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AFLATOXIN CONTAMINATION OF CASSAVA FLOUR (KOKONTE) PROCESSED BY TRADITIONAL METHODS IN GHANA

BY

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A THESIS PRESENTED IN PART FULFILMENT FOR A MASTER OF SCIENCE DEGREE IN BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON.

DEPARTMENT OF BIOCHEMISTRY,
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DECEMBER, 1978

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I CERTIFY THAT THIS WORK HAS NOT PREVIOUSLY BEEN ACCEPTED FOR ANY DEGREE AND IS NOT BEING CURRENTLY SUBMITTED IN CANDIDATURE FOR ANY DEGREE

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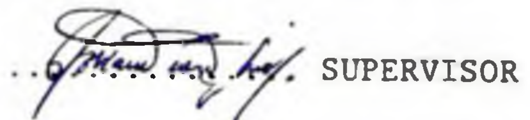 SUPERVISOR

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ABSTRACT

Aflatoxin contamination of cassava flour (kokonte) bought in the markets of Accra has been investigated. A survey of the methods of production and of processing of the cassava chips was conducted to determine the sources of contamination in the production of the flour.

Samples of the flour were analysed for aflatoxin contamination and concentrations using Thin Layer Chromatography (T.L.C.).

Known levels of aflatoxin in kokonte flour samples were fed to weanling rats to determine the effects on the rats. Serum alkaline phosphatase levels were determined in the serum samples of the individual rats.

The study revealed that:-

(1) Three types of kokonte flour are sold at the markets of Accra and these are named according to their colours. There are red, black and white kokonte types.

(2) Methods used in the processing of the cassava chips exposed the fresh roots to mould growth and hence to aflatoxin contamination.

(3) Twenty-two percent of the kokonte flour samples bought from the market were found to contain aflatoxin. The mean aflatoxin concentration was calculated to be 867µg/kg.

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(4) There were reduced rates of growth in the rats fed on aflatoxin-contaminated diets over a period of 25 days and their food intake was also found to have reduced when compared with those of the control group.

(5) The rats on aflatoxin-contaminated diets had fatty livers and there was an evidence of a growth on one of the livers.

(6) Serum alkaline phosphatase levels in rats fed aflatoxin-contaminated diets were found to be higher than those observed for the rats on control diets.

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LIST OF ABBREVIATIONS

D.N.A ---------- De oxyribonucleic acid

F.A.O ---------- FOOD AND AGRICULTURE ORGANISATION

I.D.R.C ---------- INTERNATIONAL DEVELOPMENT RESEARCH CENTRE

J.A.O.A.C. - - - - Journal of Association official Analytical chemists.

R.N.A. ---------- Ribonucleic acid

T.L.C. ---------- Thin-Layer Chromatography

T.P.I. ---------- Tropical Products Institute

UNICEF ---------- United Nations International Children Emergency Fund

W.H.O. ---------- World Health Organisation

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CHAPTER I

INTRODUCTION

The aflatoxins represent a group of secondary fungal metabolites which were discovered as contaminants of certain lot of animal feed. These compounds have a high order of acute toxicity to many animal species and have been shown to possess potent carcinogenic properties in several animal species (Lancaster *et al*, 1961; Wogan, 1965)

In June 1960, Young turkeys on poultry farms in South and East England died in the course of a few months from an apparently new disease that was termed "Turkey X disease" (Blount, 1961). Within a short period of time, a similar disease was reported in ducklings and chickens (Asplin & Carnaghan, 1961). Reports were also received from Kenya and Uganda of severe losses of ducklings from similar disease. Swine and cattle were also affected by the disease (Harding *et al*, 1963; Asplin and Carnaghan, 1961; Loosemore and Markson, 1961). Almost simultaneously in the United States, an outbreak of trout hepatoma was discovered in the spring of 1960, which was attributed to aflatoxin contamination of feed (Wolf and Jackson, 1963).

Although the toxic agent in these outbreaks was initially unknown, investigations were directed towards the possibility that the rations were contaminated with

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toxic substances. Investigations by Asplin and Carnaghan (1961) showed that the common factors were the presence of Brazilian groundnut meal in the feed. The investigations however did not demonstrate any specific known poisonous agent in samples of the meal. At this time Asplin and Carnaghan (1961) reported that examinations of liver sections from ducklings sent by a veterinary laboratory in East Africa indicated that the disease suffered by the East African ducklings, was similar to that associated with the feeding of Brazilian groundnut meal to ducklings in England. This was the first indication that it is not only Brazilian groundnut meal that could cause the disease, but groundnut meals from India, Kenya, Uganda, French West Africa, Nigeria and Ghana (Sargeant et al, 1961). A more intensive investigation of the suspect East African groundnut meal was then undertaken and it was quickly found that this groundnut meal was toxic to poults and ducklings with symptoms similar to "Turkey X disease".

Ducklings proved to be the more suceptible species (Asplin and Carnaghan, 1961). Proliferation of bile duct epithelial cells was clearly visible within a few days of ingestion of toxic meal. This afforded the basis for a

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sensitive and a relatively rapid biological method for detection of toxicity. In turn this was used to effectively monitor the extraction and concentration of toxin through classical procedures.

Sergeant et al (1961); and Allcroft et al (1961) were able to extract the toxic principle from the meal with chloroform and 250-fold concentration was achieved. Chromatography of 250-fold concentration on neutral alumina afforded a crystalline almost colourless compound (Sergeant et al, 1961). Further purification of the toxin was achieved by paper chromatography. The crystalline product when chromatographed on Whatman No.1 paper using n-butanol-5% acetic acid as developer gave a single spot of Rf value 0.7 which emitted bright blue fluorescence when illuminated with U.V. light. Further, the amount of fluorescent material as estimated visually to be present in the spots produced in this way from numerous samples, corresponded with the toxicity as determined biologically (Sergeant et al 1961). Thus for the first time, the basis for routine chemical analysis of the toxin was provided.

Speculations made during 1960 regarding the nature of the toxic factor included suggestions that the disease

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might be due to a toxin produced by a fungus. The first evidence substantiating this was provided by Austwick and Ayerst (1963) who, during a microscopic examination of a sample of Brazilian groundnut meal noted that pieces of the groundnut cotyledon tissue contained hyphae, although none was present in the sample of non toxic meal. Attempts at culture showed that these hyphae were dead (Austwick and Ayerst 1963). Later Sergeant et al (1961) succeeded in producing pure cultures of certain of the fungal species present in a highly toxic sample of groundnuts from Uganda, heavily contaminated with fungi. A chloroform extract of a culture of one of the isolates grown on Czapek's solution agar was found to contain a fluorescent material with Rf value of 0.7 when chromatographed on paper under conditions developed previously.

The toxin-producing fungus was identified by J.J. Elphick as Aspergillus flavus (Sergeant et al, 1961) and the toxin was given the name "Aflatoxin" by the Inter Departmental working party on Groundnut toxicity Research in 1962 in view of its origin.

The aflatoxins are soluble in moderately polar solvents such as methanol, chloroform and acetone. But they are virtually insoluble in non polar solvents like hexane, and petroleum ether, and also insoluble in

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water.

Initial isolation was accomplished by extraction with hot methanol (Allcroft et al 1961, Sergeant et al, 1961) and a variety of extraction solvents have since been used for various purposes. These include 55 per cent aqueous methanol, (Campbell et al, 1964; Nesheim, 1964-; Trager et al, 1964) 70 per cent aqueous acetone (Pons and Goldblatt 1964), and hexane - water - acetone azeotrope (Goldblatt 1965). In the production and isolation of quantities of aflatoxins from mould cultures on solid substrates, a convenient extraction and concentration procedure involves total extraction of the culture with chloroform and subsequent precipitation of the toxins in petroleum ether (Asao et al, 1963). The toxins produced on liquid media are quantitively extracted by partitioning in chloroform (Adye and Mateles 1964). Extracts produced by these procedures usually contain mixtures of fluorescent compounds which are separated into their individual components by chromatographic techniques.

Although several systems have been developed including the use of alumina as the support medium (Broadbent

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et al 1963), the conditions mostly used involve separation on silica gel plates developed with three to five per cent methanol in chloroform (Asao et al, 1965; de longh et al 1965; Nesheim, 1964) or with five to fifteen per cent acetone in chloroform (Eppley, 1966). When chromatograms of extracts containing aflatoxins are viewed under uv light, a complex array of fluorescent compounds is generally present. Nesbitt et al (1962) succeeded in resolving on alumina chromato-plates, by means of chloroform methanol (98.5 : 1.5) the fluorescent material described as having Rf of 0.7 on filter paper when developed with n-butanol acetic acid. They obtained two spots, one having a blue violet spot, Rf. 0.6 and the other having a lower Rf and green fluorescence. For convenience, these were referred to as aflatoxin B and aflatoxin G respectively.

Almost simultaneously, other groups introduced the use of silica gel chromatography for the separation of the aflatoxins. Smith and Makernam (1962) by chromatography on Silica gel G Merck of extracts of cultures of toxigenic strains of *A. flavus* obtained at least twelve fluorescent compounds.

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Isolation and characterisation of four closely related toxins was first reported by Hartley et al (1963). The four compounds separated on silica gel chromatoplates using chloroform methanol (98:2) as developing solvent, and were designated aflatoxins B_1 B_2 G_1 G_2 in order of decreasing Rf value. These toxins appeared to be identical with the material previously described by Nesbitt et al (1962), Sergeant et al (1961), Vander Zidjden (1962) and de longh (1962). The Molecular formula of aflatoxin B_1 was established from elemental analysis and mass spectrometric determinations as C_{17} H_{12} O_6 , aflatoxins G_1 as C_{17} H_{12} O_7. Aflatoxins B_2 G_2, were found to be hydro derivatives of the parent compounds with the formulae C_{17} H_{14} O_6 and C_{17} H_{14} O_7 respectively. (Hartley et al 1963). The structural elucidation (Figure 1) of aflatoxins was determined using ultra violet, infra red, nuclear magnetic resonance and mass spectra (Asao et al 1965)

When aflatoxin B_1 or an unseparated mixture of the aflatoxins is fed to animals certain toxins, Aflatoxin M_1 and M_2 (Figure 2), may be released from the secretion of the animals. Two groups have reported the identity of aflatoxin M_1 and M_2 from several sources and have also detected the toxins among metabolic products of A flavus.

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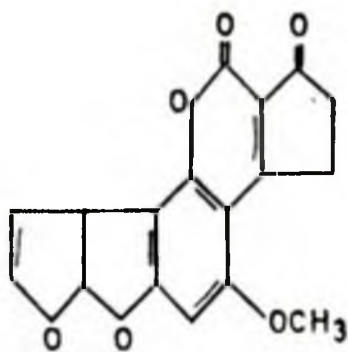
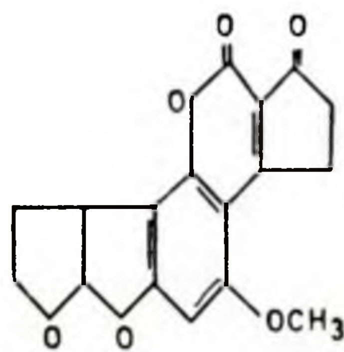
FIGURE 1

STRUCTURE OF THE AFLATOXINS

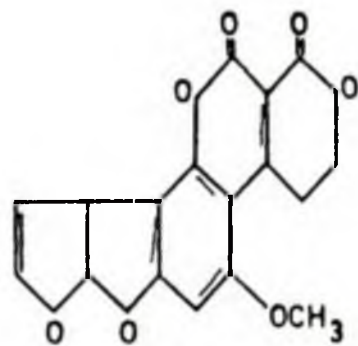
AFLATOXINS B_1 B_2 G_1 AND G_2 (ASAO *et al*. 1965)



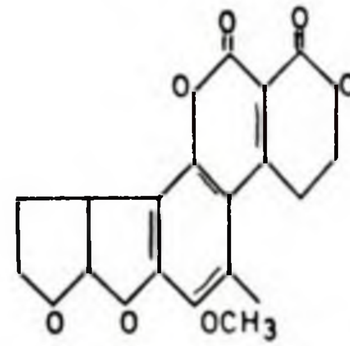
B_1



B_2



G_1



G_2

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FIGURE 2

AFLATOXINS M_1 AND M_2 (HOLZAPFEL et al, 1966)

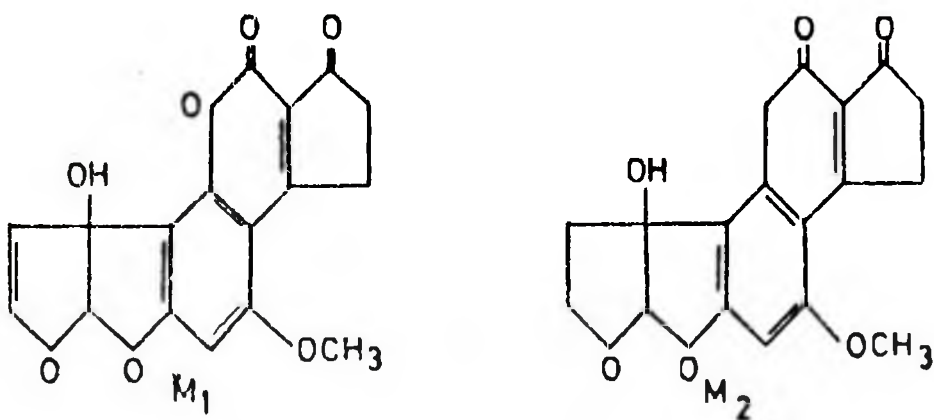

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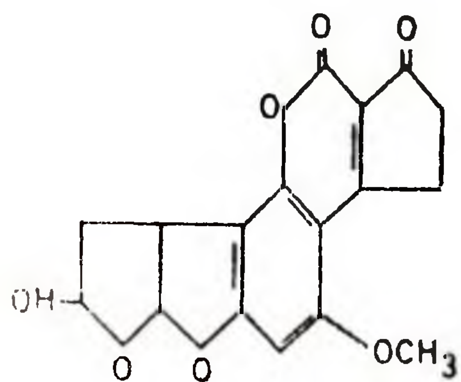
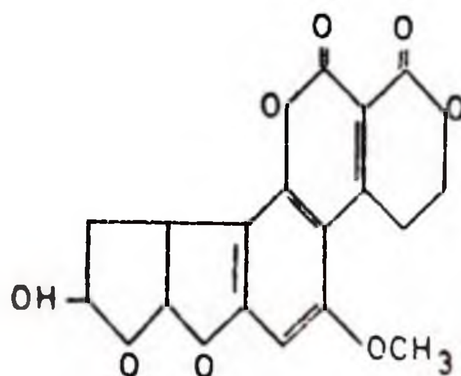
FIGURE 3

AFLATOXINS B_{2a} AND G_{2a} (Dutton and Heathcote 1969)



B 2a



G 2a

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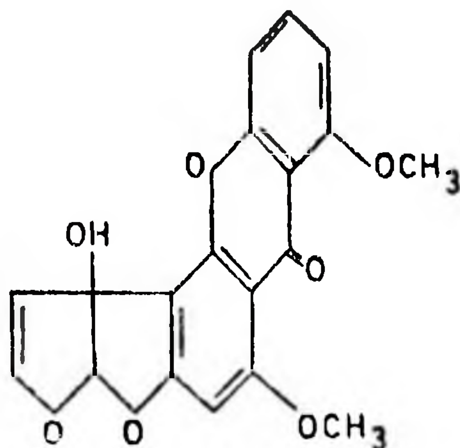
FIGURE 4

ASPERTOXIN (Rodricks et al, 1968, Waiss et al 1968)



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(Holzapfel *et al* 1966, Masri *et al*, 1967). The same groups also reported on the structures. The structures of two additional hydroxyl containing aflatoxins isolated from culture of *A flavus* and designated aflatoxin B_{2a} and G_{2a} have been reported by Dutton and Heathcote (1966). One exhibited blue fluorescence under U.V. light and the other green. Another addition to the aflatoxins, aspertoxin was reported by Rodricks *et al* (1968); Waiss *et al* 1968). (Figures 3 and 4).

The structural formulae of the most commonly occuring aflatoxins, B_1 B_2 G_1 and G_2 are shown in Fig. 1. The most reactive funtional group for ease of attack by chemical reagent are the lactone ring of aflatoxin B_1 and B_2, and the two lactone rings of Aflatoxin G_1 and G_2. These δ-lactone rings can be readily opened by hydrolysis with strong alkali such as sodium hydroxide. This results in reduction of toxicity and carcinogenicity (Fishback and Campbell 1965). The methyl ether and furan ether groups would be cleaved only by very strong acids like hydrochloric acid. The double bond of the terminal furan ring of aflatoxin B_1 and G_1 is susceptible

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to attack by electrophilic reagents and can be oxidised or reduced but B_2 and G_2 will be unaffected. Similarly reagents attacking the ketogroup of the cyclopentenone ring of aflatoxins B_1 and B_2 would be without effect on aflatoxin G_1 and G_2.

The discovery of these compounds as contaminants of animal feeds and recognition of their potency has stimulated a great deal of research concerned with their effects in various biological systems. The toxic properties of the aflatoxins manifest themselves differently depending on the test system, the dose and the duration of exposure. Thus they are lethal to animals and animal cells in culture when administered in large doses. They also cause histological changes when sublethal doses are given subcutaneously. Long term exposure for extended periods result in chronic toxicity including tumour induction in several species.

The acute toxicity and carcinogenecity of the aflatoxins to rats have been studied extensively since the recognition of the disease among farm animals. Prior to isolation of the aflatoxins, Lancaster _et al_ (1961) showed that a diet containing the same meal which was

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toxic to poultry. induced hepatic carcinomas in rats. Other workers induced carcinomas in rats when they fed the rats with diets containing groundnuts meal (Schoental, 1961; Salmon and Newberne, 1963; Butler and Barnes, 1963). When purified aflatoxins became available, these were shown to be carcinogenic (Barnes and Butler, 1964). The main difference in lesions induced by aflatoxins compared with that induced by other carcinogens was the comparative absence of fibrosis and especially of cholangio fibrosis.

Using purified aflatoxin B_1 Wogan and Newberne (1967) showed that levels as low as 0.015 ppm in the diet induced a high incidence of hepatic carcinomas.

In short term experiments Madhavan and Gopalan (1965) described an increased severity of liver lesions when aflatoxin was given to protein depleted weanling rats.

The acute toxicity of aflatoxin B_1 has now been studied. At one day old, the rat is most susceptible with an $L.D_{50}$ of 0.56 mg per kg body weight. (Wogan 1965). At weaning the $L.D._{50}$ is 5.5. mg per kg male and 7.4 mg per kg female (Wogan, 1965). The effect of aflatoxin on Guinea pigs has been studied (Peterson *et al* 1962).

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Asplin and Carnaghan (1961) studied the poisoning in ducklings. Newberne and others (1966) worked on the effect of the toxin on dogs. Madhavan and his colleagues (1965) studied the effect of aflatoxin on rhesus monkey. Two groups of monkeys were given different concentration of aflatoxin B_1 and the effects of the toxin were studied. Histological examination of the two groups of monkeys showed essentially the same changes of fatty infiltration, biliary proliferation and portal fibrosis. Biochemical findings prior to death suggested an impaired liver function (Tulpule et al 1964, Madhavan et al, 1965). The effect of dietary protein level was also investigated (Madhavan et al 1965) and it was shown that reduced protein intake increase the susceptibility to aflatoxin.

Estimates of susceptibility of most farm animals have been derived from feeding aflatoxin - containing groundnut meal. Such feeding trials have indicated a marked variation in susceptibility, but in all species so far studied, the young have proved to be much more susceptible than mature animals. There is little recorded information on the influence of sex and pregnancy on susceptibility of farm animals, however field observations

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in Weybridge have indicated that pregnancy has little influence in cattle and sheep but does increase susceptibility in pigs. There is no indication that aflatoxin - containing feed influences hatchability in hens and ducks (Asplin and Carnaghan (1961).

Ducklings are particularly susceptible, turkey poults and pheasant chicks less so, and chickens are comparatively resistant but show a reduction in growth rate. In larger farm animals, pigs, from 3 to 12 weeks of age, pregnant sows, and calves aged 1 to 6 months are very susceptible when fed rations containing highly toxic meal. Older cattle are more resistant, but sheep are remarkably resistant, (Allcroft, 1965). Allcroft (1965) reported increases in serum alkaline phosphatase activity in sheep, calves and pigs fed rations containing high levels of aflatoxins. A similar result was noted in rats treated with aflatoxin B_1 by Clifford and Rees (1967), who also found increased serum levels of isocitrate, malate, glutamate dehydrogenases in similarly treated animals. Those investigators further reported decreased activity of drug metabolising enzymes in rat liver damaged by aflatoxin B_1 (Clifford and Rees 1966).

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Aflatoxin has previously been shown to be hepatotoxic to many animal species. The mechanism of action of aflatoxin has been worked out in rat liver and other systems. Accordingly, with heteroploid human embryonic lung cells, aflatoxin (B_1 and G_1) suppressed mitosis and DNA synthesis, leading to increase in giant cell formation and cell vacuolation (Legator et al 1969). The specific effect of B_1 leading to DNA synthesis in this particular cell was shown to be an induction in the activity of thymidine kinase, a factor important in the initiation of DNA synthesis and cell replication in tissue cultures (Childs and Legator 1966). In mammalian systems for example in partially hepatectomized rats, aflatoxin B_1 (0.83mg/kg - body weight) markedly inhibited the synthesis of DNA in hepatic cells and also reduced the amount of nuclear RNA but aflatoxin B_1 did not inhibit the synthesis of all RNA species (De Recondo et al, 1965). This effect was accompanied by partial necrosis of the liver tissue, alteration of nuclear and fragmented chomatin. It was also shown that the effect of aflatoxin B_1 was not due to any effects on the enzymes involved in DNA synthesis or break down but was probably the result of B_1 acting on the

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DNA molecule and therefore, inhibiting the priming action, (De Recondo *et al* 1965). Similar decreases in RNA synthesis in the nucleus of Rat liver cells were observed with aflatoxin B_1 by other workers, (Clifford and Rees, 1967); Friedman and Wogan, 1966; Sporn *et al* 1966; Gelboin *et al*, 1966; La farge *et al*, 1966). Aflatoxin B, was found to combine with DNA *in vitro* to give rise to DNA-B_1 interaction leading to an interferance in DNA dependent RNA synthesis and more specifically to a possible reduction of RNA polymerase activity. With *in vivo* studies, aflatoxin B_1 was specifically located in the rat liver nucleus (Clifford and Rees, 1966, 1967) and the rat liver nucleus RNA polymerse activity was inhibited in aflatoxin B_1 dosed-animals, (Clifford *et al* 1967; Friedman and Wogan, 1967). Pregnancy increases the susceptibility of the female rat to aflatoxin, the susceptibility being greater in latter stages of pregnancy. Growth retardation of the foetus was also observed with aflatoxin B_1 ingestion (Butler and Wigglesworth 1966). In the Hamster, B_1 was shown to be teratogenic (Ellis & Di Paolo 1967, Di Paolo & Ellis 1967). In addition, interperitoneal administration of aflatoxin B_1 to dogs resulted in abortion or foetal

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death (Newbern *et al*, 1966) thus in the region of rapidly producing cells, the blocking of RNA synthesis by aflatoxin B_1 may affect cellular differentiation and development in foetus (Butler & Wigglesworth 1966 ; Ellis & Di Paolo, 1967).

In terms of potential human implications, it is likely that the consumption of toxic fungal metabolites is continuous, and related to specific localised factors, especially in the under developed regions of the world. These specific actions of aflatoxin merit further consideration as a potential hazard to man. Since groundnut meal is used in many animal feeds the problem arises as to whether animals fed aflatoxin-contaminated feeds contain any of the toxic materials in their body tissues. While animal tissue may not contain any aflatoxin, the milk and cheese obtained from animals, fed aflatoxin containing rations do sometimes contain a toxic metabolite; aflatoxin M which causes liver lesions in ducklings (Allcroft & Carnaghan, 1963; Vander Linde *et al* 1965; de longh *et al*, 1965; Allcroft *et al*, 1966; Nabney *et al*, 1967). Hence in man's food supply, both milk, cheese and butter could be implicated in aflatoxicoses.

Beardwood (1963) detected aflatoxins in groundnut

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samples bought from the markets in Accra (Ghana). Concentration was in the order of 1000µg/kg and contamination was 69 per cent.

An extensive survey was conducted in Ethiopia in search of hepatoxins of plant origin with special reference to fungal contamination of staple grains because of the high incidence of chronic liver disease in that country (Coady 1966). Carbohydrate sources in areas high incirrhosis and primary hepatoma were observed to have in general been stored from one harvest to another over rainy seasons, which provide ideal conditions for fungal growth on grains (Goldblatt 1969). Porridges and fermented foods have been found to contain moulds and aflatoxin (Allcroft et al, 1961; Goldblatt, 1969). These porridges are widely eaten by children and nursing mothers as well as at breakfast times; thus the use of mycotoxin contaminated grains in porridges and other foods is bound to affect man.

Aflatoxin, while primarily inducing hepatomas in animal systems may also give rise to carcinoma in other organs particularly after prolonged periods of low level ingestion

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possibly punctuated by short periods of ingestion at high levels. .

Among the Bantus of South Africa, hepatomas represent 68 per cent of carcinomas in Bantu males Mouldy corn is an important ingredient in the diet of the Bantus. (Higginson and Oetle 1960).

Aflatoxin at low doses in man probably cause lassitude and weakness. (New Scientist, 1966).

In Germany in 1973, acute aflatoxin poisoning caused the death of a man, in whose liver aflatoxin was detected (Rosenberg et al 1973).

In a case report in Czechoslovakia, aflatoxin was detected in the liver of 3 out of 5 babies. Clinical evidence suggested that they were exposed to the toxin during intra-uterine life or soon after birth (Dvorackova et al. 1975).

Hepatocarcinogenic material was also found to be present in urine specimens from humans consuming aflatoxin. The toxin was extracted with chloroform from the urine of a group of children who were fed for two days groundnut butter made in the Phillipines. The extracts produced hepacarcinogens in rainbow trout (Campbell, et al, 1975).

In another populations study in Kenya, Peers and Linsell (1973) showed that although frequency of

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contamination of diets and beers was low, there were significant associations with incidence of liver cancer and altitude.

A sample of human liver tissue obtained at biopsy for study of metabolism of aflatoxin B_1 in vitro, was found to contain aflatoxin apparently ingested. The patient, a resident in U.S. had carcinoma of the rectum and liver (Phillips *et al* 1977).

Clerk and Caurie (1968) reported that *Aspergillus flavus* was the most predominant mould found when fresh cassava chips, spread out to dry, were in Ghana.

Studies with fermented cassava starch from Benin (Dahomey) (Toury and Giyorgi, 1966) have shown presence of aflatoxin.

Aspergillus flavus, was successfully grown on cassava (Mannihot Utilissima) and high concentration of aflatoxin B_1, B_2, G_1 and G_2 were observed to have been synthesized by the mould within four weeks (Nartey, 1966).

Work done in Mozambique on methods of food production, harvesting, storage and preparations in relation to

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aflatoxin contamination showed that, aflatoxin occurs in the main carbohydrate sources, namely cassava and maize during storage (Wogan, 1968).

Studies with cassava starch in Thailand and in Hongkong also revealed the presence of aflatoxins. (Shank et al, 1972).

Brudzynski (1974) estimated aflatoxin in cassava bought in market in (Kinshasa) Zaire, Thirty three per cent of the samples were found to be contaminated with aflatoxin.

Cassava (Manihot utilissima) forms a major part of the staple diet of the people of Ghana. It is the most commonly produced root crop when compared with other root staples, plantains and cereals in Ghana. Actual figures of production from 1970 to 1974 show that cassava covered between 32 per cent to 38 per cent of the total area put under cultivation for root crops, tubers and plantain, and provided between 39 per cent to 45 per cent of the total tonnage obtained from all such staples.

Cassava contains the toxic principle, hydrogen cyanide. The toxicity is caused by the presence of cyanogenic glucoside, linamarin, which is hydrolysed in

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the presence of the enzyme linamarase to hydrogen cyanide (Coursey 1973). The safe level of hydrogen cyanide in cassava is not known but less than 50mg/kg fresh peeled root is estimated to be harmless (Coursey 1973. Coursey (1973) also classified food processes on the basis of non detoxification and detoxification. Techniques in which no specific detoxification is affectted to an appreciable extent are simple cooking, roasting or baking, frying and sundrying. Other processes such as soaking, fermentation or a combination of long soaking and fermentation however effect some detoxification.

In relation to other root crops and tubers, cassava has the most diversified modes of processing and utilization. Cassava is eaten in different forms in Ghana and is traditionally processed into these various forms. Some of these processed products are gari, tapioca, starch and kokonte (Cassava flour). Most of the traditional processes involve fermentation and drying which are mostly done in the open and thus may give rise to mould growth and possibility of aflatoxin production. One of the common cassava products, kokonte is prepared by cutting fresh peeled roots into chips and drying the chips. The dried chips are milled into flour. In the process

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the chips are alternatley exposed to dew and sunlight which allow fermentation, mould growth and dark colouration, all of which are desirable characteristics in the final products. The flour is simply stirred into boiling water and cooked into sticky paste which is usually eaten with soups such as groundnut soup and stews.

Cases of poisoning after consumption of kokonte meals have occasionally been reported in the mass media in Ghana, especially from the Northern Region. Although hydrocyanide poisoning may be suspected in these cases, this could hardly be so since the long soaking and fermentation processes involved usually effect some detoxification of hydrocyanide.

Given that in the preparation of cassava flour (kokonte) the chips are exposed to dew and sunlight which allow mould growth and that among the moulds identified to grow well on cassava could be found Aspergillus flavus, it may therefore not be surprising to suspect that some of the cases of poisoning after consumption of kokonte meals may be attributed to aflatoxin intoxication.

In an unpublished report of observation made in Uganda in 1966, a girl died after eating aflatoxin-containing mouldy cassava. She had liver damage similar

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to those seen in monkeys given sublethal doses with 3ppm.

In a field study in Uganda by Serck-Hansen in 1970, he reported aflatoxin induced hepatitis in a boy resulting in death, the diet of the boy was dry cassava meal, analysis of the meal showed 1.7mg/kg aflatoxin.

Nestel (1974) also refers to an endemic toxic type of hepatitis in the Amazon region characterised by diffuse necrosis, periportal mononuclear infiltration, and

fatty vacuolation presented to be due to aflatoxin intoxication. Samples of cassava from the same region analysed was found to contain high levels of aflatoxin.

From the references discussed above and from other references proving the presence of the *Aspergillus flavus* and aflatoxin in Ghana, we strongly suspect the presence of the toxin in kokonte processed by traditional methods.

The following project was set out to test the theory of the presence of aflatoxin in kokonte flour processed by traditional methods.

The project was divided into three parts;

A. Survey of traditional processing methods of kokonte.

B. Determination of the presence of aflatoxin in collected samples and finding the concentration present.

C. Feeding trials with aflatoxin-contaminated kokonte flour samples.

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CHAPTER II

EXPERIMENTAL PROCEDURES

A. Investigation Into Traditional Processing Methods of Cassava Flour (Kokonte).

a) A questionnaire was drawn up for this part of the project (See Appendix 1). Fifty market women and men selling kokonte chips or flour in Accra markets were interviewed in order to complete the questionnaires. The markets visited were Makola, Mamprobi, Malata, Kaneshie, Salaga, London and Osu markets. The completed questionnaire forms were later analysed and the results compiled.

b) Collection of Samples

Eighty different kokonte samples, about five hundred grams (500g) each were bought from the various markets visited. The samples were stored in well labelled polythene bags in a cold room at -20°C till needed for the various analyses. Twenty other samples were prepared in the laboratory and used as controls.

c) Preparation of Control Samples

Twenty big fresh uninjured cassava tubers were bought from the Malata market. They were washed with water and peeled separately. Each tuber was cut into small pieces and the pieces put in different trays. The pieces were dried in a drought oven at 35°C for one hour. The dried chips in each tray were milled separately and the flour

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stored in labelled polythene bags in the cold room.

d) Determination of Moisture Content of Kokonte Samples:

Percentage moisture content of kokonte samples were determined using the gravimetric method.

B. Determination of the presence and levels of Aflatoxin in kokonte flour samples purchased.

a) Carlibration of Spectrophotometer (Official methods of Analysis, 1970; Jones 1972).

The spectrophotometer, (Unicam S.P 500) was calibrated with potassium dichromate solution. The dichromate solution was prepared in 0.108N sulphuric and to give 0.4mM concentration and two accurate successive half dilutions were made of the acid dichromate with 0.018N Sulphuric acid to make 0.2mM and 0.1mM solutions respectively.

The absorbence of the three solutions were determined at 35C $_{nm}$ using the 0.018N sulphuric acid solvent blank. The absorptivity E for each of the solutions were calculated using the following given equation:-

$$E = \frac{\text{Absorbance} \times 1000}{\text{Concentration in Mm}}$$

The average of the three E values were obtained to give $\bar{E}$. The correction factor for SP. 500 spectrophotometer and cells were determined by applying the equation.

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$$CF = \frac{3.160}{E}$$

The results obtained for the three concentrations of potassium dichromate standard solutions are shown below:-

TABLE I

Concentration mM	Absorbance	Absorptivity
0.1	0.31	310
0.2	0.65	325
0.4	1.40	350

The calculated Average Absorptivity for E = 328.33

The correction factor CF for the spectrophotometer was therefore calculated as $\frac{3.160}{E} = 0.96$

According to the Official Methods of Analysis (1970) if CF is $<$ 0.95 or $>$ 1.05, the technique or the instrument should be checked to eliminate the cause. The calculated results of CF = 0.96 obtained falls within the acceptable limits given. The SP 500 was therefore considered calibrated.

b) Aflatoxin Standard Solution

Five hundred micrograms crystalline aflatoxin B_1 standard obtained from the Unilever Research Laboratories were dissolved in 10mls chloroform to give 50µg/ml concentration stock solution. An aliquot of 5mls was taken

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from the stock solution and diluted to 25mls with chloroform to give 10µg/ml working standard. The stock and working standards were kept in the refrigerator until ready for use.

The UV spectrum of the aflatoxin working standard solution was determined in the wave length region between 320 and 390 nm on the SP 500. Chloroform was used as the blank. The results obtained are shown in Table II. These values were used to draw an absorption curve as shown in Fig 5. The wavelength of maximum absorption was found to be 358, which gave absorbance reading of 0.70. The actual concentration of the working standard was later calculated using the equation:

$$\text{Concentration } (\mu g/ml) = \frac{\text{Absorbance x mol.wtxCfx1000}}{E}$$

where 0.96 obtained above was used as CF and E the absorptivity of aflatoxin B_1 in chloroform was given as 20,600 and Molecular weight of B_1 also given as 312 (Jones 1972).

Absorbance from UV spectrum = 0.70

Absorptivity E = 20,600

Molecular weight of aflatoxin B_1 = 312

CF = 0.96

∴ Concentration of standard B_1 solution

$$= \frac{0.7 \times 312 \times 096 \times 1000}{20,600}$$

$= 10.18\mu g/ml$

$= 10.00\ \mu g/ml$.

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FIGURE 5

Absorption Spectrum of Aflatoxin B_1 Standard Solution

Absorption was measured for Aflatoxin B_1 over 320-390 nm at Concentration of 10 µg/ml using Unican S.P. 500 Spectrophotometer. The blank solution was chloroform.

The results obtained below in Table were used to draw the absorption Curve in Figure 5.

TABLE 2

Wavelength nm	Absorbance	Wavelength nm	Absorbance
320	.37	358	.70
325	.43	360	.69
330	.49	365	.65
335	.54	370	.51
340	.61	375	.31
345	.65	380	.13
350	.68	385	.06
355	.69	390	.03
357	.69		

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357	.69		

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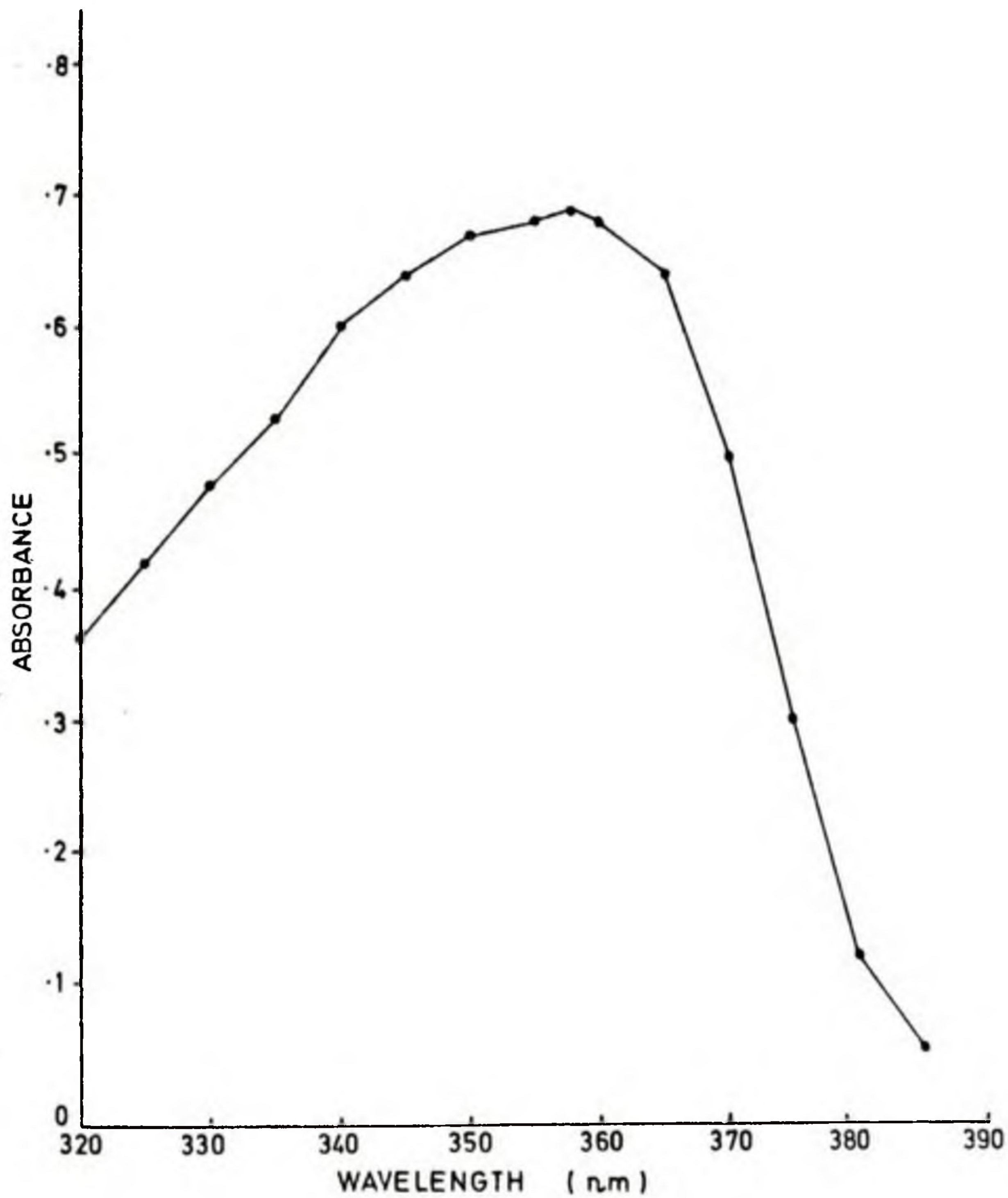
FIGURE 5

ABSORBTION SPECTRUM OF AFLATOXIN B1 STANDARD SOLUTION



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The recommended method (official methods of analysis 1970, Jones 1972) for checking stability of stored aflatoxin standards were periodically employed to make sure that there was no breakdown of the aflatoxin B_1 in the standard solution utilised during the experimental period.

c) Thin Layer Chromatography (T.L.C.) for Estimation of Aflatoxin B_1.

Method: A modification of the method by Jones (1972) was used in the preparation of the plates. Fifty grams kieselgel G (silicagel G) were mixed with 100ml of water and stirred continuously for about 5 minutes until the slurry could thickly coat the sides of the glass container. The thin layer plates were coated with the resultant slurry and the plates were left in racks in a dust free atmosphere for one hour. The plates were dried for a further hour in a forced draught oven at 105°C, and were then cooled in a dust free atmosphere for 30 minutes. The plates were then stored in a dessiccator plate cabinet till needed.

d) Determination of Purity of Standard Aflatoxin B_1 Solution.

The purity of aflatoxin B_1 standard solution was determined by spotting on a kiesel gel G chromato plate, 5µl of aflatoxin B_1 standard solution, at 2 cm intervals. A qualitative standard solution containing a mixture of

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aflatoxins B_1, B_2, G_1 and G_2 was also spotted alongside the B_1 standard spots. The chromatoplate was developed in chloroform: methanol system (95:5). Two distinct spots, one of blue colour and the other giving a green colour of aflatoxin G were obtained. The spots of aflatoxin B_1 showed no other aflatoxin spots and the standard was therefore considered to be pure aflatoxin B_1.

e) Column Chromatography

Since column chromatography had to be used for the purification of the sample extracts, chromatographic columns were prepared and the efficiency of the column was checked by doing aflatoxin B_1 recovery test on them. Since the recommended column specifications were found to be too big, new chromatographic columns were drawn to our own specifications which consisted of

(1) A reservoir at top, 95 mm long and 30mm diameter converging into

(2) Absorption tube, 145mm long, 6mm diameter and at the lower end

(3) The tube is drawn into a capillary of 35mm long and of such diameter that when the tube is charged, rate of flow was 1ml/minute.

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Method: Silica gel was activated by drying at $105^{O}C$ for one hour. 1% water was added to the cooled dried silica gel. This was thoroughly mixed and stored in an air tight container.

A ball of glass wool was placed loosely at the bottom of the absorption tube. One gram of anhydrous sodium sulphate was added to give an even base to the silica gel. Chloroform was added to the column until the column was about half full. Ten grams of activated silica gel made into a slurry with chloroform was added. The chloroform was drawn up to the top of the sodium sulphate and as soon as the level entered the sodium sulphate, 1 ml of aflatoxin B_1 standard solution containing 10µg of aflatoxin B_1 was added to the column and then washed with 40mls of hexane, followed by 40mls of anhydrous diethyl ether. The adsorbed toxin was finally eluted from the column with chloroform-methanol mixture (97:3v/v) and collected into a flask. The eluate was evaporated to dryness on a water bath under a stream of nitrogen and the residue was dissolved in 1 ml chloroform. Serial dilutions were made of aflatoxin B_1 standard solution (10.0µg/ml) to give concentrations of 9.0µg/ml, 8.0µg/ml, 7.0µg/ml, 6.0µg/ml and 5.0µg/ml.

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Duplicate samples of 5µl of the aflatoxin were spotted on thin layer chromatoplate. At 2cm interval were also spotted 5µl of the 10.0, 9.0, 8.0, 7.0, 6.0 and 5.0µg/ml aflatoxin B_1 standard. This was repeated with 10µl of the recovered aflatoxin alongside 10µl of each of the prepared standard B_1 solutions. The chromatoplates were developed in chloroform: methanol (95:5v/v) and were later examined under UV light in a dark room. All the spots emmitted blue fluorescence. The intensity of the fluorescence emmitted by the 5µl recovered aflatoxin B_1 spot, could not be differentiated from the intensities of the fluorescence produced by the 5ul spots of the 10.0µg/ml, 9.0µg/ml, 8.0µg/ml concentration of the aflatoxin B_1 standard solutions. The 10µl spots also showed similar results when compared with the 10ul spots of the same standard concentration. The fluorescence emitted by both the 5 and 10µl spots of the 7.0µg, 6µg and 5µg/ml B_1 standard solutions were however distinctly lower in intensities when compared with the recovered B_1 standard spots. It can therefore be said that more than 80% of the aflatoxin B_1 standard was recovered from the column. The columns were therefore considered to be efficient.

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f) Extraction of Aflatoxin From Cassava Flour Samples

The standard procedure of Jones (1972) as recommended by Tropical Products Institute (T.P.I.) for extraction of aflatoxins from low fat or fat free material was utilised for the aflatoxin extraction from the 80 bought kokonte samples and the 20 laboratory prepared control samples.

Method:

Twenty grams of each kokonte sample were weighed into 500ml conical flasks. Two hundred millilitres of chloroform were added to the flask and mixed thoroughly with a glass rod. Twenty millilitres of water were added to the flask, and the flask was stoppered with rubber bung wrapped in aluminium foil, to protect the rubber from exposure to chloroform. The flask was shaken by a wrist action shaker for 30 minutes to extract the toxin and the mixture filtered through Whatman No.1 filter paper into a 200ml. volumetric flask. The residue was washed several times with chloroform. The filtrate was then made up to volume with chloroform. The flask was stoppered, labelled and stored in the cold room till required for analysis. All extractions were done in duplicate.

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g) <u>Screening of the Sample Extracts For The Presence of Aflatoxins</u>:

Screening of the kokonte sample extracts was done by concentrating 20ml of each filtered extract to 5ml. Ten microlitre portions of the concentrates were spotted on silica gel G Chromatoplates together with a spot of standard aflatoxin B_1. The chromatoplate was developed in chloroform: Methanol (95:5v/v) solvent. The dry developed plate was examined under UV. light in a dark room. The presence of a blue fluorescent spot from sample spot, corresponding to the standard B_1 spot and having the same R_f as the standard spot was interpreted as positive aflatoxin contamination. The absence of blue fluoresecnt spot with the same R_f as the aflatoxin B_1 spot was interpreted as negative aflatoxin.

The results obtained from screening of the samples are given in Table 6. Screening revealed 29 samples [illegible] contaminated with aflatoxin. Although the remaining samples showed some fluorescent spots, none of these corresponded with the R_f values of the standard B_1 spots. Extracts from cassava are known to contain naturally occuring extraneous fluorescent compounds which can be confused with the aflatoxins. To get rid of these interfering compounds, a further 20ml of each sample extract were evaporated to dryness on a water bath under a stream

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of nitrogen. The residue was picked up in 3ml chloroform and then transferred to a chromatographic column for cleaning. The adsorbed aflatoxins were finally eluted with chloroform: methanol (97:3v/v) solvent. The eluate was evaporated to dryness on a water bath, under a stream of nitrogen. The cleaned residue was then picked up in 5ml of chloroform. Five microlitres and 10µl of this cleaned solution were spotted on fresh chromatoplates alongside aflatoxin B_1 standard spot. The chromatoplate was developed and examined in a dark room under UV. light. It was observed that most of the other blue fluorescent spots were eliminated except a few faint blue ones whose R_f Values did not correspond with the standard B_1 spot. Those that corresponded with the B_1 standard spots were very faint, therefore, the eluate after evaporating to dryness, was picked up in 1ml chloroform instead of 5ml and this dilution gave clear fluorescent spots. Five microlitres and two spots of 10µl of the above eluate were spotted successively on silica gel G Chromatoplate, along with the B_1 standard. Another 10µl of 5µg/ml of B_1 was spotted on top of one of the two 10µl sample spot as internal standard. This was to check if there was difference in rate of migration of the sample spot and the B_1 standard spot. The chromatoplate was developed in

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chloroform: methanol (95:5v/v) solvent, dried and examined under UV. light.

The R_{fs} of the samples spots were compared with those of the standard spots. Eighteen out of the twenty-nine positive contaminated samples remained positive. The remaining eleven sample extract spots either separated from their internal standards and therefore registered lower R_{fs} or did not show any fluorescence.

h) Confirmatory Test for Aflatoxin B_1

Additional tests were needed to confirm the presence of aflatoxin in the remaining 18 samples. The method used involved treating the suspected aflatoxin containing extract with concentrated hydrochloric acid and water, or with concentrated hydrochloric acid and acetic anhydride to give the acetate. (Official Methods of Analysis, 1970; Jones 1972)

Twenty millilitres of a filtered sample extract were evaporated to dryness on a water bath under a stream of nitrogen and picked up in 3mls chloroform which was transferred to a column for a clean up. The adsorbed sample was eluted with chloroform - methanol (97:3v/v) and the eluate evaporated to dryness on a water bath under a stream of nitrogen. The cleaned residue was dissolved in 250ul of chloroform and the solution applied to T.L.C. plate

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(kiesel gel G) using 10µl spots at 5mm interval. Five microlitre spots of standard aflatoxin B_1 solution were placed at each end, 1cm beyond the initial and final spots. The plate was developed in a chloroform: methanol (95:5v/v) solvent, dried and examined under UV. lamp in a dark room. The area containing aflatoxin B_1 was carefully outlined with a needle. The silica gel was scraped from the marked area and washed thoroughly with chloroform: methanol (2:1v/v) to remove the aflatoxins. The mixture was filtered and the filtrate evaporated to dryness on a water bath under a stream of nitrogen. The residue was dissolved in 250µl of chloroform and 100µl was transferred to each of two small tubes A and B. Ten microlitres of water and a drop of concentrated hydrochloric acid was added to tube A while 250µl of acetic anhydride and one drop of hydrochloric acid were added to tube B. Tube A and B were covered with foil lined caps and were both shaken vigorously. They were heated for ten minutes in a water bath, shaking occasionally. The solutions were then evaporated under a stream of nitrogen on a water bath.

For the control, 100µl aflatoxin B_1 standard was used to prepare each of the derivatives. Each derivative was dissolved in 20µl of chloroform. Ten microlitres of

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FIGURE 5b

Thin-layer chromatogram of Aflatoxin B_1 derivatives obtained during Confirmatory tests for Aflatoxin B_1 in kokonte flour sample extracts

For Figure 5b

(a) - Tube A reaction products (Sample extract)

(b) - Tube A Control (Aflatoxin B_1)

(c) - Tube B reaction products (Sample extract)

(d) - Tube B Control (Aflatoxin B_1)

(e) - Kokonte flour sample extract

(f) - Aflatoxin B_1 standard

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FIGURE 5b

CHEMICAL CONFIRMATORY TESTS FOR THE PRESENCE OF AFLATOXIN B_1 IN KOKONTE SAMPLE EXTRACTS.

Photograph taken at the end of the experiment showing thin-layer chromatogram of sample extracts and Aflatoxin standard B_1 derivatives.

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each derivative was spotted on a silica gel chromatoplate in the following sequence: Tube A reaction product, Tube A control, Tube B reaction product, tube B control. A spot from the extract and standard were also added and the plate developed in chloroform: methanol (95:5v/v) solvent. When the developed plate was examined, it was found that the spots of the reaction products of the derivatives compared in size, intensity and R_f with the spots of the control derivatives. Spots of Tube A and Tube B control gave bluish spot of R_f 0.72 while tube B and Tube B control each produced two bluish green spots of R_{fs} 0.50 and 0.73.(Fig 5b)

This test confirmed the presence of aflatoxins in the remaining eighteen samples.

i) Quantitative Analysis of Aflatoxin B_1 In Sample Extracts:

Ten micrograms per millilitre concentration of aflatoxin B_1 solution was serially diluted to give working standard of 10.0µg/ml, 7.0µg/ml, 5.0µg/ml, 3.0µg/ml and 1.0µg/m. concentrations. These were prepared before each quantitative estimation.

Twenty millilitres each of the extracts of the 18 remaining samples were evaporated to dryness on a water bath, under a stream of nitrogen. The residue was picked

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up in 3mls of chloroform and transferred to the column. The adsorbed aflatoxin was eluted with 40mls chloroform: Methanol (97:3v/v) solvent. The eluate was evaporated to dryness under a stream of nitrogen on a water bath. The cleaned residue of the sample extract was picked up in 1ml chloroform and 3µl, 5µl and 10µl of the cleaned sample were spotted successively on silica gel G chromatoplate. Two microlitres, 5µl and 10µl of each of B_1 standard of 10.0µg/ml, 7.0µg/ml, 5.0µg/ml, 3.0µg/ml and 1.0µg/ml were spotted along with the sample extract on the same chromatoplate. The plates were developed in chloroform: methanol (95:5v/v). The spots were observed under UV. lamp in a dark room and the fluorescence intensities and R_f of the sample spots were compared with the standard B_1 spots. The sample spot matching those of the standards spots in intensity were visually estimated and noted.

j) Calculation of Aflatoxin B_1 concentration In Sample Extracts:

The concentration of aflatoxin B_1 in the contaminated samples in µg/kg was calculated from the formula:

$$\text{Aflatoxin } B_1 \text{ content } (\mu g/kg) = \frac{S \times Y \times V}{W \times Z}$$

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where S = µl of aflatoxin B_1 standard equal to that of material being evaluated on plate

Y = Concentration of aflatoxin B_1 standard in µg/ml.

W = Weight in grams of original sample contained in final extract

Z = µl of sample extract spotted to give fluorescence intensities equal to S, the B_1 standard

V = µl of solvent required to dilute final extract.

C. Animals and Diets

a) Rats

Male hooded weanling rats were kept for seven days on normal rat diet. The rats weighing between 33 to 40 gms were distributed into two groups of four rats in such a way that the average weights was the same for each group. The rats were kept separately in a cage. The groups were put on control diet 1 and experimental diet II. Daily food intake was recorded per rat per group. Each rat had free access to water and was weighed every other day.

In preparing the experimental diets, the cassava flour used in diet I was pooled samples of the laboratory prepared control cassava flour which was found not to have

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been contaminated with aflatoxin. The cassava flour used in diet II was pooled samples of the remainder of the 18 samples shown to be contaminated with aflatoxin, the content being estimated to be 761µg/kg.

The Composition of the diets is shown in Table 3

TABLE 3

Percentage Composition of Diets

	Control Diet I gms	Experimental Diet II gms
Dried Skimmed Milk ..	45.7	45.7
Groundnut Oil	14.0	14.0
Sugar	10.0	10.0
Mineral Mixture ..	4.0	4.0
Cod Liver Oil	1.0	1.0
Yeast	1.0	1.0
Cassava Flour	24.3	24.3

The flour was treated in the same way as normally prepared in Ghanaian homes before eating. One hundred grams of each diet was weighed out and stirred into 200mls of boiling water for 5 minutes. The sticky mass of food was then cooled, cut into cubes and kept in labelled polythene bags in the refrigerator. The cubes were weighed and given to the rats each day. The experiment was carried

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out for 25 days because of the shortage of the control diets. The rats were anaesthsized with chloroform and blood collected by cardiac puncture. The samples were centrifuged to obtain the serum which was kept at $-20^{o}C$ in the deep freezer. Alkaline phosphatase levels were later determined on the serum samples. The state of the heart, liver, kidneys spleen and body cavity was observed and noted.

b) Determination of Alkaline Phosphatase:

Alkaline phosphatase levels in rat serum samples were determined utilising the method of Wooton (1964). This determination utilises the fact that in the presence of alkaline oxidising agent, 4-amino-antipyrine gives a red purple colour with compounds containing phenolic groups. Colour development although rapid is also stable for at least an hour out of bright light.

Reagents:

Bicarbonate buffer pH 10 (.01M)

Substrate buffer, disodium phenyl phosphate - 0.01M

Stock phenol standard solution 10mg/litre

Sodium hydroxide - 0.5N

4 amino antipyrite - 6gm/litre

Potassium ferricyanide - 24gm/litre.

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One millilitre of buffer was placed in a 10ml test tube followed by lml of phenyl phospate substrate. The tube was placed in a water bath at 37°C for three minutes. At the end of this period, 0.lml of serum was added and incubated for a further three minutes. The reaction was stopped by addition of 0.8ml of 0.5N NaoH, A control(c), a standard(s) and a blank(b) were set up for each serum sample. The control contained lml buffer, lml phenyl phosphate substrate and 0.8ml of NaoH mixed together in a test tube followed by 0.lml of serum. The standard contained 1.lml of buffer, lml of phenol standard and o.8ml of 0.5N NaoH mixed together. The blank also contained 1.lml of buffer, lml of water, 0.8ml of 0.5N NaoH, all mixed together. At the end of the incubation period, 1.2ml of 0.5N sodium bicarbonate, followed by lml amino antipyrine solution and lml potassium ferricyanide solution were added to each mixture, shaking each tube after each addition. Each sample was measured at 510nm. 10µg of phenol was present in standard, therefore phenol produced in test was

$$\frac{\text{Test - control}}{\text{Standard-blank}} \times 10$$

Hence 100mls of serum will liberate

$$\frac{\text{T - c x 10mg}}{\text{S - B}} \text{ phenol}$$

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Since 1 King Amstrong unit is the production of lmg of phenol in 15 minutes under conditions of reference alkaline phosphatase.

$$\text{K A Units/100ml} = \frac{T - C}{B - B} \times 10$$

N.B.

Silica gel G, developed in Chloroform-Methanol (95:5) separates the Aflatoxins into two spots of Aflatoxins B and Aflatoxins G. It is assumed in this study that Aflatoxins B_1 and B_2 have the same fluorescent intensity per unit weight and Aflatoxin B_1 spots of the standard can be compared to B_2 spots of the extract. Therefore the spots of the sample extracts observed on the chromatograms having the same R_{fs} as the standard Aflatoxin B_1 could be the combination of Aflatoxins B_1 and B_2. Consequently, Aflatoxin B_1 as used in this study pre-supposes the presence of Aflatoxin B_2 as well.

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CHAPTER III

RESULTS

A. (a) Survey

A survey was carried out to collect background information on the production, processing, marketing and storage of kokonte. This work was carried out in order to monitor sources of contamination to the cassava chips. The survey covered all the eight major markets in Accra. Accra was chosen for the survey because most of the commercial producers and middlemen send their products from all over the country to be retailed in Accra. Fifty sellers consisting of forty-nine females and one male were interviewed. All of them obtained their products from different sources. Thirty of the subjects interviewed said they only retailed the commodity while the remaining twenty produced as well as retailed. Thirty-eight sold the kokonte flour alone, ten sold only the chips while the remaining two sold both the chips and the flour.

Table 4 gives the distribution of subjects among the markets.

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TABLE 4

Table Showing Location of Market and Subjects Interviewed

Name of Market	Location	No. of People Interviewed
London ..	Accra Central	2
Makola ..	Accra Central	6
Salaga ..	Accra Central	6
Mamprobi ..	Accra West	4
Kaneshie ..	Accra West	17
Malata ..	Accra North	6
Osu ..	Accra East	5
Tema ..	Tema District Council Area	4

The survey took about one and half months to complete. A few problems were encountered during the course of the survey. Most of the sellers were suspicious of the exercise and refused to give their identities - only seven out of the fifty people interviewed gave their names.
A lot of people always clustered around the subjects being interviewed and tried to explain the questions in their own way, thus confusing the exercise at times. Other people did not want to co-operate and some refused to answer the questions but did not mind selling samples for analysis.

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Most of them had little or vague ideas about details of the processing methods.

b) Marketing

Fifty-six per cent of the sellers interviewed answered that they obtained their commodities from the markets, meaning suppliers brought the chips to the respective markets from elsewhere. Forty per cent of the sellers produced the chips themselves while the remaining four per cent bought them outside the markets. Fifty per cent of the sellers obtained their commodity through middlemen. Analysis of the answers showed that 28 per cent of the kokonte chips was produced in the Eastern Region, 21 per cent in the Brong-Ahafo Region, 14 per cent in Ashanti Region and 7 per cent from each of the following Regions: Upper, Volta, Greater-Accra and Central Regions. There were no samples obtained from the Western Region.

c) Processing

The type of cassava used for the production of kokonte was found to be the Ankra or ordinary type. Forty per cent of the sellers answered that their kokonte was prepared using fresh cassava. Thirty-two per cent answered that the cassava used were old discards, bad ones, or left-overs from sale while twenty-eight per cent did not know the state of the cassava used for the preparation.

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The results also showed that most of the producers, 82 per cent dried their chips between 1 - 10 days, while twelve (12) per cent dried chips for 11 - 15 days. 16 per cent of the sellers did not know the duration of the drying process. The majority of the sellers (92 per cent) answered that the chips were normally spread out to dry and were mixed uniformly everyday or removed altogether indoors on rainy days. The remaining 8 per cent did not know what was done. The sun was utilised for the drying process and this was done where there were open spaces, for example, on roof tops, road sides, and on tabl tops, away from rodents, reptiles, goats and other pests. Drying by application of heat was also mentioned. Sheds were constructed at different levels considering the quantity of chips to be dried. Fire is prepared under the sheds and the heat together with the smoke cause the chips to dry quickly. Quality of the chips was determined by breaking. If the chips are good and dry, they are normall brittle, hard and easily broken. They are also fluffy inside and of uniform colour. The bad ones are generally soft and rotten, mouldy and infested with insects.

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40 per cent of the sellers interviewed said they removed the bad ones before milling the chips into flour since mouldy chips normally gave dark colouring to the flour which was considered unacceptable for food.
About 60 per cent of the subjects said they did not normally remove the bad ones as these made up only a small proportion of the bulk of the product.

d) Storage

Most of the sellers (74 per cent) stored their commodities between 1 - 7 days. 14 per cent had stored them for 8 - 14 days while the remaining 12 per cent had stored them between 15 - 90 days. Once the chips were dry, they could be stored for up to six months and made into flour anytime the flour was needed. The flour could be stored for a long time provided it was dried periodically. Six per cent of the subjects interviewed said they usually stored the flour for only a month or two. The kokonte chips were normally stored in large jute bags and left in the markets under sheds. They could be stored under this condition for a long time provided the chips were of good quality before packaging. The flour was stored in aluminium or enamel bowls. For storing, the flour should

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not be pressed too hard when warm, otherwise it caked up. It should also be dry to prevent mould growth which is considered undesirable because it produces bad chips whic subsequently give darker flour. Buyers normally judged the quality of the chips by breaking them to see if they are brittle. The quality of the flour is determined by colour, texture, smell and dryness. There are three types of flour sold at the markets; these are named according to their colour. The method of processing and brightness of the sun determine the final colour of the product. There are red, white and black types of kokonte at the markets and samples of each type were purchased an used for the analysis.

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ANALYTICAL RESULTS

A. a) Moisture Content of Kokonte Flour Samples

Ranges, mean standard deviations for moisture content and distribution of the kokonte samples under specific moisture ranges are shown in tables 5a and 5b.

TABLE 5a

Table Showing the Ranges, Mean Moisture Content and Standard Deviations of Various Kokonte Flour Samples

Sample	No	Mean $\pm$ SD %	Ranges %
Control	20	10.50 $\pm$ 0.38	10.20 - 11.50
White	10	12.24 $\pm$ 1.68	11.46 - 16.96
Black	20	12.05 $\pm$ 1.80	10.68 - 13.35
Red	50	12.07 $\pm$ 1.09	10.19 - 14.68

TABLE 5b

Table Showing Distribution of Samples Into the Various Moisture Ranges

Sample	No. of Samples	Number of Samples with Moisture Content Shown In Percentage						
		10.0-10.9	11.0-11.9	12.0-12.9	13.0-13.9	14.0-14.9	15.0-15.9	16.0-16.9
Control	20	18	2	-	-	-	-	-
White	10	-	6	3	-	-	-	1
Black	20	3	6	8	3	-	-	-
Red	50	5	12	20	8	4	1	-
Total	100	26	26	31	11	4	1	1

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Eighty-eight per cent of the market kokonte flour samples was found to have percentage moisture content ranging from 11.0 - 14.0% which falls within the moisture ranges of 7-14% recommended for storage of most grains and some oil seeds.

B. a) Screening of Kokonte Flour Samples for Aflatoxin:

Results obtained from the screening of 100 kokonte flour sample extracts for the presence or absence of aflatoxin are shown in Table 6.

TABLE 6

Table Showing the Presence or Absence of aflatoxin in Kokonte Flour Sample Extracts

SAMPLE	NO. OF SAMPLES	NEGATIVE AFLATOXIN	POSITIVE AFLATOXIN
White	10	10	-
Black	20	16	4
Red	50	25	25
Control	20	20	-
Total	100	71	29

A total of 29 samples gave positive results

This was confirmed in 18 out of the 29 Samples

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Fourteen of these were from red kokonte group with the remaining four coming from the black group.

Results obtained for the determination of aflatoxin concentration in the remaining eighteen confirmed samples are shown in Tables 7a and 7b.

TABLE 7a

Distribution of Aflatoxin Concentration in the "Positive" Kokonte Flour Samples that gave Positive Results in the Qualitative Test

Aflatoxin Concentration(μg/kg)	Black Samples	Red Samples	Control Samples	White Samples
200 - 400	-	4	-	-
401 - 600	-	-	-	-
601 - 800	3	5	-	-
801 - 1000	-	-	-	-
1001 - 1200	-	-	-	-
1201 - 1400	1	3	-	-
1401 - 1600	-	2	-	-
Total	4	14	-	-

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TABLE 7b

Ranges Mean and Standard Deviation of Aflatoxin Concentration in the "Positive" Kokonte Flour SAmples

Sample	No.	Ranges (µg/kg)	Mean µg/kg	SD
Black	4	762.5 - 1272.5	890.0	255.0
Red	14	255.0 - 1525.0	843.0	462.0

The actual aflatoxin concentration as obtained from each positive sample is illustrated in the histogram in Figure 6.

C. RESULTS OF ANIMAL EXPERIMENT

1. Food Intake:

As previously indicated, two different diets were fed to two groups of four rats; an experimental diet containing aflatoxin contaminated kokonte flour and a control diet containing no aflatoxin. Records of intake showed that the daily average food intake of the two groups did not vary during the first twelve days of the experiment. The amount eaten per rat per day was around 16 grams. The experimental group increased their average food intake from 16 grams per rat per day to 19 grams per rat per day after the

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twelveth day and maintained this level of intake to the end of the experiment. The control group increased their food intake around the same time from 16 grams per rat per day to 22 grams per rat per day and maintained this level of intake till the end of the experiment.

At the end of the experiment, the total food eaten by the control group was 504.0 grams and that of the experimental group was 457.0 grams.
The calculated total amount of aflatoxin in the final amount of food eaten by the experimental group was 63 µg.

Figure 7 shows the cumulative average food intake of the two groups of rats during the experimental period.

II. Growth

Weight measurements taken of the two groups of rats during the experimental period showed that growth of both groups remained the same from the beginning of the experiment up to the 10th day, as shown in Figure 8, after which the control groups proceeded to overtake the experimental group. The experimental group trailed behind till the end of the experiment. The average weight gained by the control group was found to be 8o grams as compared with 58.6 grams gained by the experimental group.

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FIGURE 6

A HISTOGRAM SHOWING CONCENTRATION AS OBTAINED FROM EACH 'POSITIVE' SAMPLE

Figures B_1 - B_{13} refer to Sample numbers of black kokonte flour Samples.

Figures R_1 - R_{47} refer to Sample numbers of red kokonte flour Samples.

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FIGURE 6

ILLUSTRATION OF CONCENTRATION OF AFLATOXIN IN BLACK AND RED KOKONTE FLOUR SAMPLE

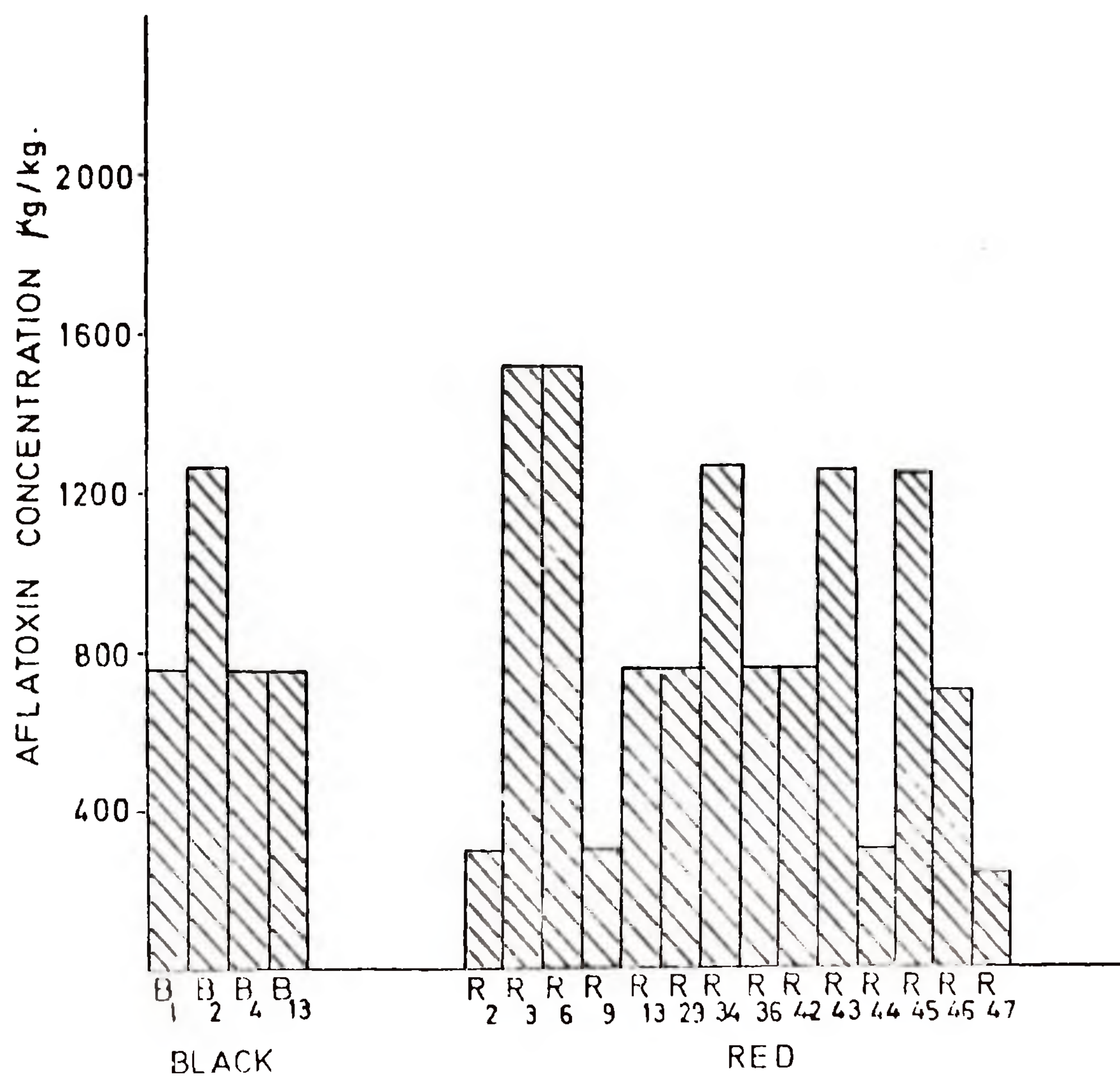

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FIGURE 7

Two different diets were fed to two groups of four rats. The diet for the experimental group contained aflatoxin contaminated kokonte. The diet for the control group contained no aflatoxin.

For Figure 7

•——•——•——•——• Experimental Group

x-----x-----x-----x-----x Control Group

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FIGURE 7

THE EFFECT OF FEEDING AFLATOXIN CONTAINING KOKONTE FLOUR ON GROWTH OF THE RATS

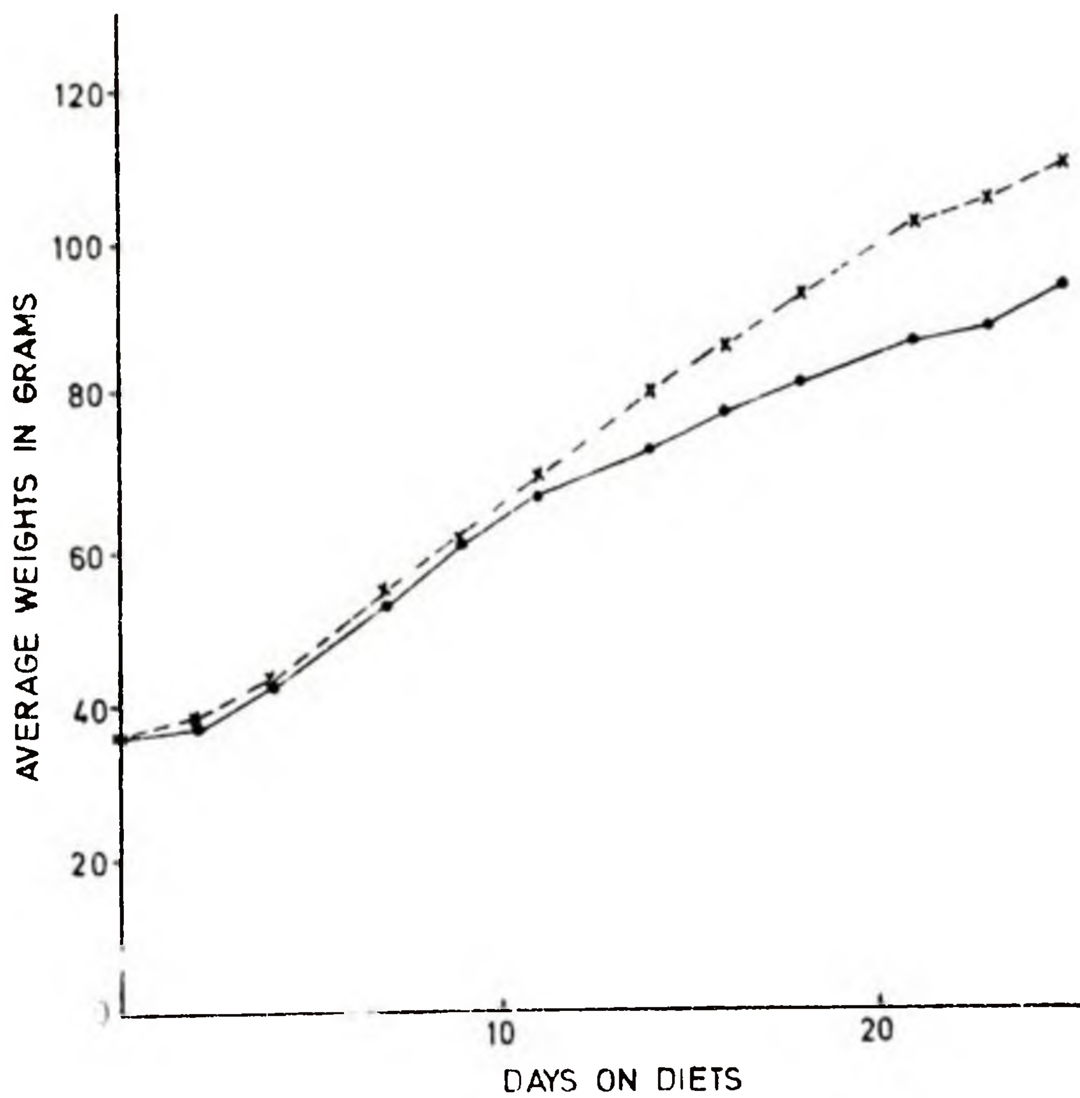

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FIGURE 8

CUMULATIVE FOOD INTAKE BY THE TWO GROUPS OF RATS

For Figure 8

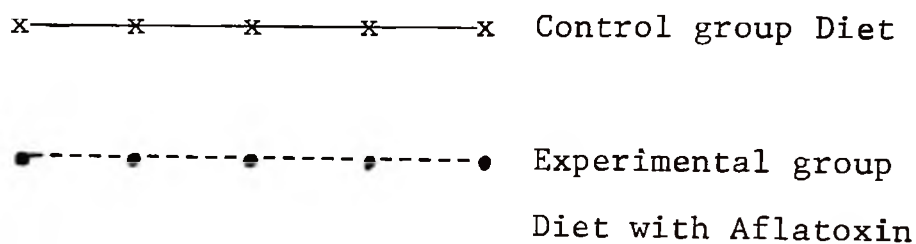

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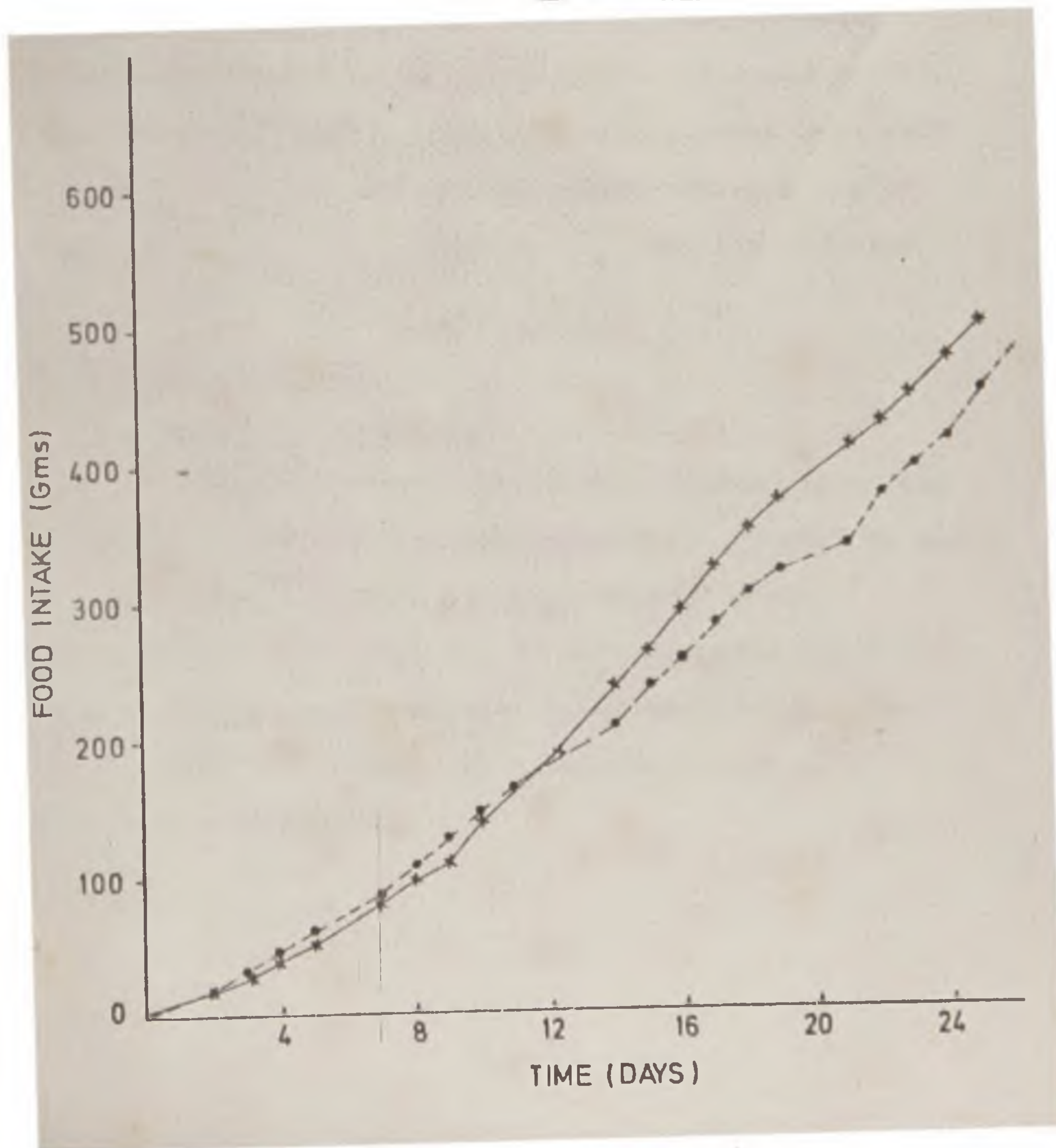
FIGURE 8

CUMULATIVE FOOD INTAKE BY THE TWO GROUPS OF RATS



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III . General Observations:

Post-mortem examinations made on the rats from both groups showed that the heart, spleen, adrenal, urogenital tract and intestinal tracts appeared normal. The livers from the experimental rats however were pale and very fatty with a brownish nodular area on one of them. The livers from the control group did not show any obvious paleness.

D. BIOCHEMICAL FINDING

I. Alkaline Phospatase:

Alkaline phosphatase levels determined in the serum of each of the rats are tabulated in Tables 8a and 8b. They were higher in the experimental group (150 ± 34 KA units) than in the control group (70 ± 10 KA units). Both results however lie within normal ranges for the rat. The individual levels are shown in a histogram in Figure 9.

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TABLE 8a

SAMPLE	OPTICAL DENSITIES TEST - CONTROL (T - C)	RESULTS (K.A. units)
1a	37	84.00
1b	26	59.00
1c	31	70.45
1d	30	68.18

Mean Alkaline Phosphatase level for

Control group = 70 ± 10.32

TABLE 8b

SAMPLE	OPTICAL DENSITIES TEST - CONTROL (T - C)	RESULTS (K.A. units)
2a	76	172.72
2b	68	154.54
2c	76	172.72
2d	44	100.00

Mean Alkaline Phosphatase levels for

Experimental group = 150.00 ± 34.41

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FIGURE 9

EFFECT OF AFLATOXIN CONTAINING DIET ON RAT SERUM ALKALINE PHOSPHATASE

Figures 1a - 1d refer to Control group individual rat alkaline phosphatase levels.

Figures 2a - 2 d represent experimental group individual rat alkaline phosphatase levels.

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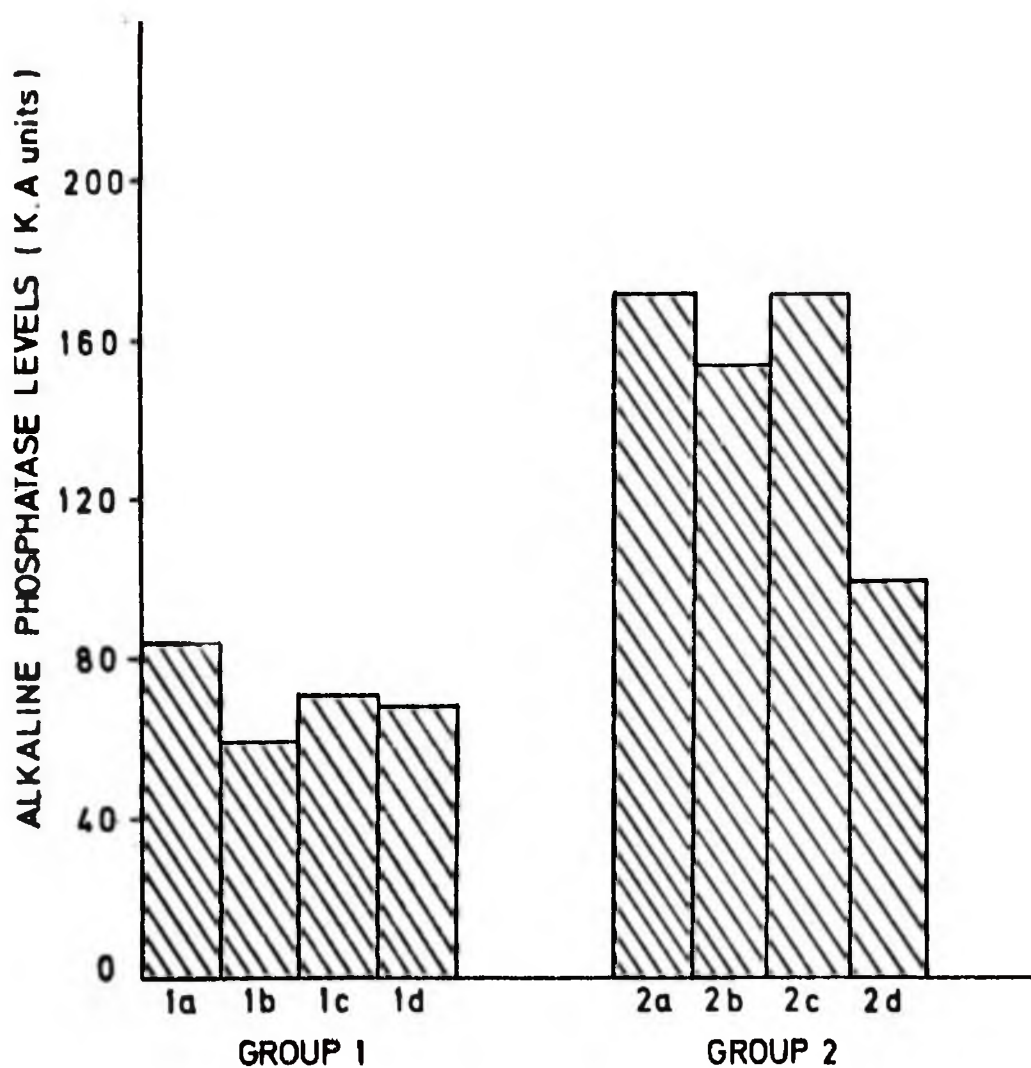
FIGURE 9

EFFECT OF FEEDING AFLATOXIN CONTAINING DIET ON RAT SERUM ALKALINE PHOSPHATASE



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CHAPTER FOUR

DISCUSSION

Processing Method and Mould Growth:

From the viewpoint of public health, one of the most remarkable contributions to the aflatoxin literature is the topical survey by Barnes (1970), who pointed out that there is little point in trying to analyse food eaten at any particular time in order to find out whether it contains aflatoxin because changes play too great a part in determining the degree of contamination. Information must be obtained at a much earlier stage with adequate survey of food harvesting and storage, so that the population that may be most heavily exposed to food regularly or intermittently contaminated with fungi may be identified.

Some interesting facts from the survey conducted on traditional processing methods of kokonte in this study were revealed. It was observed that three types of kokonte flour were sold at the markets of Ghana namely red kokonte, black kokonte and white kokonte flours. There were however intermediate shades of these colours. The red type was the most abundant being sold in the market. During the processing of this type of kokonte,

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mild heat with smoke drying was practiced and the application of dry heat helped in the development of the brown colour. Grace (1971) reported that when cassava roots were not sorted, peeled and washed, the chips were usually brown in colour. This was not confirmed during the survey.

The black type of kokonte chips were sundried but poor drying conditions, especially when there was not sufficient solar energy or when rain fell on the chips, created the condition for mould growth with a resulting darkening of the product.

It is interesting to note that none of the processors of cassava chips interviewed considered the presence of moulds on the chips as toxic. They did not like moulds because the moulds were responsible for the darkening of the end product. The white type of kokonte was the type not produced in large commercial quantities. The chips were usually sundried but in this case care was taken to prevent mould growth.

Any of the varieties of cassava grown in the country could be used for kokonte production. It takes on the average up to ten days to thoroughly dry the kokonte chips although a few people quoted up to fifteen days, the longer time being attributed to lack of adequate sunshine.

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Clerk and Caurie (1968) reported five days of drying time in sunlight. Grace (1971) gave two to three sunny days as the drying period. Rawnsley (1969) made it up to 20 days depending on the weather. Ingram and Humphries (1972) in their review on cassava storage gave three to ten days as the usual drying time but gave one to two days in ideal weather.

Properly dried chips were easily broken and this was found in this study to be the method used by the processors to test the degree of dryness of the product.

Moisture Content:

The moisture content to which the chips were normally dried varied between 12 to 16%. Grace (1971) gave moisture content of well dried good quality chips to be between 13 - 15 per cent. Clerk and Caurie (1968) gave a figure of 16%. Rawnsley (1969) reported the moisture content required for chips to be stable, to be 12 per cent which coincided with the mean moisture value obtained for the samples analysed in this study. The traditional method of breaking open the chips to test for dryness could therefore be reasonably assumed to give accurate indication of moisture levels.

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Aflatoxin Determination:

Visual estimation of aflatoxin on thin layer plates under ideal conditions had been reported to have a precision range of ± 20 per cent. If conditions were not ideal, the precision had been known to be about 30 per cent. Even under ideal conditions, the precision of visual measurements of aflatoxin were conditioned by limitations of observer's acuity. Beckwith and Stoloff (1968) gave data which established the visual estimation at ± 20 to 28%, under ideal conditions. Calculations of the estimation errors in four visual aflatoxin procedures (de longh *et al*, 1964, Nesheim, 1964; Pons *et al*,1966; Eppley, 1966;) gave measurements of ± 30 to 50%, when a given unknown was judged to match with one or two adjacent standards.

Campbell and Funkhouser (1966) reported a collaborative study by 13 laboratories on the recovery of aflatoxin B. They concluded that at a contaminated level of 10 µg/kg, aflatoxin tended to be over estimated whereas at high levels, the recovery was about 75%. Pons (1969) also reported a collaborative study by 12 laboratories on estimation of aflatoxin B. The average recovery by visual estimation on thin layer plates was found to be 85%.

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The method used for the determination of aflatoxin in this study gave an average recovery by visual estimation on thin layer plates of about 80 per cent and could not detect anything less than 1µg/ml concentration. Out of the 100 samples analysed only 18 samples gave positive aflatoxin contamination. It might be possible that many of the samples considered to be negative might have contained aflatoxin levels either too low to be detected because of the factors discussed above or because of the limitations of the method used.

During the aflatoxin determination on thin layer plates in this study an aflatoxin-like factor of slightly lower Rf value was also found to be present. A similar factor with blue fluorescence had been reported by Nagarajan et al (1973) to be present in heavily mouldy cassava flour samples. De longh *et al* (1964) also reported that a lot of samples suspected to contain aflatoxin because they were heavily mouldy contained another fluorescent compound with slightly lower R_f. There is a naturally occuring blue fluorescent compound in cassava and cocoyam of slightly lower R_f than aflatoxin. This compound is called scopoletin (T.P.I. 1972).

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This might be the factor being discussed above or another factor altogether that needs to be investigated.

Dickens and Welty (1967) also noted that there is a high correllation between aflatoxin content and presence of *Aspergillus flavus* mould. Although our black samples were very mouldy, only 27% contained aflatoxin.

Aflatoxin Levels:

Regulatory agencies in certain countries have established acceptable tolerance levels for aflatoxin in peanut and peanut products, and in other crops harvested or stored under conditions likely to result in fungal attack. These tolerance levels currently in the range of five to 30 μg per kg, are based on toxicological considerations and on limits of sensitivity of analytical methods, employing principally, thin layer chromatography.

The protein advisory group of the United Nations (FAO/WHO/UNICEF) also concerned with the problem of aflatoxin recommended that for protein supplement consumed by infants, the aflatoxin limit must be set at 30 μg/kg but that this could be reduced further with improvements in agricultural practices.