had normal livers. Ascorbic acid therefore, inhibited tumour induction in 80% of the rats but failed to do so in 20% during this period of 22 months.

The rats in group four were fed on mice pellets fortified with reduced glutathione for seven days. They were each challenged by 500 µg/kg body weight of aflatoxin B₁ and left to continue on the fortified food for three more weeks. Two rats died in the early stages of the experiment after receiving aflatoxin B₁. Their livers were found to be macroscopically normal. Pieces from the livers were taken for histological examination and found to be normal. The surviving four rats were sacrificed after 22 months and three other rats had normal livers macroscopically. Pieces of
the livers which were sent for histological examination, confirmed the liver from one rat to have hepatocellular carcinoma while the livers from the other three rats were found to be normal. Dysplasia was not observed in any of the livers. Thus in the four surviving rats, reduced glutathione suppressed liver tumour induction in 75% of the rats while 25% developed liver tumour.

The six rats in group V were fed on mice pellets fortified with vitamin E (tocopherol) for seven days. They were then each challenged by 500 µg/kg body weight of aflatoxin B₁. Two rats died a few days after receiving aflatoxin and their livers were found to be normal both macroscopically and histologically. Another rat died after 18 months and when it was opened up, it had liver tumour. A piece of the liver tumour was sent for histology and was confirmed to be hepatocellular carcinoma. When the three surviving rats of this group were sacrificed after 22 months, one rat had liver tumour while the other two had liver lesions. Pieces of the livers were
sent for histological examination. All together, two rats were confirmed to have hepatocellular carcinoma while the other two showed only moderate dysplasia. No single rat in this group had a completely normal liver after the 22 months. Vitamin E failed to suppress liver tumour induction in 50% of the rats. In the other 50% only dysplasia was observed.

The six rats in group six were fed on mice pellets fortified with selenium for seven days. They were each challenged by 500 \( \mu \text{g/kg} \) body weight of aflatoxin B\(_1\). One rat died during the first week of aflatoxin administration but its liver was found to be normal both macroscopically and histologically. Another rat died in the 21st Month of the experiment and was found to have liver tumour. Pieces of the liver tumour were taken for histological examination. The surviving four rats were sacrificed after 22 months and two rats had liver lesions while two had normal livers macroscopically. Pieces of their livers
were taken for histological examination which showed that one rat had hepatocellular carcinoma, two had dysplasia while the other two had normal livers. Selenium therefore, suppressed liver tumour induction in 80% of the rats but failed to do so in 20% of the rats. 40% of the rats had normal livers while another 40% had dysplasia.

The rats in the 7th group were fed on mice pellets fortified with uric acid for seven days then they were each challenged by 500 μg/kg body weight of aflatoxin B₁. Four rats died during the first two weeks after receiving aflatoxin B₁. When two surviving rats were sacrificed after 22 months, their livers appeared grossly normal. Pieces were taken from the livers and sent for histological examination and the livers were confirmed to be normal. Although a very high proportion of the rats died probably due to aflatoxin B₁ toxicity, the two rats that survived had 100% protection against aflatoxin tumourogenicity by uric acid. Table VII shows the inhibitory effects of B-carotene, ascorbic
<table>
<thead>
<tr>
<th>PROTOCOL</th>
<th>INITIAL NUMBER</th>
<th>DEATHS</th>
<th>SURVIVORS</th>
<th>TUMOURS</th>
<th>NON TUMOURS</th>
<th>% TUMOUR SUPPRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB₁ ALONE</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>20%</td>
</tr>
<tr>
<td>AFB₁ + 1mg B Carotene</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>AFB₁ + 10mg Ascorbic Acid</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>80%</td>
</tr>
<tr>
<td>AFB₁ + 1mg GSH</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>75%</td>
</tr>
<tr>
<td>AFB₁ + 1mg Vitamin E</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>AFB₁ + 1mg Selenium</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>80%</td>
</tr>
<tr>
<td>AFB₁ + 1mg Uric acid</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>100%</td>
</tr>
</tbody>
</table>
DISCUSSION:

In recent years there have been a number of reports concerning the ability of antioxidants to inhibit chemical carcinogenesis when the antioxidants were administered either prior or concommitantly with the carcinogen (Novi, 1981, Ames, 1983). When aflatoxin B₁ was given to rats in the absence of any inhibitor or anti-carcinogen, a high percentage (80%) of the rats developed cancer and in a shorter period compared to those which received anti-Carcinogens. Liver tumours developed in the group of rats that did not receive anti-carcinogen as early as by the 18th month whereas they appeared later in the groups that received anticarcinogens.

In the group of rats that received high amount of B-carotene, all the rats survived for the entire experimental period and no single rat developed liver tumour. There was therefore a complete inhibition of aflatoxin hepatocarcinogenesis and toxicity by β-carotene. β-carotene exerts its anticarcinogenic activity by quenching
the excitation energy of singlet oxygen and also by trapping certain organic free radicals in the cell membrane and body fat. Singlet oxygen is a very reactive form of oxygen which is mutagenic and particularly effective at causing liquid peroxidation (Ames 1983). It has been suggested that anticancer effects may be produced by deactivation of these chemical species or by preventing the lipid peroxidation or other oxidative damage which they might cause (Peto et al., 1981). It is possible that β-carotene may alter the metabolic fate of carcinogens, such as aflatoxins, by modulating the activities of the drug metabolizing enzymes and thus prevent initiation. They may also modify the promotion phase or preneoplastic state, since they are known to exert profound control on cellular differentiation and growth in the epithelial tissues (Peto et al., 1981). Their ability to control cell differentiation in epithelial cells is of major importance in their suppression of carcinogenesis. The inverse association between dietary β-carotene and incidence of
certain types of cancer that has been observed in some populations may be due to a genuine protective effect of \( \beta \)-carotene against the onset of cancer. Age, social and class-standardized mortality rates for cancers at all sites and cancers of the stomach and lung by frequency of green-yellow vegetable intake revealed a significant association and suggested a possible beneficial effect of \( \beta \)-carotene intake in preventing major cancers in man (Hirayama, 1982). \( \beta \)-carotene is present in carrots and in all foods that contain chlorophyll. Certain diets were found to be important cancer risk modifiers. In particular daily intake of green-yellow vegetables was found to reduce the risk of cancer at all sites and at specific sites (Hirayama, 1982).

The results of this experiment coupled with the findings that people who increase the frequency of consumption of green-yellow vegetables have a reduced risk of stomach cancer, both strengthen the hypothesis of a beneficial effect of \( \beta \)-carotene and points to the advisability of increasing the frequency of intake of carrots and other
B-carotene containing vegetables with the aim of reducing the risk of liver cancer and other cancers in our population.

The group of rats that received high amounts of ascorbic acid revealed that ascorbic acid inhibited induction/or development of liver tumour in 80% of the rats, which is a very high rate of inhibition. This then means that ascorbic acid offers a high degree of protection against liver carcinogenic agents, particularly aflatoxins. Ascorbic acid is an important antioxidant and carries out its inhibitory activity on tumour induction by scavenging the reactive species of carcinogens, thus protecting cellular constituents from attack. In addition ascorbic acid exerts a scavenging effect on radicals generated by tumour promoters (slaga, 1984). Ascorbic acid increases the levels of enzymes that are important in detoxifying radicals, for instance, by reducing carcinogen induced peroxidation (Cameron et al., 1979). Ascorbic acid protects against carcinogenesis by altering
liver microsomal metabolism so that the reactive species are not generated. Like other antioxidants, ascorbic acid has been shown to protect against carcinogen-induced chromosomal breakage (Cameron et al., 1979). Ascorbic acid has been shown to be anticarcinogenic in rodents treated with ultraviolet radiation, benz(a)anthracene plus croton oil. Benedict et al. (1980) reported that ascorbic acid inhibited the transformation of C3H/10T1/2 cells by methylc~holanthrene. The inhibitory effect of ascorbic acid in that study was observed in some cases, long after the carcinogen was given indicating that ascorbic acid was acting during the promotional stage or progression. Ascorbic acid has also been shown to be inversely associated with human uterine cervical dysplasia (Ames, 1983). Kallistratos and Fosseke (1980) also reported that high doses of ascorbic acid administered in the drinking water significantly decreased the induction of sarcomas by benz(a)pyrene. Ascorbic acid is essential for the integrity of the intercellular matrix and its resistance to malignant infiltrative growth, and there
is strong evidence that it is involved in the inhibition of invasive tumour enzymes. There is good evidence that high intakes of ascorbic acid potentiate the immune system against cancer. Collective evidence suggests that increasing ascorbic acid intake could produce significant benefits in both the prevention and the treatment of cancer. Since ascorbic acid is readily available in certain dietary foods such as fruits and it is almost completely safe and harmless to humans even when given in sustained high doses for prolonged periods of time, the public should be urged to consume more of these fruits more frequently in order to make use of the protection that these fruits offer, especially to the high risk individuals.

In the group of rats that received high amounts of reduced glutathione, a 75% protection effect against aflatoxin hepatocarcinogenesis was observed. This is a quite strong inhibitory effect and fully agrees with the observations of Novi (1981), who reported that reduced glutathione produced
effective protection against aflatoxin tumourigenicity. Apart from binding to macromolecules, AFB₁ 2,3-oxide can undergo hydrolysis to aflatoxin 2,3-dihydrodiol and conjugation with glutathione to form AFB-SG. This glutathione conjugate is a major biliary metabolite that is formed through the catalytic action of a family of isozymes the glutathione S-transferases, and is considered to be a detoxication product (Kensler et al., 1986). The induction of this family of glutathione conjugating isozymes is felt to be an effective mechanism for ameliorating aflatoxin toxicity and carcinogenicity. Elevation of glutathione S-transferase activities enhances the detoxication and elimination of the ultimate electrophilic form of aflatoxin, the 2,3-oxide and thus inhibits the formation of aflatoxin DNA adducts in the target tissues. The glutathione transferases, some of which have peroxidase activity, are major defenses against oxidative and alkylating carcinogens. The enzyme glutathione peroxidase (GSHP) functions to detoxify hydrogen peroxide and hydroperoxides within the cells (Ames, 1983). Glutathione peroxidase probably
lowers the level of potentially damaging peroxide radicals that might be generated from various carcinogenic and promoting chemicals. Glutathione is present in foods such as cruciferous vegetables, for example cabbage, cauliflower and brussels sprouts. Epidemiological evidence suggests that consumption of these vegetables is associated with a reduction in the incidence of cancer in human (Kensler et al., 1987).

In the group of rats that received high doses of vitamin E (α-tocopheral) as an anti-carcinogen, 50% of the rats that survived the entire experimental period developed liver cancer while the other 50% had dysplasia. There was no single rat with normal liver thus demonstrating that vitamin E is not a very effective anti-carcinogen for AFB-induced liver carcinogenesis. It however, managed to delay the development of liver tumour in some rats. Ames (1983) reported that vitamin E was the major radical trap in lipid membranes and had been used clinically in a variety of oxidation-related diseases. Vitamin E ameliorates
both cardiac damage and carcinogenicity of the quinones, adriamycin and daunomycin which are mutagenic, carcinogenic and appear to be toxic because of free radical generation (Wang et al, 1982). Protective effects of tocopherols against radiation-induced DNA damage and mutation and dimethylhydrazine-induced carcinogenesis have also been observed (Ames, 1983).

In the group of rats that received selenium fortified food, there was an 80% protection of rats against aflatoxin hepatocarcinogenesis. High dietary selenium has been reported to inhibit or delay carcinogenesis (Le Boeuf et al, 1985). Selenium has also been reported to significantly inhibit the induction of skin, liver, colon and mammary tumours in experimental animals by a number of different carcinogens including viruses (Ames, 1983). Selenium is a necessary cofactor for the enzyme glutathione peroxidase (GSHP), an enzyme which functions to detoxify hydrogen peroxide and lipid hydroperoxides within the cells. Selenium dependent glutathione peroxidase lowers the level of potentially damaging peroxide
radicals that might be generated from various carcinogenic and promoting chemicals (Sla9a and Digiovanini 1984). The overall result is a decrease in the amount of active carcinogen that reaches critical cellular targets such as DNA.

In the group of rats that received uric acid the most outstanding feature was that a very high proportion of these rats died early in the experiment presumably due to the toxicity of aflatoxin. However, the few rats that survived were well protected by uric acid against aflatoxin hepatocarcinogenicity. None of the surviving rats developed liver cancer. Uric acid is a strong antioxidant present in high concentrations in blood of humans (Ames, 1983). It's concentration in the blood can be increased by dietary purines however, too much of it causes gout. Uric acid appears to inhibit carcinogenesis by inducing cytochrome P-450 and other metabolic enzymes (Ames, 1983).

Taken together, the results of this experiment strongly suggest that β-carotene, ascorbic acid, reduced glutathione, vitamin E, selenium and uric acid when taken frequently in sufficient
amounts, can produce strong inhibitory effects against chemical carcinogens especially aflatoxins. Since these compounds are often found in the foods commonly eaten by human, and are not harmful, the public should be advised to take these foods more frequently and in increased amounts in order to reduce the risk of cancers particularly liver cancer in our population. A carefully planned combination of some of these inhibitors might produce a more effective way of preventing the induction or development of cancer in our population.
CHAPTER 7:

GENERAL DISCUSSION:

The cause of hepatocellular carcinoma still remains unresolved although major effort has been made in research to identify it. The prospects of identifying the cause of this killer disease are very high so far. Although a multifactorial aetiology has been implicated, two causative factors that stand out predominantly are chronic hepatitis B virus infection and aflatoxin exposure.

Hepatitis B Virus Infection:

Overwhelming evidence has accumulated over the last decade, consistently indicating that chronic infection with hepatitis B virus as indicated by the presence in sera of the hepatitis B surface antigen (HBsAg) is causally related to the development of PHC (Peers et al., 1987). It has been estimated that up to 80% of PHC cases are attributable to HBV infection in the World (WHO report, 1983).
Strong arguments in favour of causal relationship between HBV and PHC were obtained along four lines namely:

(a) Prospective studies by Beasley and Hwang (1984) in Taiwan and by Lu et al. (1983) in China, demonstrated that HBV carriers had several-fold excess risk of developing PHC relative to non-carriers. There is need to carry out such a vertical study in this country that will include studying hepatitis B virus carrier rate, aflatoxin exposure and the prevalence of PHC to clarify the relationship between these parameters.

(b) A geographical correlation has been established between the prevalence of HBV carriers and the frequency of PHC in several countries. However, the results of this study did not demonstrate any association between hepatitis B virus infection and PHC. Similar situations
of anomalous relationship between HB Ag carrier rate and the prevalence of PHC have been reported by Bagshawe et al., (1975), Melbye et al (1984) and Van Rensburg et al (1985). Furthermore a study by Kew et al., (1983) concluded that HBV status could not account for the differences in the frequency and age at onset of PHC in rural and urban populations. The lack of association between the incidence of HBV carrier status and the prevalence of PHC may indicate that HBV infection acting alone has little carcinogenic effect but it needs to be potentiated by other factors.

Hepatitis B virus infection is common in this country as was observed from the results of this study. Although the average frequency rate in the 12 districts analysed was 9.5% for HB Ag and 56% for anti-HBC antibody, the frequency rate varied widely from one ethnic group to another. Turkana, for instance, had the highest frequencies (30.7% for HB Ag and 96% for anti-HBc AB) while Kericho District had low
frequencies (4.7% for HBs Ag and 40.6% for anti HB Ab).

The main route of primary exposure to HBV that commonly leads to persistent or chronic HBV infection is by vertical perinatal transmission from mother to child. The transmission of HBV from infected mothers to their children early in the life of the children results in persistent HBV infection that carries a high risk for the development of PHC (Nishioka, 1985). Although vertical transmission from mother to child has been found to be of major importance in South East Asia, a low rate of HBV infection in children was evident in this study. In this country the main route of transmission is the parenteral route involving contact with infected blood and other body fluids.

c) The association between HBV and PHC is supported also by the demonstration of integration of HBV-DNA into the genome of hepatoma cells in culture, in specimens of hepatocellular carcinoma and also
in the non-tumourous hepatic tissue of the hepatoma patients and long-term HBsAg carriers. However, no study has demonstrated that HBV as such has neoplastic transforming activity in culture cells.

d) Additional evidence linking HBV and PHC has come from the discovery that features of the HBV genome and virus morphology are shared by hepatitis-like viruses found in animals such as the woodchuck, Beechey ground squirrel and Pekin ducks. Like humans with HBV, a large proportion of these species are carriers of their unique viruses and PHC occurs in many of their chronic carriers (Beasley and Hwang, 1984). However, in China a study was carried out involving the feeding of aflatoxin contaminated food to domestic ducks known to be frequently infected with duck hepatitis virus and at high risk of developing
PHC. The ducks receiving the special aflatoxin-free food did not develop PHC unlike their domestically fed counterparts on aflatoxin contaminated food (I.A.R.C. Internal technical report No.84/003 1984). Furthermore, in Guangxi region in China, the mortality rate of PHC in HBVAg positive individuals was higher when the individuals lived in villages with high aflatoxin exposure (Henderson, 1985). The lack of parallelism between HBV carrier rate and PHC prevalence in the results of this study indicates that other factors including aflatoxin exposure may be important in the causation of liver cancer in this country.

**Aflatoxin Exposure:**

The results of this study did not show any positive correlation between aflatoxin exposure and liver cancer in the 12 districts covered. However, earlier studies by Peers and Linsell (1973) demonstrated a positive correlation between the two parameters. The geographical distribution of liver cancer in the people's Republic of China appeared to correlate more closely to the possible exposure to aflatoxins rather than
to HBV infection (Sun and Chu, 1984). Van Reinsburg et al. (1985) reported that a major determinant of variation in the frequency of PHC both within Mozambique and between Mozambique and other areas of the World was aflatoxin intake. Furthermore, the multivariate statistical analysis seemed to implicate aflatoxin rather than HBV in the regional variation of PHC in Swaziland (Peers et al., 1987). Aflatoxin is now known to be a well-established liver carcinogen in many animal experiments including non-human primates and in human cell system (Garner et al., 1985). However, only a few animal species are susceptible to an HBV-like infection and the consequent chronic liver damage (Peers et al., 1987).

In humans, exposure to HBV can be reliably measured by immunological methods. However, aflatoxin exposure is rather difficult to measure at individual level. Tests for DNA-adducts are useful for measuring recent exposure but they cannot be used to measure long-term exposure to aflatoxin. Only longitudinal studies using long-term exposure detection methods of individuals at high risk
of PHC will decisively establish aflatoxin as a human carcinogen.

The sources of aflatoxin exposure are aflatoxin contaminated maize, ground nuts, cassava, sorghum and millet but milk from cows feeding on aflatoxin contaminated feeds and forage, may also be important sources.

Furthermore no significant correlation was demonstrated between the joint effects of HBV and aflatoxin on PHC prevalence in this study. This means then that some other factors may be involved in the causation of liver cancer in humans.

**Modulating Factors:**

Neoplastic transformation is multifactorial in origin and is a multiphase process and hence subject to the influence of modulating or modifying factors. The multiphase process of carcinogenesis can be enhanced or inhibited by different factors. Tumour promoters, for instance, can markedly
enhance tumour yield when applied repeatedly following a low or suboptimal dose of a carcinogen. The results of the animal experiment on tumour promotion clearly demonstrated that the presence of tumour promoters substantially increased the overall cancer risk and/or shortened the latency period of tumour development. Some of these tumour promoters are commonly encountered in the human environment as vegetable foods, food additives and drugs. The presence or absence of tumour promoters in certain localities may as well be responsible for the variation of liver cancer incidences in those areas. Tumour promoters in the human environment have very much complicated extrapolation of carcinogenesis data from animal experiments to humans. Therefore, identification and control of tumour promoters in our environment is an area that should be embarked on seriously if we have to effectively control the risks of liver cancer in humans.

Co-carcinogenic agents including parasitic infestations such as Schistosomiasis should be viewed seriously as carcinogenic risks in the human environment in certain high risk areas.
Posed against the impact of human exposures to environmental carcinogenic, co-carcinogenic and tumour promotion risks is the presence of agents that inhibit or suppress the effects of the risks. This study clearly demonstrated in an animal model that compounds such as B-carotene, ascorbic acid, reduced glutathione, vitamin E (tocopherol) and selenium have strong inhibitory or suppressive effects against aflatoxin hepatocarcinogenicity. Since these anti-carcinogenic compounds are present in vegetables such as cabbages and brussels sprouts and carrots and fruits such as oranges, lemons, pineapples, normally eaten by humans and show no adverse effects even when consumed in large amounts, our people should be encouraged to eat them more often in sufficient amounts to reduce or control liver cancer risks.

CONCLUSION

1. This is one of the first studies of its kind to measure HBV infection and AFB exposure at individual level in the same population group. The incidence of HBV infection
was measured by serum HBsAg and the rate of exposure to AFB by measuring the urinary excretion of AFB-Gua.

2. No significant correlation was established between hepatitis B virus carrier rate and primary hepatocellular carcinoma in the aetiology of liver cancer study in humans. Similarly no significant association was established between aflatoxin exposure and liver cancer.

3. The combined effects of HBV and aflatoxin still gave no significant association with PHC.

4. AFM -metabolite secreted by lactating rat mothers dosed with AFB, induced hepatocellular carcinoma in the offsprings suckling their mothers.

5. Current results indicate that tumour promoters such as phenobarbitone amplify the effects of carcinogens.
6. It was further found that AFB$_1$-induced liver tumours can be inhibited by nutritional modifiers such as vitamins and other accessory food factors.

CONCLUDING REMARKS.

1. More work should be carried out to prove that hepatitis B virus is oncogenic in humans. This can be done by using for instance human cell culture system when one becomes available.

2. There is an urgent need to develop a method that can measure aflatoxin long-term exposure in humans that can be used epidemiologically.

3. To define clearly what roles hepatitis B virus infection and aflatoxin exposure play in the causation
of liver cancer, a carefully planned prospective study of HBV infection, AFB-DNA adducts and PHC should be carried out in selected small high risk population groups.

4. Since accessory food factors such as vitamins and minerals possess anti-carcinogenic properties, people should be encouraged to consume them more frequently in order to reduce the frequency of liver cancer in this country.

5. There is real need to improve the reporting of liver cancer cases in our hospitals.
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