THE EFFECT OF DIET ON THE METABOLISM OF AFLATOXIN IN THE MAMMAL

A THESIS

Presented By

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A B S T R A C T

The metabolic fate of aflatoxin is of interest in connection with studies related to the mode of action of hepatotoxins in the induction of liver tumours. The ability of the toxin to interact with sub-cellular particles and the rate of excretion of the carcinogen by a particular species, may be important in interpreting its gross effect on an animal.

In some studies described in this thesis, the rate of metabolism of drugs in the normal rat and in the poisoned animal has been compared. It is a more useful information when the effect of a drug on animals under different dietary treatments are evaluated, since this will reflect susceptibility to those drugs by animals with nutritional deficiencies.

Evidence is here presented to show that rats on low protein diets are more susceptible to aflatoxin-poisoning, because they are unable to metabolise the drugs as rapidly as rats on high-protein diets. This finding is supported by histological evidence.

In order to facilitate the identification of metabolic products of the aflatoxins, use was made of C^{14}-labelled aflatoxin. This material was produced by incorporation of labelled isotopes into cultures of Aspergillus flavus on Czapek-Dox media. The utilisation of Sodium Acetate-1-C^{14}, Sodium Acetate-2-C^{14}, in the biogenesis of the aflatoxins is reported.

After a given dose, aflatoxin or its metabolites were absent from the heart and muscles of animals examined.

The other major part of the work described in this thesis consists of studies on the metabolism of labelled aflatoxins in mammals fed

on high or low-protein diets. Urine and bile samples obtained from experimental rats and rabbits were analysed for the presence of aflatoxins or its metabolites. Bile samples were obtained after the establishment of biliary fistulae. For the collection of urine samples, from animals under light anaesthesia, diuresis was stimulated by implantation of a polyethylene cannula into the external jugular vein followed by an infusion of 5% mannitol in saline at 0.75 ml per minute for rabbit and 0.2 ml per minute for rat.

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CHAPTER ONE

I N T R O D U C T I O N

(i) Discovery of Aflatoxin.

Legumes are used as common ingredients in animal feeds as well as in food for human consumption. These grains are susceptible to attack by a wide variety of fungi in the soil or after harvest. Forgacs, Koch, Carll and White-Stevens (1962) have warned that increased attention must be given to diseases of animals and men caused by fungi that have grown and produced toxic substances in foods. The "poisoning of the host following entrance into the body of toxin(s) of fungal origin" has been described by J. Forgacs (1962) as "Mycotoxicosis".

Farmers have always been doubtful about the wisdom of feeding mouldy foodstuffs to their animals. Nevertheless, illness and mortality caused by such practices have been cited in the

literature. A more recent example is the disease produced by peanut meal contaminated by a strain of Aspergillus flavus (Burnside, Sippel, Forgacs, Carll, Atwood and Doll, 1957); Stevens, Saunders and Spence (1960), Smith (1960). This disease was later shown to be caused by aflatoxin, a metabolite of the fungus.

(ii) Occurrence in Natural Products.

Stevens, Saunders and Spence (1960) reported occurrences of a disease among young turkey in forty-five different farms in Great Britain. It was estimated that about 100,000 turkey died during this period when imported groundnut was used as supplement in animal feeds (Blount, 1961; Gibson and Harris, 1961). In a particular case, one turkey farmer divided his intake of poults into two halves, kept side by side, but fed on different brands of food. One batch remained

perfectly healthy throughout and the other suffered a severe attack (Smith, 1960). At this time it was not possible to associate any micro-organism or virus with this disease, since all attempts by different laboratories to identify the causal agent(s) were unsuccessful. It was, however, concluded that the disease probably originated from a preformed toxin. This suggestion was contained in the report of an Inter-departmental Working Party on Groundnut toxicity. (Tropical Product Institute Report, 1962).

Groundnut is imported into Great Britain from tropical countries, such as Brazil in South America; Uganda and Rhodesia in East Africa; Senegal, Gambia and Nigeria in West Africa. In these 'producing countries' the seed is planted in loose clay soil and it matures within 140 to 150 days into a large seeded runner, with dark green foliage. Groundnuts are usually considered ready for harvest when the leaves turn yellow (Smartt, 1960). After harvest, the plants are stacked together until the haulms

are dried and the kernels begin to rattle in the shells. This process is called curing. The nuts are then threshed from the haulms and dried in the sun. McDonald and Brook (1963) suggested that the use of artificial driers would be more effective in preventing fungal attack at this stage, since the moisture content of the nuts could thus be brought below 8 per cent. During storage, however, the moisture content of stored products may rise, hence adequate precaution is necessary to ensure the quality of these materials.

A number of Aspergillii species were found by Deiner and his associates (1960) in the microflora of mouldy nuts. The presence of Aspergillus flavus in stored groundnut materials has been confirmed by Jackson (1964). Fig. 1 shows photomicrographs of infected groundnuts, first published by Spensley (1963). McDonald and Brook (1963) regarded temperature and moisture content of stored groundnuts as two important factors which control fungal growth.

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Fig.1: Photomicrographs of infected groundnuts (Spensley, 1963). The fungi attach the seed coat and penetrate into the cotyledons. The upper picture shows a higher magnification (X25) of mould infested nut. The other picture shows low magnification (X2.5) of a group of infected nuts.

Fig 1.

Damage to the pods or insect attack may also facilitate deterioration.

Clegg and Bryson (1962) examined samples of produce from Brazil, Uganda and Tanganyika. A consignment of groundnuts from Uganda was found to be heavily contaminated with fungi. W.P. Blount (1961) had earlier suspected Brazilian groundnut as the causal agent of outbreaks of 'turkey-X-disease'. K. Sargeant and his collaborators (1961) obtained pure cultures of some of the fungal species present in these samples. Eight isolates were grown for seven days at 27°C on Czapek's solution agar. Chloroform extracts of these cultures were tested for biological potency and the toxin producing fungus was thus identified as Aspergillus flavus Link ex Fries. This fungus was later isolated from other sources than groundnut and was associated with outbreaks of the new disease of farm animals called 'turkey-X-disease'. In view of its origin the toxin was named AFLATOXIN (Tropical Product Institute Report, 1962).

The main interest of research workers on aflatoxin at this time was to study the incidence of fungal attack on groundnuts in the 'producing countries' and to suggest methods of preventing future epidemics. Investigations on methods of detection of toxic nuts and of making toxic meals safe for consumption were encouraged.

McDonald and Harkness (1964) working with a team of research workers at the Samaru experimental station in Northern Nigeria, observed that when pods were hand-picked from the haulm and sun dried in a layer, with protection from rain, the rate of drying was high and the crop was toxin free. Dickens and Pattee (1966) also noted that aflatoxin was not likely to develop during curing if recommended practices were followed.

(iii) Biological Assays.

Reports from farms and feeding trials in the laboratories have indicated that many farm animals are susceptible to 'turkey-X-disease', but some are more so than others (Asplin and Carnaghan, 1961). Among the large farm animals, pigs from three to twelve weeks old and pregnant sows are the most commonly affected (Loosemore and Harding, 1961). Calves from one to six months of age are highly susceptible but becoming more tolerant with age (Loosemore and Markson, 1961). In a long-term feeding trial, heifers were as clinically affected as monkeys (Tulpule, Madhavan and Gopalan, 1964) while sheep are comparatively resistant.

Siller and Ostler (1961) described the clinical and macroscopic features of diseases in turkey poults fed on mouldy groundnuts. The histological changes are primarily hepatic and showed remarkable consistency. These were summarized by Loosemore and Markson (1961)

as follows:

"(i) there is considerable proliferation of bile duct epithelium to form numerous structures resembling small bile ducts,

(ii) chronic obliteration of centrilobular and hepatic veins, and

(iii) a wide variation in size and shape of parenchymal cells, many of which contain abnormally large, coarse and densely baso-phylic nuclei and finally

(iv) diffuse fibrosis which disrupt lobular structures."

(a) Duckling Test.

This biological test was developed at the Central Veterinary Laboratory, Weybridge, England and can be used for qualitative and quantitative assessment of toxic groundnut meals and extracts.

The test depends on the rapidity with which the proliferation of bile duct epithelium occurs in ducklings after ingestion of aflatoxin. The intensely basophilic properties of these cells when stained with haematoxylin and eosin render histological examination and confirmation of disease more rapid and easier than in turkey poults and chickens.

In the 'duckling test', aqueous extract, or suspensions of toxic samples, are administered individually to groups of day-old khaki-Campbell ducklings. The sample is introduced into the lower part of the oesophagus through a thin polythene tube attached to an hypodermic syringe. After dosing, the birds are kept away from their food and water for one hour to avoid regurgitation into their common food supply. This procedure is repeated for five consecutive days. Survivors are sacrificed on the eighth day. The greater susceptibility of ducklings to aflatoxin poisoning compared to other farm animals is notable.

Klimes and Kruza (1962) observed that ducklings are more susceptible to nitrofurazone intoxication, particularly at two to four weeks of age, when they consume more nitrofurazone per pound of body weight than chickens of a comparable age and size. In field outbreaks of 'turkey-X-disease', the highest mortality was recorded among this age group of birds (Asplin and Carnaghan, 1961). There is, however, no record of food consumption in these reports to justify this assumption. In any case, sensitization of the birds to toxins may depend on a number of factors, one of which is the composition of the diet given to these farm animals.

(b) Egg Test

Noxious influences often have a greater effect, the younger the subject, and maximal effects can then be expected during embryonic development. Tests performed on poultry or other laboratory animals are laborious and expensive, but the introduction of test substances into the yolk of hen's egg is simple. Furthermore, the embryonic development of the chick takes place within a closed egg shell, permitting no elimination. Thus the 'egg assays' may be expected to give results of highest sensitivity. Platt, Stewart and Gupta (1962) used five-day old chick embryos. These were injected with varying doses of aflatoxin extracts dissolved in water and examined after two days. As little as 0.3 µg of the more potent samples caused death of the embryo. The controls were not affected by this treatment. The teratogenic effect of aflatoxin was therefore rated higher than that of β-amino-propionitrile (Morcos and Platt, 1962) or

thalidomide (Kemper, 1962). When aflatoxin B_1 was administered at levels of 0.1 µg per egg, 10 per cent mortality was recorded in 12 days (Diener, Davis, Hayes and Eldridge, 1966), but the teratogenic effect was still detectable over longer periods at doses between 0.01 to 0.05 µg per egg. It was therefore suggested that suspected rations should not be permitted in animal feeds until tests have shown them to be harmless for the most sensitive tissues. The Ministry of Health (1962) in Paris has, however, advised against the use of this method because the chick embryo, lacking a placenta, cannot be compared with the mammalian embryo.

(c) Tissue Culture Test.

Biological assay methods involving the administration of aflatoxin either into the embryo (chick) or into young animals are all indirect procedures for assessing toxicity of materials to tissues. Wolff and Haffen (1951) proposed direct administration of teratogens to the tissues or organs excised and cultivated in vitro. This method enables an organ to be explanted and, at a certain stage of its development, to be placed in direct contact with the toxin. In this way it is possible to localize the toxic action by finding out whether its application to a particular organ can induce specific lesion.

The successful maintenance of cells in tissue culture depends on the availability of a suitable nutrient medium which provides the aminoacids, vitamins and trace elements essential for the growth of cells. Jutesz and Greczi (1964) described a tissue culture test for aflatoxin.

In this assay, methanol extract of infected groundnut was inoculated into calf-kidney monolayers. After 48 hours incubation, toxicity was evaluated by the degree of cell destruction observed in the tissue culture. Toxin at concentration of 0.1 to 0.5 parts per million caused cell destruction up to a dilution of 10^{-4}. The quantity of aflatoxin required for an indication of toxicity was one-thousandth of that used in the egg test and about 10^{-6} of the LD_{50} for day-old ducklings. The nature of the maintenance medium used in this experiment was not stated by the authors in their paper. Nevertheless, the usefulness of this technique as a quick check on toxicity of infected groundnut samples is obvious.

(d) Albinism Test.

One other test that is of interest is the inhibition of development of green colouration in plant cells by fungal metabolites. Shoental and White (1965) pointed out that a solution of aflatoxin (10 mg/ml) inhibited the formation of green colour in cress seedlings. For this test, twenty seeds of cress, Lepidium Sativum were explanted on circles of surgical gauze placed in two-inch petridishes which contained 4 ml of distilled water. Solutions of aflatoxin B_1 (10 mg/ml) or groundnut extract (25 mg/ml) was then used in place of distilled water. The development of colour was delayed in the test preparations, but the controls behaved normally. The occurrence of albinism in these plants is associated with the interaction of aflatoxin with R.N.A., which may alter the genetic characteristic of the organism. This interpretation is in agreement with the view that development of colour in leaves is a gene-controlled character (Koehler and Woodworth, 1938).

(iv) Physico-Chemical Assays.

In an attempt to provide a quick and reproducible method for checking the toxicity of imported groundnuts and animal feed stuffs, a physico-chemical test was developed at the Unilever Laboratories, London in 1962. This test is based on an earlier observation by Allcroft and her associates (1961), that a chloroform extract from a toxic meal gave a characteristic blue fluorescence when viewed under ultraviolet light. This work which was started at the Weybridge Central Veterinary Research Station was later extended in collaboration with the Tropical Product Institute, London. Several extracts were prepared, using different solvents, and screened for toxicity by individual administration to young ducklings. The level of aflatoxin in agricultural products is generally very low, of the order of one part per million. Hence the assay procedure must not only ensure complete extraction of toxin, but must also be very sensitive and capable of

detecting the presence of a fraction of a microgram of the toxin in diets.

The following solvents have been found suitable for the extraction of aflatoxins: aqueous methanol (Nesheim, Campbell, Stoloff and Barnes, 1964), chloroform (Lee, 1965) and hexane-acetone water mixtures (Wogan, 1966). The next advance was the development of a simple micro-method which could be used for the resolution of mixtures of fluorescent metabolites from fungi.

(a) Column Chromatography.

Column chromatographic techniques have been used for the separation of aflatoxin from other fluorescent substances produced by Aspergillus flavus (Sargeant, et al, 1961). Deactivated alumina was found suitable by Allcroft et al (1962) for the chromatography of aflatoxins when petroleum-ether-methanol mixtures were used as solvent. A blue-violet fluorescent band on the column was associated with the toxicity of the groundnut meal investigated.

Chromatography on silica-gel columns revealed a fraction which contained this material and induced proliferation of bile duct in ducklings, (De Iongh, Vles and van Pelt, 1964). The solvent system used by these authors was a solution of two percent methanol in chloroform. Sargeant et al (1961) prepared crystalline aflatoxin from a petroleum ether, methanol water extract. After fractionation on neutral alumina this substance gave an almost colourless product.

A column chromatographic technique can be used as a preparative method for isolation of aflatoxins from other fungal metabolites. But the resolution of aflatoxins B_1 and B_2 or G_1 and G_2, however has not been achieved by this method. The limitations to further fractionation of the aflatoxins are the length of the column and the particle size of the adsorbent. The choice of a suitable solvent is also important. Purification of the aflatoxins has been achieved by paper and

thin-layer chromatographic techniques. (Coomes and Saunders, 1963; Coomes, Crowther, Francis and Shone, 1964).

(b) Paper Chromatography.

Paper chromatography was used by McLarnon (1962), who found that the intensity of the blue fluorescence on paper appeared to be correlated with the toxicity of the samples examined. This was then developed as a quantitative assay procedure by Coomes and Saunders (1963). These authors proposed a descending paper chromatographic technique, using benzene, toluene, cyclohexane, ethanol, water (3:3:5:8:5 v/v) as solvent. Coomes et al (1964) later pointed out that the resolution of the aflatoxins was poor and incomplete by this method. Rf. values were not reproducible on paper chromatography, except on strict adherence to specific conditions such as, time allowed for saturation of tank by the solvent; temperature and grade of paper used. These difficulties can be avoided if a

standard sample is run along with the test. This procedure was adopted by Broadbent et al (1963). An assessment of the amount of toxin present in the sample is made by a technique of serial dilution until visual extinction of the fluorescence occurs. The minimum detectable amount of aflatoxin was stated as 0.1 to 0.2 µg . This method has been criticized by Nabney and Nesbitt (1965), since it is subject to certain errors, such as quenching of fluorescence by impurities accompanying the aflatoxin spot (Lijinski and Butler, 1966).

(c) Thin-Layer Chromatography.

Thin-layer chromatography can be used as a preparative as well as an analytical method. It also has the advantage that the overall time for analysis is considerably reduced. An extract containing aflatoxin is applied onto a plate, which is later developed with two to five percent methanol in chloroform as solvent. The chromatogram is viewed under ultraviolet light at 363mµ .

A complex array of fluorescent compounds is generally present, (Nesbitt et al , 1963). The known aflatoxins comprise four of these components. Two of them emit blue-violet light and the other two give yellow-green fluorescence. On silica gel plates developed in three per cent methanol in chloroform, Asao et al (1963) recorded the following Rf. values for aflatoxins B_1, B_2, G_1 and G_2 :-

Aflatoxins	B_1	B_2	G_1	G_2
Mol. Wt.	312	314	328	330
Rf. Value	0.56	0.53	0.48	0.46

In our laboratories we have noticed that the resolution of the aflatoxins is best when freshly prepared plates are used. Rf. values are more reproducible on plate than on paper and can be used for the identification of the toxins. Lijinsky and Butler (1966), however, noted that the green fluorescence often observed for aflatoxin G_1 is due to the presence of yellow impurities

accompanying the toxin during chromatography. When acetic anhydride was added to this solvent, these impurities were removed. Similar improvement in resolution has been achieved by Adye and Mateles (1964).

In order to be able to estimate the concentration of the different levels of aflatoxin present in peanut meals, Nesheim et al (1964) used standard solutions of pure aflatoxin and compared the fluorescence of known amounts of the standard with that of the test samples. This method has also been adopted by de Iongh et al (1964). There are two possible objections to this method. Firstly, it is known that the fluorescence of methanolic solutions of aflatoxin varies with time; secondly the activity of the plates and the choice of developing solvents appear to be critical for the success of the determination. The method is however valuable for routine determination of levels of aflatoxin in groundnut products.

(d) Spectrophotometry.

Chromatographic analysis of aflatoxin followed by visual or photographic examination of fluorescence are known to be subjective and has been criticized by many investigators, (Coomes et al 1965; Lee, 1965). The absorption and emission spectra of the aflatoxins were observed to be similar (Sargeant et al , 1961; de Iongh et al , 1962). On exposure to ultraviolet light, aflatoxin B_1 is excited, and a characteristic blue fluorescence is emitted. Other fluorescent materials present in chloroform extracts obtained from natural products may prevent a precise measurement of toxicity. It is also desirable that the recording of the intensity of fluorescence should be non-subjective. Furthermore, Lijinski, Raha and Chestnut (1961) have suggested that successful application of spectrofluorimetry as an analytical technique requires the measurement of emission intensities under carefully controlled conditions.

Carnaghan, Hartley and O'Kelly (1963) recorded the fluorescence emission maximum for aflatoxin B_1 or B_2 at 425mμ and that for G_1 or G_2 at 450mμ. These authors also observed wide differences in the intensities of light emitted from equi-molar solutions of these four substances. When compared on an arbitrary unit called KQ, the following values were recorded for aflatoxins B_1 (0.5); B_2 (4.0); G_1 (2.5) and G_2 (6.5). The KQ value represents the fluorescence intensity of the test substance, relative to that of quinine sulphate. The reciprocal of this value is a measure of the concentration of the substance in micrograms per millilitre, that will give the same fluorescence intensity as one microgram of quinine sulphate per millilitre of solution.

The above method can therefore be used for the estimation of low levels of aflatoxin provided a suitable separation of the toxins one from the other has been achieved. Lijinsky and Butler (1966) observed that during chromatography of extracts of

spoiled diets, the green fluorescence given by aflatoxin G_1 was due to the presence of yellow impurities. Hence reliance on green fluorescence as a means of identification and estimation of this material may be misleading. The presence of non-fluorescent impurities may also reduce the intensities of fluorescence of those substances considerably.

Nesbitt, Hartley, and O'Kelly (1963) recorded the ultraviolet spectra of aflatoxin B_1, B_2, G_1 and G_2 in methanol. The authors observed that all the aflatoxins showed peak absorption at 223, 265 and 363mμ . Using a 2-cm cell, Nabney and Nesbitt (1964) measured the optical density of methanolic solutions of the aflatoxins at 363mμ and used this as a basis for quantitative assessment of the concentration of these substances in solution. The relationship between optical density and concentration is known to be linear over a wide range. In practice the optical density at 363mμ minus that at 420mμ is used for the calculation

of extinction coefficient. This takes into account the fact that the shape and position of this peak in the ultraviolet spectrum does not vary with time and also eliminates the background effect caused by the presence of some other substances which may be present in the methanolic extract.

The biological and chemical tests have generally been in good agreement with one another. Physico-chemical tests, with better separation of components on thin layers of silica-gel G are, however, more reliable. Spensley (1963) suggested that the presence of a non-toxic substance having similar chromatographic and fluorescent properties must not be overlooked. For this reason, laborn-tories undertaking chemical tests for aflatoxin are advised to cross-check their 'positives' by biological assays. There is no doubt that if the chemical test is negative, the sample is aflatoxin-free, at least down to the level of sensitivity of the test.

(v) The Structure of Aflatoxin.

The elucidation of the structure of the aflatoxins posed a challenge to the earlier investigators. Van der Zijden et al (1962) obtained a crystalline toxin from synthetic culture medium on which Aspergillus flavus had grown. Chromatographic analysis showed that the crystalline toxin contained a number of components which were separable by counter current distribution, (Nesbitt et al , 1963). Two fractions were distinguishable; a blue fluorescent material, now called aflatoxin B, was obtained after re-crystallization from methanol. It formed irregularly shaped plates which melted with decomposition at 270°C with prior softening at 250°C to 260°C. The other component, aflatoxin G, formed fine colourless needles with melting point at 247°C to 250°C. These crystals were later shown to contain impurities, which may be difficult to remove by solvent extraction on repeated re-crystallization. Structural studies on complex molecules require the application of

sophisticated analytical techniques. These include mass spectroscopy; ultraviolet and infra-red spectroscopy. Recently, nuclear magnetic resonance spectra of molecules have revealed the dispositions of protons or orientation of end groups in stereo-isomers (Van der Merwe et al, 1964; Lado et al, 1965). Mass spectroscopic examination revealed that the molecular weight of the blue fluorescent material (aflatoxin B_1) was 312 and that of the green fluorescent compound (aflatoxin G), 328. Elementary analysis showed that the aflatoxins contained C, H and O only. This was in agreement with an earlier observation by Sargeant et al (1961), that this toxin was different from pyrolizidine alkaloids which contain nitrogenous bases. An emperical formula of $C_x(H_2O)_y$ was obtained for aflatoxin B. On this basis the molecular formula for aflatoxin B was found to be $C_{17}H_{12}O_6$. A molecular formula for aflatoxin G, $C_{17}H_{12}O_7$, was in agreement with available data.

The strong absorption of light shown by the compounds in the ultraviolet region revealed the presence of chromophoric groups. The ultraviolet spectrum of the aflatoxins is characteristic of compounds containing conjugated double bonds and the presence of loosely bound electrons or lone pairs. The similarity in the spectra of aflatoxin B_1 obtained from different sources also suggest that the composition of the molecular species is the same (de Iongh et al , 1962; Nesbitt and O'Kelly, 1963).

The infra-red spectrum is characteristic of the molecule that gives rise to it. Hence the examination of infra-red patterns or 'finger prints' is a useful aid in the identification of compounds or closely related substances It is noteworthy that there is a striking similarity in the infra-red spectra from the four aflatoxins. Nesbitt and O'Kelly (1963) were the first to recognise the presence of lactone groups; a dimethylene group attached to an oxygen atom and the existence of an

unsaturated function in the aflatoxins. It was, however, difficult at this stage, to suggest the structural arrangement of the groups because of insufficient data. The ultraviolet and infra-red spectra were also not readily interpretable.

A new approach to structural elucidation of the aflatoxins was made by a group of investigators at the Massachusetts Institute of Technology in 1963 (Asao, Buchi, Abdel-Kader, Chang, Wick and Wogan, 1963). In their studies, aflatoxin was extracted from Aspergillus flavus cultures grown on crushed wheat. The identity of this compound with that described earlier by British workers was confirmed. On catalytic reduction of aflatoxin B_1 in ethanol over palladized charcoal, three moles of hydrogen were absorbed. The spectral characteristics of the reduction product was then compared with that of other organic compounds derived from natural and synthetic coumarins, such as dihydrobergaptene or sterigmatocystin. This confirmed

that aflatoxin B_1 is a derivative of a furo-coumarin. The structure of dihydrobergaptene was published by Van Dorp et al (1963); and that of sterigmatocystin was elucidated by Bullock et al (1962). The structures and physical properties of aflatoxin and that of derivatives of coumarins are summarized in table one on page 37. In proposing these structures for aflatoxin, it was argued that the emperical change in the ultraviolet and infrared spectra, accompanying the catalytic reduction of aflatoxin B_1, demanded the presence of olefinic double bonds in conjugation with either a double bond or with the coumarin ring.

Nesbitt and O'Kelly (1963) reported a marked difference in the nuclear magnetic resonance (n.m.r) spectrum of aflatoxin B_1 and that of aflatoxin G_1. These authors also suggested the presence of a dihydrofuran ring in aflatoxin B_1. The n.m.r. spectrum also revealed the presence of a methoxy group, accounting for three out of the twelve protons identified.

TABLE 1: PHYSICAL PROPERTIES OF AFLATOXIN AND RELATED COUMARIN DERIVATIVES.

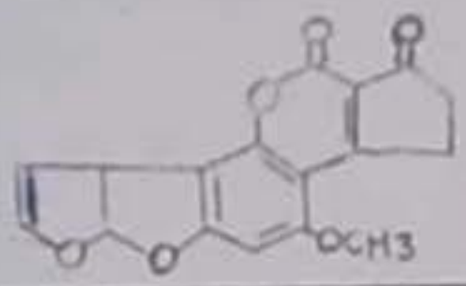
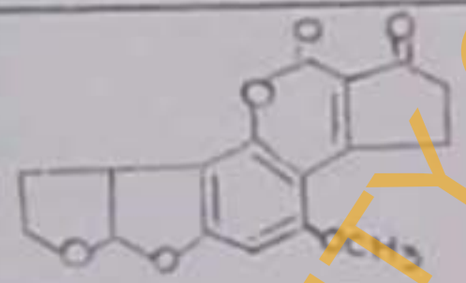
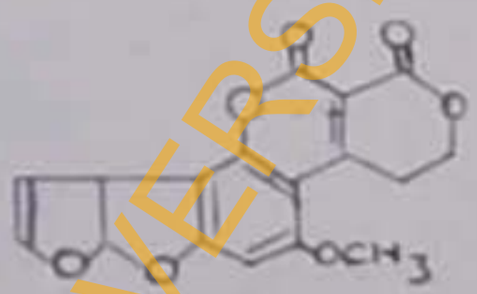
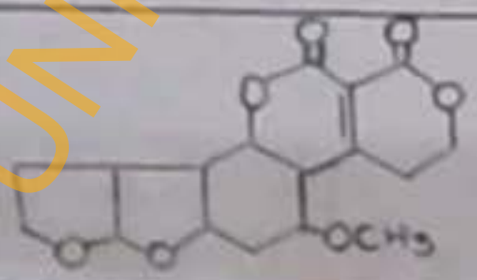
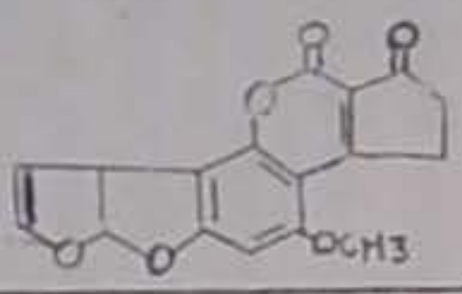
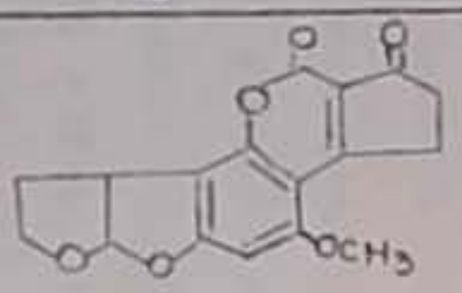
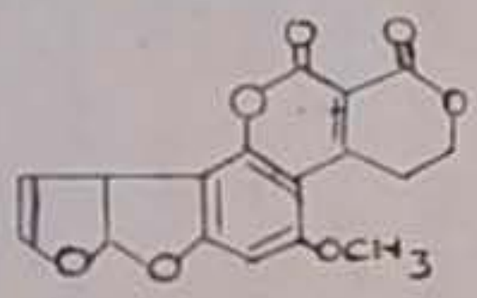
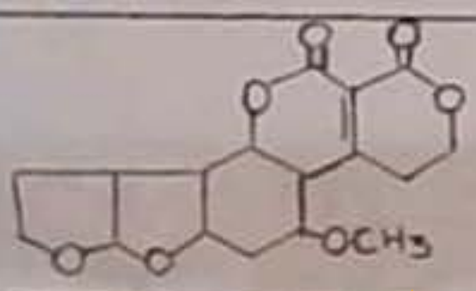
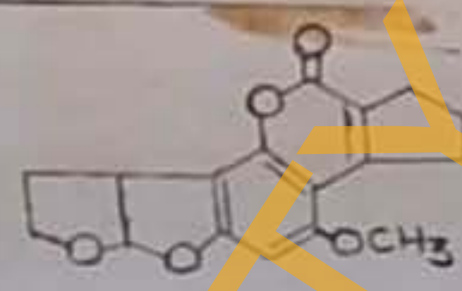
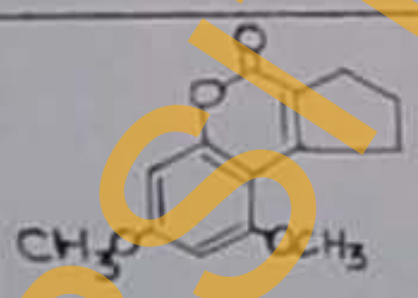
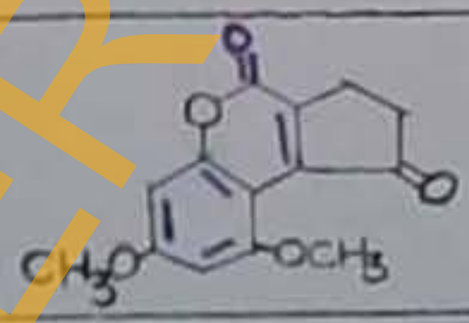
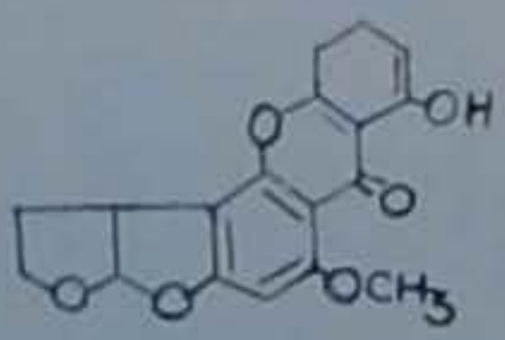
SUBSTANCE	Mass Spec. Mol. Wt.	Mol. Formula	Structure	UV λmax	ε	Infra Red ν cm-1	Reference
AFLATOXIN B_1	312	$C_{17}H_{12}O_6$		223 265 362	25,600 13,400 21,800	1760-1684 1632 1598 1562	Asao et.al. 1963 1965
AFLATOXIN B_2	314	$C_{17}H_{14}O_6$		220 265 362	19,600 9,200 14,700	1760 1685 1625 1600	Cuang et.al. 1963
AFLATOXIN G_1	328	$C_{17}H_{12}O_7$		243 257 264 362	11,500 9,900 10,000 16,100	1760 1695 1630 1595 1545	Asao et.al. 1963 1965
AFLATOXIN G_2	330	$C_{17}H_{14}O_7$		217 245 265 363	28,800 12,900 11,200 19,300		

TABLE 1: PHYSICAL PROPERTIES OF AFLATOXIN AND RELATED COUMARIN DERIVATIVES.

SUBSTANCE	Mass Spec. Mol. Wt.	Mol. Formula	Structure	UV m λmax	E	Infra Red ν cm-1	Reference
AFLATOXIN B_1	312	$C_{17}H_{12}O_6$		223 265 362	25,600 13,400 21,800	1760-1684 1632 1598 1562	Asao et.al. 1963 1965
AFLATOXIN B_2	314	$C_{17}H_{14}O_6$		220 265 362	19,600 9,200 14,700	1760 1685 1625 1600	Chang et.al. 1963
AFLATOXIN G_1	328	$C_{17}H_{12}O_7$		243 257 264 362	11,500 9,900 10,000 16,100	1760 1695 1630 1595 1545	Asao et.al. 1963 1965
AFLATOXIN G_2	330	$C_{17}H_{14}O_7$		217 245 265 363	28,900 12,900 11,200 19,300	-	
TETRAHYDRO-DESOXY AFLATOXIN B_1	300	-		255 264 332	8,500 9,200 13,900	1705 1625 1580	Asao, et al, 1965
5,7-DIMETHOXY CYCLOPENTENO (C) COUMARIN	-	$C_{14}H_{14}O_4$		248 257 325	7,700 7,000 16,100	1706 1608 1567	Asao et al, 1965
5,7-DIMETHOXY CYCLOPENTE-DION (3,2-C) COUMARIN	-	$C_{14}H_{12}O_5$		245 268 356	13,200 8,700 9,000	1726 1614 1556	Asao et al, 1965
5,7, DIMETHO-CYCLOPENTE-DION (2,3-C) COUMARIN	-	$C_{14}H_{12}O_5$		215 237 257 345 355	22,200 14,600 9,650 25,800 26,800	1759 1685 1614 1594 1550	Asao et al, 1965
STERIGMATO-CYSTIN	-	$C_{17}H_{10}O_5$ OMe		235 249 329	24,800 27,600 13,100	1650 1627 1590	J.E. Davis, 1960 Bullock, 1962 " , 1963

NOTE: UV = Ultraviolet Spectroscopy. E = Extinction Coefficient.

The proposed structure for aflatoxin B_1 has now been confirmed by Buchi et al (1966) when a total synthesis of racemic aflatoxin B was achieved. Another confirmatory evidence was reported from X-ray chrystallographic studies. Cheung and Sim (1964) observed that aflatoxin G_1 crystallized from benzene as well formed prismatic crystals containing benzene of solvation. In this way, one crystal unit would consist of two molecules of aflatoxin G_1 and one molecule of benzene. Using the isomorphous replacement technique, bromobenzene and bromothiophene solvates were also prepared. X-ray crystallography of these crystals showed three dimensional electron density patterns. These were displayed in contoured sections on sheets of glass and staked on a frame. The final picture which emerged confirmed the proposed structure for aflatoxin G_1.

A variant of the structures of aflatoxin G_1 had been proposed by Van der Merwe, Fourie and Scott (1963). In their view, the position of the

dihydrofuran rings in relation to that of the lactone rings were reversed. This proposal is not supported by the available evidence. It is now known, that aflatoxin B_1 contains an αβ-unsaturated δ-lactone and a cyclopentenone ring in which the two carbonyl groups are cross-conjugated with the double bonds, while aflatoxin G_1 with its additional oxygen atom, has two cross-conjugated αβ-unsaturated δ-lactonic rings.

(vi) Toxicity of Aflatoxin.

(a) LD_{50} of Aflatoxin in Different Species.

In table 2 the results of LD_{50} studies on aflatoxin reported by different laboratories show slight variations in details but support the assertion that aflatoxin is a very potent carcinogen. The LD_{50} for aflatoxin B_1 to day-old ducklings varies from 0.37 mg/kg (Carnaghan et al, 1963) to 0.56 mg/kg (Asao et al, 1965). Aflatoxin G_1 has a third of the potency of B_1, whilst aflatoxins

TABLE 2: TOXICITY OF AFLATOXINS B_1, B_2, G_1 AND G_2 TO DUCKLINGS AND RATS

Animal	Age or Weight	Sex	Route of Administration	Aflatoxin used	Dose (μg)	LD_{50} mg/kg	Reference
Duckling	1 day	M	Oral	B_1	18.2	0.37	Carnaghan, 1963
Duckling	1 day	M	Oral	B_2	34.8	1.85	" "
Duckling	1 day	M	Oral	G_1	39.2	0.90	" "
Duckling	1 day	M	Oral	G_2	17.25	3.97	" "
Duckling	1 day	M	Oral	B_1	17.5	0.335	Lijinsky and Butler, 1966
Duckling	1 day	M	Oral	G_1	54.08	0.95	" "
				Pure G_1	45.7	0.785	" "
Duckling	1 day	M	Oral	B_1	28.2	0.56	Asao et al ,1965
				G_1	90.0	1.80	" "
Duckling	1 day	M-F	Oral	B_1	12	-	Holzapfell et al 1966
Duckling	1 day	M-F	Oral	M_1	16.6	-	"
				M_2	62.0	-	"
Rats	1 day	M-F	Oral	B_1	-	1.0	Asao et al 1965
	21 days	M	Oral	B_1	-	5.5	" "
	21 days	F	Oral	B_1	720	7.2	Butler, 1964
	100 g	M	ip	B_1	600	6.0	"
	150 g	F	Oral	B_1	1193	17.9	"

B_2 and G_2 are relatively much less toxic. In the rat, the toxicity of aflatoxin decreases rapidly with age, and weight increase (Butler, 1964; Asao et al , 1965). This may be related to the relative underdevelopment of drug-metabolising mechanisms in the very young animal. It has, however, been reported by Holzapfel, Steyn and Purchase (1966) that aflatoxin M_1, a metabolite of aflatoxin B_1 is also a potent carcinogen. The species differences in the toxicity of aflatoxins to animals have not been fully investigated. An explanation of the species differences will also require understanding of the metabolism and detoxication of these toxins in vivo.

(b) Injury to Animal Tissues.

Postmortem examination of organs extracted from dead poults in field outbreaks always revealed the following four features :-

(1) there is considerable proliferation of bile duct epithelium to form numerous structures resembling small bile ducts;

(2) chronic, frequently obliterating, endophlebitis of centrilobular and hepatic veins;

(3) wide variation in size and shape of parenchymal cells, many of which contain abnormally large, coarse and densely basophilic nuclei and

(4) diffuse fibrosis which disrupts lobular structure.

Lancaster, Jenkins and Philp (1961) fed toxic groundnut meals to rats for a period of six months and observed the presence of liver tumours, but cirrhosis, cell necrosis or cellular infiltration was absent. This suggests that the toxic agent acts directly on hepatic cells.

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The diet used in this experiment contained 20.15 per cent casein. A more severe attack was reported by Shoental (1961) in guinea pigs fed on diets containing 15 per cent groundnut meal. Butler and Barnes (1963) fed rats and guinea pigs on commercial rations and showed that the changes in the pathological pictures in these animals are similar. These include the proliferation of small bile duct epithelium, which extended to other lobules progressively. These authors also demonstrated that as little as 0.4 parts per million of aflatoxin induced hepatic tumours in five out of six rats and that when the diet was supplemented with extra choline or methionine, the incidence of hepatoma was not decreased. This shows that aflatoxin is 1,500 times more toxic than other chemical carcinogens, or methylating agents like butter yellow and that its mechanism of action is probably different.

It is significant that a single oral dose of aflatoxin B_1 (346 mg/kg) gives rise to a

periportal lesion which remains a permanent feature of the damaged liver (Butler, 1965). It is therefore informative to recount the sequence of events leading to gross liver damage in aflatoxin poisoning. Within the first twenty-four hours, there is a loss of glycogen in the liver, which is accompanied by immediate arrest of regeneration of hepatic and kupffer cells. This is then followed in the next day by peripheral zone necrosis and fatty infiltration of parenchymal cells with pyknotic nuclei. On the third day there is well developed biliary proliferation extending into the zone of necrosis. These progressive changes result in distortion of the lobular pattern. The long term effect of aflatoxin is a permanent damage of the liver cells. Butler and Barnes (1964) found hepatoma in rats which were given diets containing aflatoxin (1.75 p.p.m.) for 89 days but returned to a commercial diet for another 316 days. The situation progressively deteriorated with time, even after a change of food habits.

(c) Protein Synthesis.

Histological changes in the liver of rats after poisoning with aflatoxin B_1 is accompanied by a fall in the activities of hepatic enzymes and a corresponding elevation of serum enzyme activities (Bassir, 1964). Clifford and Rees (1967) confirmed that the serum levels of isocitrate dehydrogenase, glutamate dehydrogenase and malate dehydrogenase were raised twenty-four hours after administration of aflatoxin B_1 to rats. This was then followed by a rise in bilirubin concentration and alkaline phosphatase activity. The hepatotoxic action of aflatoxin results in a release of hepatic enzyme into the serum.

The biochemical changes following the development of experimentally induced liver injury and necrosis may be preceded by damage to subcellular particles or alteration in enzyme systems involved in maintaining the energy supply and ion transport mechanisms of the cells. Dickens

and Jones (1965) demonstrated that aflatoxin B_1, unlike other carcinogenic lactones reacted very slowly with the sulphydryl groups of cystein in vitro. The carcinogenic potency of aflatoxin is also greater than that of other closely related substances which react chemically with sulphydryl groups quite readily, whereby their activity is lost (Dickens, et al, 1966). It is also considered that the very high activity of the aflatoxins may be a consequence of other chemical features of the molecule.

Clifford and Rees (1966) proposed that the biochemical changes underlying the development of liver necrosis in the rat after administration of aflatoxin B_1 were as follows: The toxin interracts with DNA. This interraction prevented the RNA polymerase transcribing the DNA and, inhibited the formation of messenger RNA (mRNA). A failure in mRNA formation resulted in an inhibition of protein synthesis which the authors considered to be the cause of liver necrosis.

This view is based on a number of experimental observations.

(1) The absorption spectrum of aflatoxin B_1 was altered on addition of various concentrations of calf thymus DNA (Wogan et al 1966; Rees and Clifford, 1966).

(2) The incorporation of [^{14}C]-leucine into proteins (Smith, 1964) and [^{14}C]-orotic acid into the RNA of liver slices (Clifford, Rees, and Stevens, 1967) were inhibited by aflatoxin B_1.

(3) The change in absorption spectrum of aflatoxin B_1 was similar to the difference in spectrum given by actinomycin D in the presence of calf-thymus DNA (Clifford and Rees, 1967).

(d) Carcinogenesis.

The effects of aflatoxin on different organs in rat have been reported by Butler and Barnes (1965). In the kidney the proximal convoluted tubule shows cytoplasmic swelling and pyknotic nuclei, similar to that found in the liver within twenty-four hours. The glomeruli are normal, but cells with hyperchromatic nuclei are seen in the loops of Henle. Of the three zones of the adrenal cortex, only the zona reticularis is affected. The other two zones, the zona glomerulosa and zona fasciculata are known to be the centre of synthetic activity for the adrenal steroids. The lungs, pancreas and the alimentary canals were least affected. The heart showed small areas of myocardial fibrosis and the red pulp of the spleen were necrotic in appearance.

Theron, Liebenber and Joubert (1965) reported on the electron micrographs of liver cells obtained from rats given acute doses of aflatoxin B_1. In these pictures, the mitochondria were swollen and showed evidence of dissolution of external limiting

membrane. The cisternae of the endoplasmic reticulum in contact with the red-blood cells appear dilated and filled with a finely granular material. The endoplasmic reticulum of the liver of control animals showed the long slender profiles. It was also noticed that the morphological changes in the liver cell organelles were always more severe in the vicinity of extravasated red-blood cells. This suggests that the toxic principle (aflatoxin B_1 or possibly a closely related substance) was transported by the red-blood cells.

The foregoing evidence point to the fact that aflatoxin reaching the liver through the portal system exert a cytotoxic effect on the membranes of intracytoplasmic structures. Reynolds (1963) has stated that alterations of the cell membranes may result in functional changes in enzyme activity. The induction of hepatoma in aflatoxin poisoning could therefore be a consequence of metabolic injury caused by the derangement of close relationships between multi-enzyme systems.

The high incidence of hepatomas in hatchery-reared rainbow trout (Salmo-gairdnerii) may be related to the diet used (Wolf and Jackson, 1963). It is now thought that aflatoxins present in the diet are responsible for this incidence. Thus, crystalline aflatoxin, purified diets to which aflatoxin had been added, and commercial trout rations in which aflatoxins were found, all produced hepatoma in rainbow trout (Ashley, Halver; Gardner Jr. and Wogan, 1965; Sinnhuber Wales, and Lee, 1966).

(e) Teratogenesis.

The action of aflatoxin on the cells and its interraction with subcellular particles has been reported by Legator and Withrow (1964); Legator, Zuffante and Harp (1965). There was suppression of mitosis in diploid and heteroploid lung cell cultures on the addition of aflatoxin B_1. In these cells, synthesis of DNA was decreased. Zuckerman and Fulton

(1966); Rees, Inman and Pelts (1967) compared the cytotoxic effects of aflatoxin on human and rat embryonic liver cells. These authors concluded that there was a striking similarity in the action of the toxins on these cells.

In pregnant mammals, aflatoxin poisoning may show dismal effects in the offspring. Such maternal influences may result in defects or malformations in the foetus. Elis and Di-Paolo (1967) administered aflatoxin B_1 to pregnant rats between ninth and fourteenth day of the development of the foetus. These authors noticed signs of chromosomal rearrangements in the nuclei of the rapidly differentiating cells. Aflatoxin may also have a secondary effect on the metabolism of the foetus. There was, however, a decrease in the incidence of malformations following the injection of aflatoxin B_1-DNA mixture to rats (Rees, Clifford and Stevens 1967). This is in support of the view expressed by Rees and Clifford (1966) that the teratological effects may be a consequence of the binding of aflatoxin to DNA and inhibition of the DNA-dependent RNA synthesis.

C H A P T E R T W O.

M A T E R I A L S

(i) Aflatoxin Working Standard

5 ml of a chloroform solution of aflatoxin was presented to us by the U.S. Department of Agriculture, Louisiana. This working standard contained the following :-

Aflatoxin	B_1	0.0038	per µl
"	B_2	0.001	" "
"	G_1	0.0032	" "
"	G_2	0.0005	" "

The above solution was analysed on silica gel G thin-layer plate, developed in 3 per cent methanol in chloroform. The following substances were identified when the plate was examined in ultraviolet light.

(a) aflatoxin B_1 gave intense bluish fluorescent spot at Rf. 0.48;

(b) aflatoxin B_2 : a very faint bluish spot at Rf. 0.43;

(c) an intense greenish spot at Rf. 0.38 and a faint greenish spot at Rf. 0.34 correspond to aflatoxins G_1 and G_2 respectively.

(ii) Composition of Czapek-Dox Medium

(Dox and Thom Modification, Morris 1960)

30 g Sucrose,

2 g Sodium Nitrate,

1 g Dipotassium Hydrogen Phosphate,

0.5 g Magnesium Sulphate,

0.5 g Potassium Chloride,

and 0.01 g Ferrous Sulphate,

were dissolved in tap water to make a litre. Tap water was used for the preparation of the medium in order to provide the necessary trace elements (Visser 1967a).

(iii) Fungal Isolates.

The following fungal isolates were supplied by Dr. S.O. Alasoadura, Department of Botany, University of Ibadan.

Aspergillus Ochraceus (39)

This was isolated from locust fruits. It belongs to the Aspergillus Ochraceus group, possessing septate mycelium and white spores. The spore diameter was approximately 0.25 to 0.3μ .

Aspergillus Flavus (75)

This was isolated from the soil in Ibadan. It is a member of the Aspergillus flavus group, which is commonly found in the mycroflora of stored products. The mycelium was septate and the conidia were green in colour. The spore diameter was 0.45 to 0.55μ .

Aspergillus Flavus (81)

This was a strain of Aspergillus flavus Link ex Fries, originally obtained from mouldy groundnuts.

This fungus was presented to the Mycology Laboratories, in the Department of Botany, University of Ibadan by the Tropical Product Institute, London. The morphological characteristics are similar to those of Aspergillus flavus (75) described above.

The numbers indicated in parenthesis were the serial numbers given to these cultures.

(iv) Composition of Experimental Diets for Rats.

The following ingredients were weighed and mixed together in a vat:-

Casein : Supplied by B.D.H. Limited, England contained less than 5% moisture.

Fat : Contained 10 g of Danish Butter melted in 100 g groundnut oil.

Salt : Mixture was prepared according to Hubbell, Mendel and Wakeman (1937).

ABIDEC : A multivitamin mixture manufactured by Parke, Davis and Company, London

Composition of Diets in g per kg.

Diet	Protein (g)	Fat (g)	Sucrose (g)	Cellulose (g)	Salt Mixture (g)	Vitamins (g)
(a) High-protein diet	250	80	610	30	20	10
(b) Low protein diet	40	80	820	30	20	10
(c) Normal diet	150	80	710	30	20	10

One killogram of each diet was prepared and stored at 4°C until required.

(v) Commercial Diet for Laboratory Animals.

(a) Rat: Supplied by Livestock Feed Ltd., England.

Analysis: Crude-protein 21.0%; Fibre 4.0% and Oil 3.5%.

(b) Rabbit: Supplied by Livestock Feed Ltd., England.

Analysis: Crude-protein 20.0%, Fibre 3.4% and Oil 3.7%.

(vi) Radioactive Isotopes.

All radioactive isotopes used were purchased from the Radiochemical Centre, Amersham, England.

Sodium acetate-1-C^{14}, specific activity 29 mc/mM.

Sodium acetate-2-C^{14}, specific activity 27.4 mc/mM, batch 49.

D-glucose-C^{14}(U), specific activity 123 mc/mM, batch 27.

Sucrose-C^{14}(U), specific activity 185 mc/mM, batch 4.

Glycine-C^{14}(U), specific activity 108 mc/mM, batch 13.

Taurine-S-35, specific activity 12.4 mc/mM, S3.42.

The total activities were checked just before use.

Except for Taurine-S-35, a stock solution of each of the labelled materials above was prepared to give 100µc per ml of solution.

A standard solution of taurine-S-35 was prepared and used immediately afterwards.

(vii) Composition of Krebs-Ringer Phosphate and and Bicarbonate Solutions.

1. NaCl (0.154M)
2. KCl (0.154M)
3. $CaCl_2$ (0.11M)
4. KH_2PO_4 (0.154M)
5. $MgSO_4 7H_2O$ (0.154M)
6. $NaHCO_3$ (0.154M).

The above solutions were mixed in the following proportions :-

100 parts of solution 1
plus 4 parts of solution 2
plus 3 parts of solution 3
plus 1 part of solution 4
plus 1 part of solution 5
plus 21 parts of solution 6.

The solutions after mixing was gassed with air for ten minutes.

(viii) Liquid Scintillation Phosphors.

2, 5, diphenyl oxazole, Packard Instruments Ltd. (scintillation grade).

5g was dissolved in a litre of scintillation grade toluene.

N.E. 220 liquid scintillator for use with aqueous solutions - Nuclear Enterprises Ltd.

CHAPTER THREE

METHODS.

Culture Techniques.

The growth of Aspergillus flavus on 65 different substrates as carbon sources was compared with that on sucrose (Visser, 1967a). The mycelial dry weight increases with increase in sucrose concentration, but this was not parallelled by the amounts of aflatoxin produced (Davis, Diener and Eldridge, 1966). It was therefore necessary to carry out preliminary experiments to determine the conditions for the growth of Aspergillus flavus and production of Aflatoxin in cultures. using synthetic media previously described by Osiyemi, Bababunmi and Bassir (1967). A modification of Czapek-Dox medium was found suitable for the subsequent

experiments, described in this section on the growth and production of aflatoxin by Aspergillus flavus.

(i) Preparation of Inoculum.

Fungal spores were scraped loose from cultures on agar slant with a platinum loop and transferred into 10 ml of sterile distilled water. A drop of teepol was added and then mixed thoroughly to give a uniform distribution of spores. In order to free the suspension from pieces of mycelium, the preparation was filtered through sterile absorbent cotton wool. Counting of the conidia present in the filtrate was carried out with the aid of a haemocytometer. The size of inoculum was then adjusted to 2×10^6 conidia per 1.0 ml, by adding sterile distilled water to the suspension.

(ii) Fermentation Procedure.

Fermentations were carried out in 250 ml culture flasks. 100 ml of Czapek-Dox solution

was poured into each flask and autoclaved at 15 p.s.i. for one hour. On cooling to room temperature the solution was inoculated with 1.0 ml. of spore inoculum containing approximately 2×10^6 fungal spores. The flask was left to rest in a slanting position, in order to provide a wide area for the surface culture. The incubation period was seven days at room temperature (approximately 27°C).

(iii) Growth of Aspergillus Species on Czapek-Dox Medium.

Different procedures have been used for in vitro culture of Aspergillus species on natural and synthetic media (Tropical Product Institute Report No. 6, 1964). Toxin-producing fungi were reported to grow on peanut meals (Codner, Sargeant and Yeo, 1963); Crushed wheat (Chang, Abdel-Kadir, Wick and Wogan, 1963); Corn-meal (Merwe, Fourie and Scott, 1963) and Rice (Shotwell, Hesseltine, Stable-Field and Sorenson, 1966). Unlike these

Fig.2: Growth of three fungal isolates under the same condition in culture flasks containing 100 ml Czapek-Dox medium. The surface culture was incubated at room temperature ± 27°C. Each point represents the mean dry-weight of three determinations.

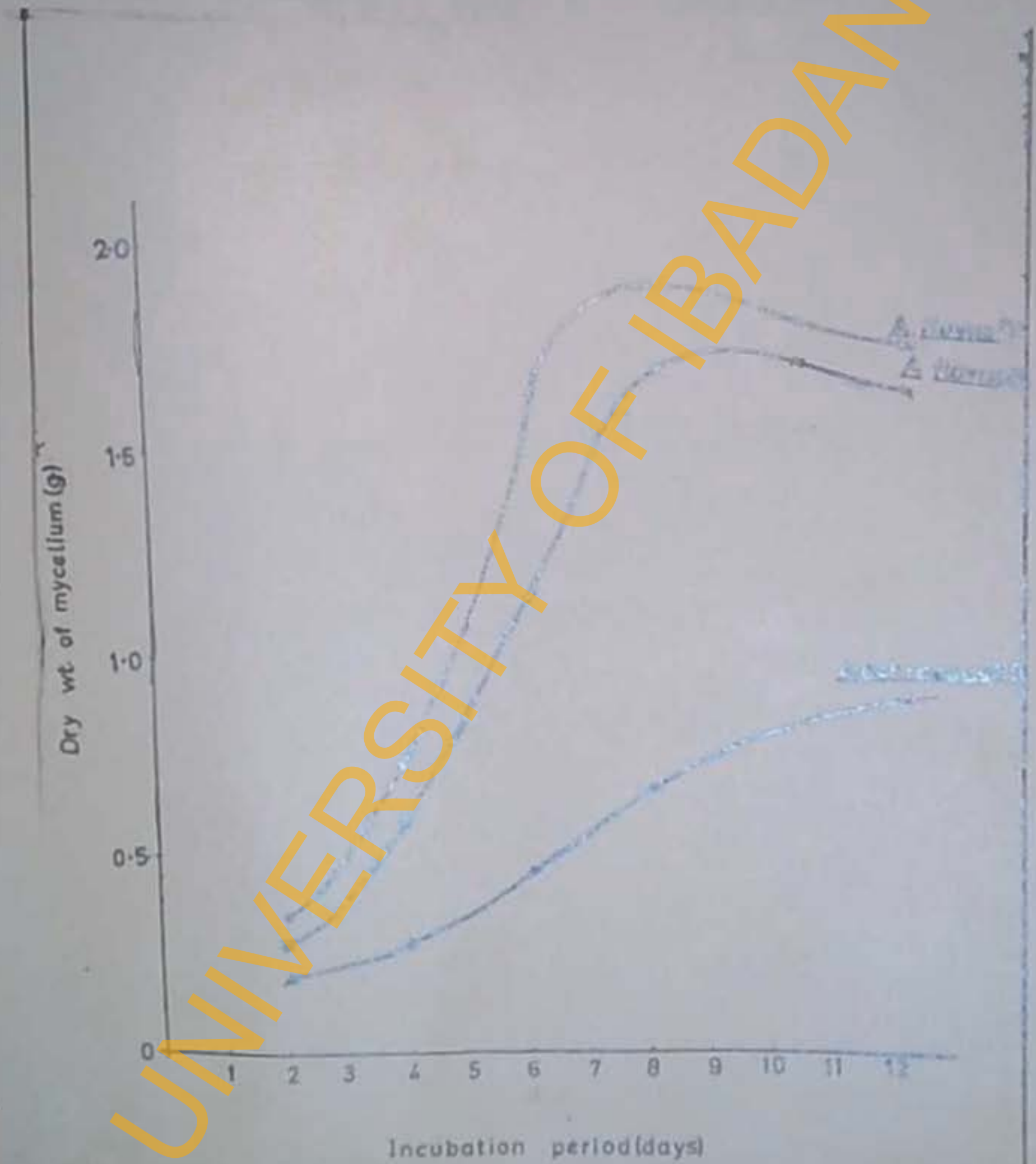

Fig. 2.

Fig.3: Dry weights of mycelia produced by fungal isolates grown on aerated Czapek-Dox medium. Each point is the mean dry weight of three determinations.

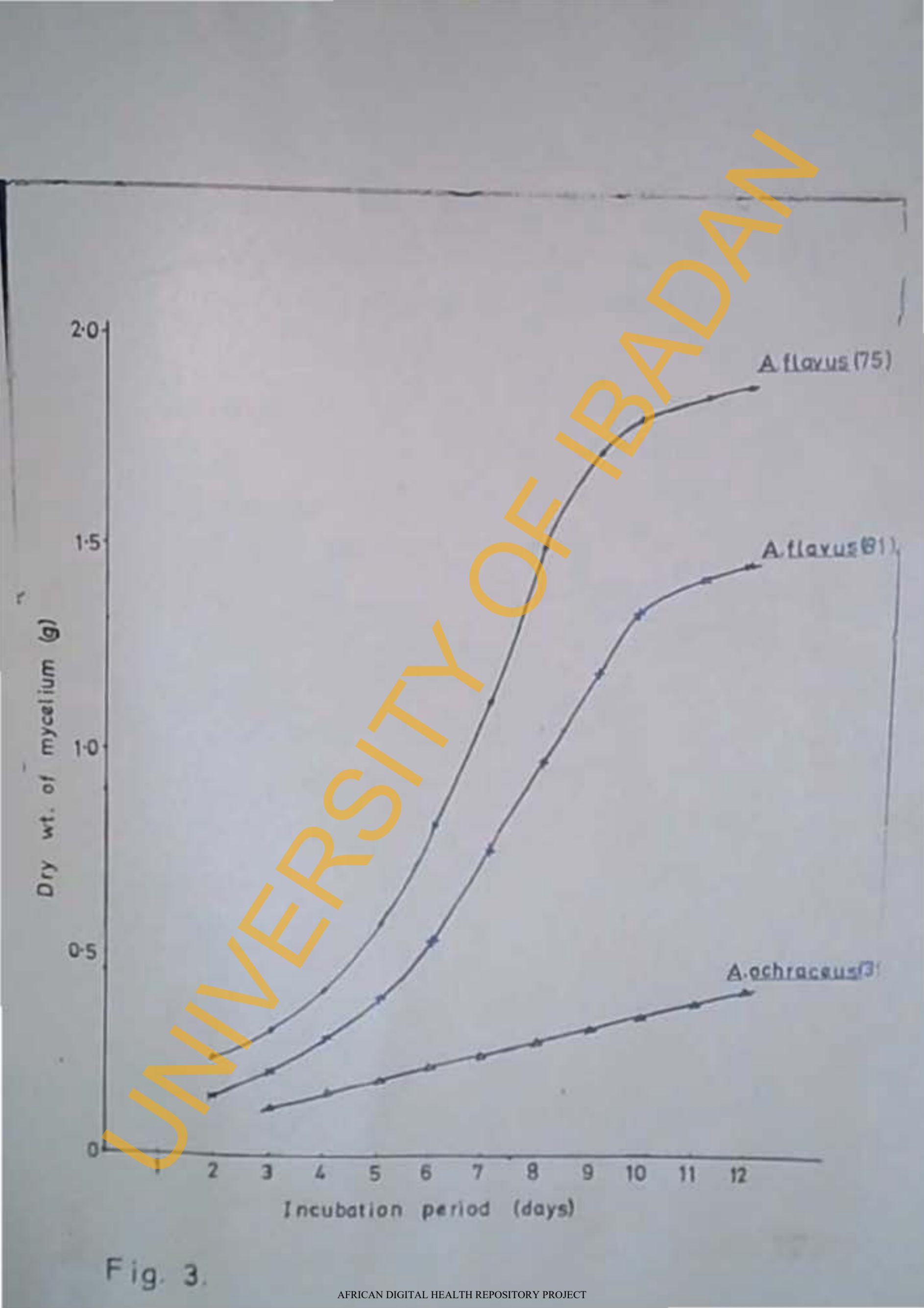

Fig. 3.

complex substrates, synthetic media contain pure compounds of known composition and concentration. These were found suitable in biogenetic studies by Adye and Mateles (1964). Addition of corn-steep liquor (Shroeder, 1965) or two per cent yeast extract (Davis, Diener and Eldridge, 1966) was found to increase the rate of growth of fungi and the production of aflatoxin. In the experiments described in this thesis, three fungi of the Aspergillus group, commonly found in Nigerian soils were selected for study. These were classified as described previously on page 54 . Fungal spores were grown either on surface culture or in aerated culture medium.

Surface Culture.

1 ml of spore inoculum was added to 100 ml Czapek-Dox solution (100 ml) autoclaved and later inoculated with fungal spores as described in the fermentation procedure on page 61 . At the end of the incubation period, the content of the flask

was filtered into a Buchner-funnel, using vacuum filtration. The mycelium was washed twice with distilled water on the funnel, and then transferred into a weighed porcelain crucible. This preparation was left to dry in a forced draft oven, at 105°C, to a constant weight. The dry-weight of mycelium was recorded. The growth rates of Aspergillus ochraceus (39), Aspergillus flavus (75) and Aspergillus flavus (81) on surface cultures are recorded in Fig. 2.

Aerated Culture.

Sterile air was passed into the fermentation flask at a constant rate of three bubbles per second. The procedure used was a modification of the method of Arnstein and Grant (1954). The CO_2 present in emergent gas stream was passed via a gas trap into three sets of saturated Barium hydroxide solutions. The dry weight of the mycelium from the cultures of Aspergillus ochraceus (39), Aspergillus flavus (75) and Aspergillus flavus (81) were determined as

Fig.4: Thin-layer chromatogram showing the production of aflatoxin by Aspergillus flavus (75) on Czapek-Dox medium. Aspergillus Ochraceus and Aspergillus flavus (81) did not produce aflatoxin under the same condition.

Fig. 4.

described above and they are recorded in Fig. 3. Analysis of the filtrate and mycelium from these cultures, carried out according to the procedure described in Tropical Product Institute Report No. G6 (1964), revealed that Aspergillus ochraceus (39) and Aspergillus flavus (81) produce blue fluorescent materials which remained at the origin during chromatography. Aflatoxin was not detected in these cultures under the experimental condition. Aspergillus flavus (75) produced good yield of aflatoxins B_1 and G_1 (see Fig. 4). This observation is in agreement with the findings of Dalner et al (1963); Codner et al (1963), that the production of aflatoxin by different strains of Aspergillus species varies considerably. Wildman, Stoloff and Jacobs (1967) recorded in a survey on Aspergillus flavus that "out of three-hundred-and-sixty-three strains of Aspergillus flavus reported in the literature, only one-hundred-and-one were toxin-producing".

In order to develop a suitable method for the production of large amounts of aflatoxin, the following procedure was adopted. Aspergillus flavus (75) was cultured on Czapek-Dox medium as described previously on page 65 . The culture was harvested daily and the amount of aflatoxin produced was assayed by a modification of the method described by Lee (1965). This is a 'wet extraction' procedure, followed by thin-layer chromatography of the chloroform extract.

The production of aflatoxin under two different experimental conditions was investigated. In table 2, is recorded the amount of toxins produced in surface or aerated cultures. In the surface culture, aflatoxin was detectable in the medium on the third day, whilst the toxin was found in the aerated culture twenty-four hours later. The production of aflatoxin progressed with increase in incubation time, after an initial latent period.

TABLE 2: PRODUCTION OF AFLATOXIN BY ASPERGILLUS FLAVUS (75)

Incubation Period in Days	AFLATOXIN PRODUCED (µg/L)					
	Surface Cultures			Aerated Cultures		
	B_1	G_1	Total	B_1	G_1	Total
2	-	-	-	-	-	-
3	9	15	24	-	-	-
4	25	52	77	3	4	7
5	150	167	217	10	14	24
6	200	218	418	32	35	67
7	221	255	476	71	98	169
8	225	236	451	135	152	287
9	216	224	430	147	182	329
10	181	172	353	155	193	348
11	180	170	350	162	200	362
12	180	171	351	167	208	375

(iv) Incorporation of Isotopes into Cultures of Aspergillus Flavus (75) and (81) Growing on Czapek-Dox Medium.

In the next experiment, biogenesis of aflatoxin from acetate $[1\text{-}C^{14}]$ and acetate $[2\text{-}C^{14}]$ was investigated. It was desirable to develop a suitable procedure for the synthesis of C^{14}-labelled aflatoxin, with high specific activity, which can be used in metabolic studies.

The biosynthetic procedure adopted was a modification of that described by Hunter and Hechenhull (1955) for the incorporation of C^{14}-labelled compounds into streptomycin. A sterile solution of $[1\text{-}C^{14}]$ acetate or $[2\text{-}C^{14}]$ acetate (50 µM) in distilled water was added aseptically into a culture of Aspergillus flavus (75). The surface culture was incubated at room temperature for seven days. At the end of this period, the mycelial growth, covering the surface of the culture was transferred into buckner funnel, rinsed twice with distilled water

and then dried to a constant weight in a forced draft oven at 105°C. The radioactivity in the dry mycelium was determined by counting portions of it on weighed planchetts in an end-window Geiger Muller counter at infinite thickness.

The washing from the mycelium was added to the filtrate in a standard flask (100 ml) and made up to mark with distilled water. Radioactive content of aliquots of this solution was estimated by the liquid scintillation technique. The aflatoxin content was determined by the method of null-fluorescence technique (Osiyemi, Babalunmi and Bassir, 1967). Specific activities of the C^{14}-labelled aflatoxins were determined and expressed in $m\mu C$ per mM. Sufficient counts to give a standard error of ± 2 per cent were made in all determinations.

When $[1\text{-}C^{14}]$ acetate was added to the culture medium, 4 per cent of the radioactivity was present in the mycelium and 10 per cent in the filtrate. A higher proportion of the radioactivity was retained when $[2\text{-}C^{14}]$ acetate was used.

The specific activity of the aflatoxin produced by Aspergillus flavus (75) was less than half the value reported by Adye and Mateles (1964) using a resting cell method. It was suggested that a considerable portion of the isotope was oxidised or used largely for the synthesis of mycelium instead of being accumulated for aflatoxin production. The distribution of labelled isotopes in the fungal cultures is shown in table 3.

In the next experiment the utilization of labelled isotope by the fungal cultures was investigated.

1.0 ml of spore inoculum was added to 100 ml of sterile Czapek-Dox solution. To the mixture was added 1.0 ml of a stock solution of labelled isotope. The culture was then distributed equally into 34 graduated centrifuged tubes (10 ml), plugged with cotton wool, under aseptic conditions. The preparation was left in a slanting position at room temperature and harvested periodically afterwards as follows :-

The content of each tube was centrifuged at 1,000 g for 30 minutes. The supernatant was decanted and the residue, washed with sterile distilled water.

TABLE 3

INCORPORATION OF [1-C^{14}] ACETATE AND [2-C^{14}] ACETATE INTO AFLATOXIN BY ASPERGILLUS FLAVUS (75) AND ASPERGILLUS FLAVUS (81)

Item	A. flavus (75)		A. flavus (81)	
	[1-C^{14}] Acetate	[2-C^{14}] Acetate	[1-C^{14}] Acetate	[2-C^{14}] Acetate
Weight of fungus (mg)	365	412	373	450
Count Rate per mg. of fungus (Counts per 100 secs. per mg)	634	1517	518	1134
Total counts in mycelium (Counts per 100 secs)	231,500	624,900	233,000	423,000
Total counts in filtrate (Counts per 100 secs)	627,500	851,250	655,830	909,150
Specific activities in n µC per mM:				
Aflatoxin B_1 (221 µg)	3.0	5.4	-	-
Aflatoxin G_1 (255 µg)	4.3	5.9	-	-

NOTE: Total radioactivity supplied to each sample was 50 µM acetate, approximately 6×10^6 counts per 100 secs.

Fig. 5: Biogenesis of aflatoxin by Aspergillus flavus (75) on Czapek-Dox medium in the presence of [1-C^{14}] acetate. The surface culture was incubated at room temperature approximately 27°C.

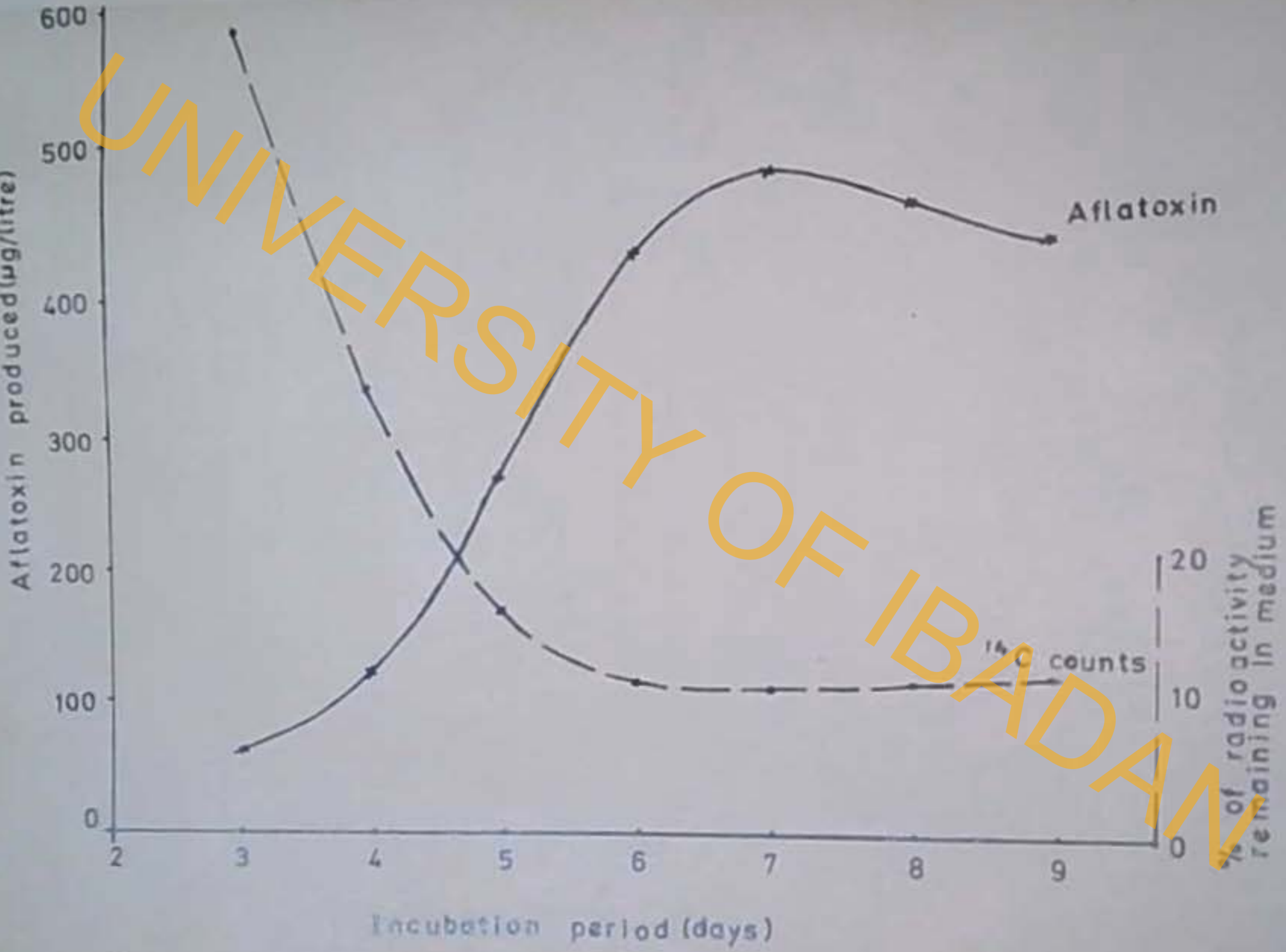

Fig 5

The washing was added to the supernatant and then made up to 10 ml in a standard flask. Aliquots of this solution was counted in the liquid scintillation counter. The residue was transferred into a mortar and ground into a paste. A portion of this was then collected into a weighed plantchett and dried on a hot plate to constant weight. The dry-weight of mycelium on the plantchett was determined by difference. The radioactivity in the mycelium was estimated by counting at 'infinite thickness' in a Geiger-Muller counter. The amounts of radioactivity remaining in the medium were recorded as shown in Fig. 5. The figure also shows the quantities of aflatoxin(s) produced in the cultures during the same period.

Very little activity was found in the aflatoxin produced by the above method in which labelled acetate was added to the culture medium before incubation commensed. A 'replacement culture' procedure adopted by Adye and Mateles (1964) also gave a low yield of labelled aflatoxin starting from $[1\text{-}^{14}C]$-acetate as precursor.

In the next experiment, a surface culture was grown on a non-radioactive medium. At a given time interval after the beginning of incubation a sterile solution of the radioactive isotope was added to the culture medium aseptically.

On the seventh day, just before sporulation started, the culture was transferred into a separating funnel and shaken with equal volume of chloroform. The solvent, in the lower layer, was drained off. A fresh chloroform was added to the mixture and this extraction procedure was repeated three times. The chloroform extracts were pooled together and filtered through a layer of anhydrous sodium sulphate. The dry chloroform extract was concentrated to a small volume (approximately 10 ml) and chromatographed on thin-layer of silica-gel G. The specific activities of aflatoxin produced was recorded in table 4. In subsequent cultures, addition of radioactive isotope to the medium was delayed for 60 hours.

TABLE 5

CHANGES IN SPECIFIC ACTIVITY OF AFLATOXIN WITH TIME OF ADMINISTRATION OF [2-C^{14}]-ACETATE TO CULTURES OF ASPERGILLUS FLAVUS (75) ON CZAPEK-DOX MEDIUM

Time after start of culture (hours)	AFLATOXIN B_1		AFLATOXIN G_1	
	Specific activity (m μ C/mM)	R. I. C.	Specific activity (m μ C/mM)	R. I. C
0	5.4	0.022	5.9	0.023
24	11.5	0.036	18.7	0.059
36	17.0	0.053	19.0	0.060
48	28.0	0.088	22.4	0.070
60	30.0	0.094	28.0	0.088
72	26.0	0.081	24.0	0.075
92	21.0	0.066	18.0	0.056

NOTE: Relative isotopic content (R.I.C.) = the ratio of the specific activity of aflatoxin to the specific activity of the precursor.

Section II: Methods for the Estimation of Aflatoxin.

The Physico-chemical test for aflatoxin described in the report of Tropical Product Institute, London, No. G6, (1964) was adopted. The test is based on visual assessment of fluorescence of aflatoxin when compared with that of standard solution of the same substance under prescribed conditions. This is called the 'visual assay technique'. An improvement on this method, which does not depend on the application of a standard reference substance was described by Coomes, Crowther, Francis and Stevens (1965) for routine assessment of toxicity due to aflatoxin B_1 in groundnut and groundnut materials. This is called the 'Null-fluorescence technique'. A modification of this procedure, which is suitable for the determination of aflatoxins B and G has been reported by Osiyemi, Bababunmi and Bassir (1967). This method which is simple and reproducible has now been extended for quantitative assays of aflatoxins B_1, B_2, G_1 and G_2.

Aflatoxin levels were determined by a dilution technique and the quantity of aflatoxin present in test sample was expressed as µg per litre of culture medium used.

(a) Visual Assay Technique.

10 ml of dry chloroform extract of test sample was applied onto a chromatoplate of kiesel-gel G. An equal volume of each of a set of standard solutions containing aflatoxins B_1, B_2; G_1 or G_2 was spotted on the same plate. The chromatogram was run in a tank containing two per cent methanol in chloroform and then viewed in a dark room illuminated by ultraviolet lamp situated 30 cm from the plate. A photographic record of the plate was produced by using a Leica Camera, fitted with a 2A (yellow) filter. After development of the plate, the intensity of fluorescence was used for the assessment of the concentration of the aflatoxins present in the test sample. A comparison of the intensities of fluorescence of the test and that of the standard solutions was made at each determination.

Fig. 6: Photo-record of fluorescence of aflatoxin B_1 on thin-layer of silica-gel G. A serial dilution of aflatoxin B_1 in chloroform was prepared. Equal volume (0.2 ml) of each dilution was spotted on the chromatogram.

Fig. 6.

(b) Null-Fluorescence Technique.

A set of dilutions of test sample extracts in chloroform was prepared, such that the dilution factor ranged from 2 to 2^{10}. 0.2 ml portions of these solutions were transferred onto Kiesel-gel G thin-layer plates. The diameter of each spot being less than 1 cm. The chromatogram was run in 2 per cent methanol in chloroform and viewed in the ultraviolet light under the conditions described above. The dilution factor of the solution with just visible fluorescence was noted. The smallest weight of aflatoxin B_1 and G_1 observable were 0.004 µg and 0.003 µg respectively (Coomes, Crowther, Francis and Stevens, 1965). By adopting a similar experimental procedure the minimum quantities of aflatoxins B_2 and G_2 observable were determined. The visual limits for the detection of aflatoxin B_2 and G_2 were found to be 0.005 µg and 0.003 µg respectively. A photo-record of the fluorescent spots on chromatoplates is shown in Fig. 6.

The concentration of aflatoxin present in the extract was calculated as follows :-

If i = visual limit for detection of aflatoxin in µg,

L = dilution factor,

k = volume applied to plate (0.2 ml),

v = volume of test sample extract (10 ml),

W = Dry weight of material extracted (g),

C = concentration of aflatoxin in µg per litre,

then $C = i \times \frac{v}{k} \times \frac{w}{10^6} \times L$

$= \underline{i \times w \times L \times 5 \times 10^{-5} \mu g \text{ per litre}}.$

(c) Densitometric Measurement of the Aflatoxins.

A developed chromatogram containing fluorescent spots was copied on an Ilford HP3 photographic plate as follows :-

A Leica camera was fitted with a 2A (yellow) photographic gel filter, which transmits only wavelengths longer than 410 mμ and eliminates stray light and ultraviolet rays. The plate was placed at a fixed distance from the U.V. source and the exposure was timed. A copy of the developed photograph is shown in Fig. 7.

Densitometric measurement was recorded on the Chromoscan (Joyce Loebls Co. Ltd.). A linear relationship was found between recorder reading and the quanties of aflatoxin. This method was however less sensitive than the null fluorescence technique.

(d) Spectrophotometry

Ultraviolet spectra were determined with the Perkin-Elmer (137 UV) spectrophotometer. Chloroform was used as solvent.

Infra-red spectra were recorded on the Perkin-Elmer (infra-cord) spectrophotometer. Sodium chloride-crystal cells were used.

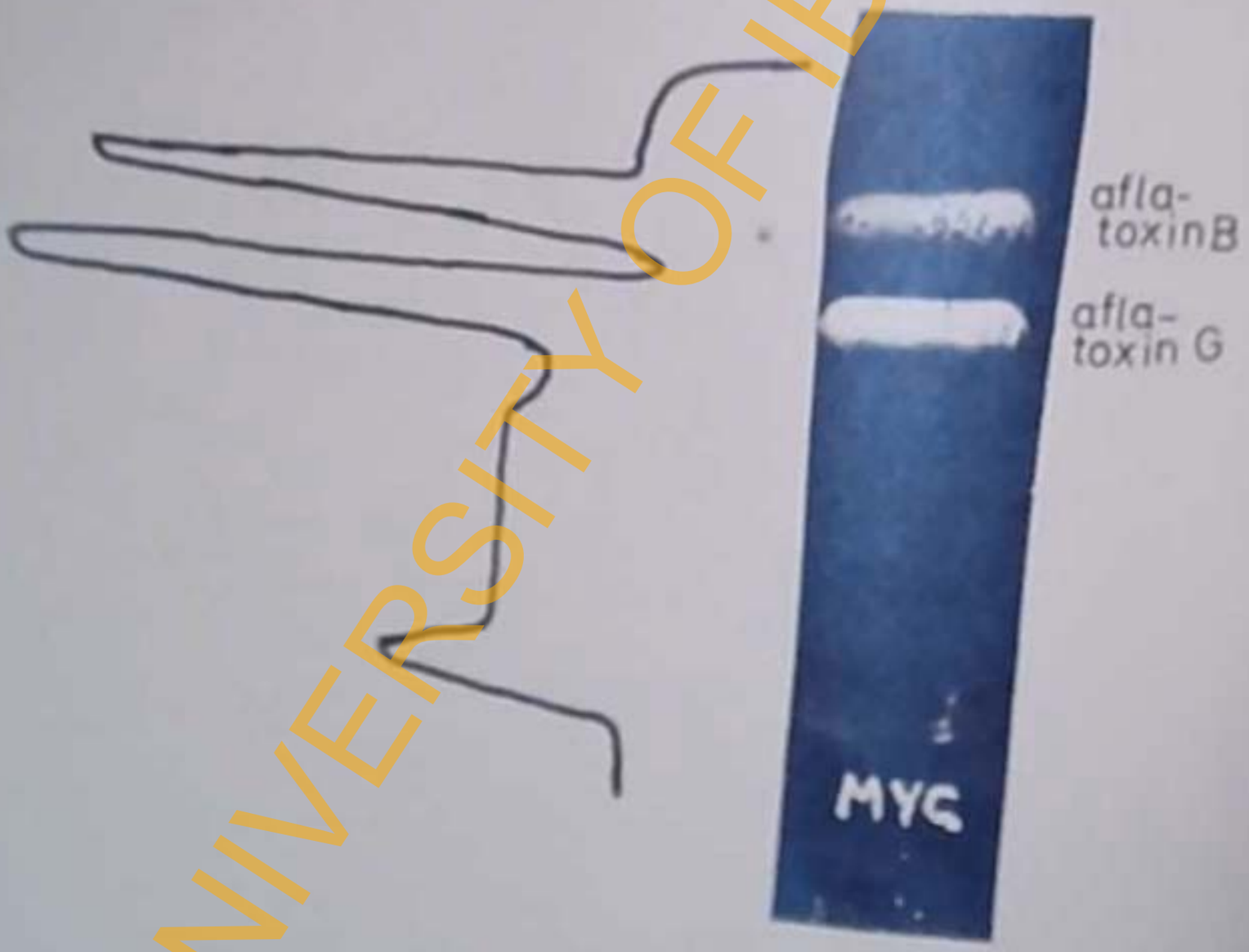

Fig 7.

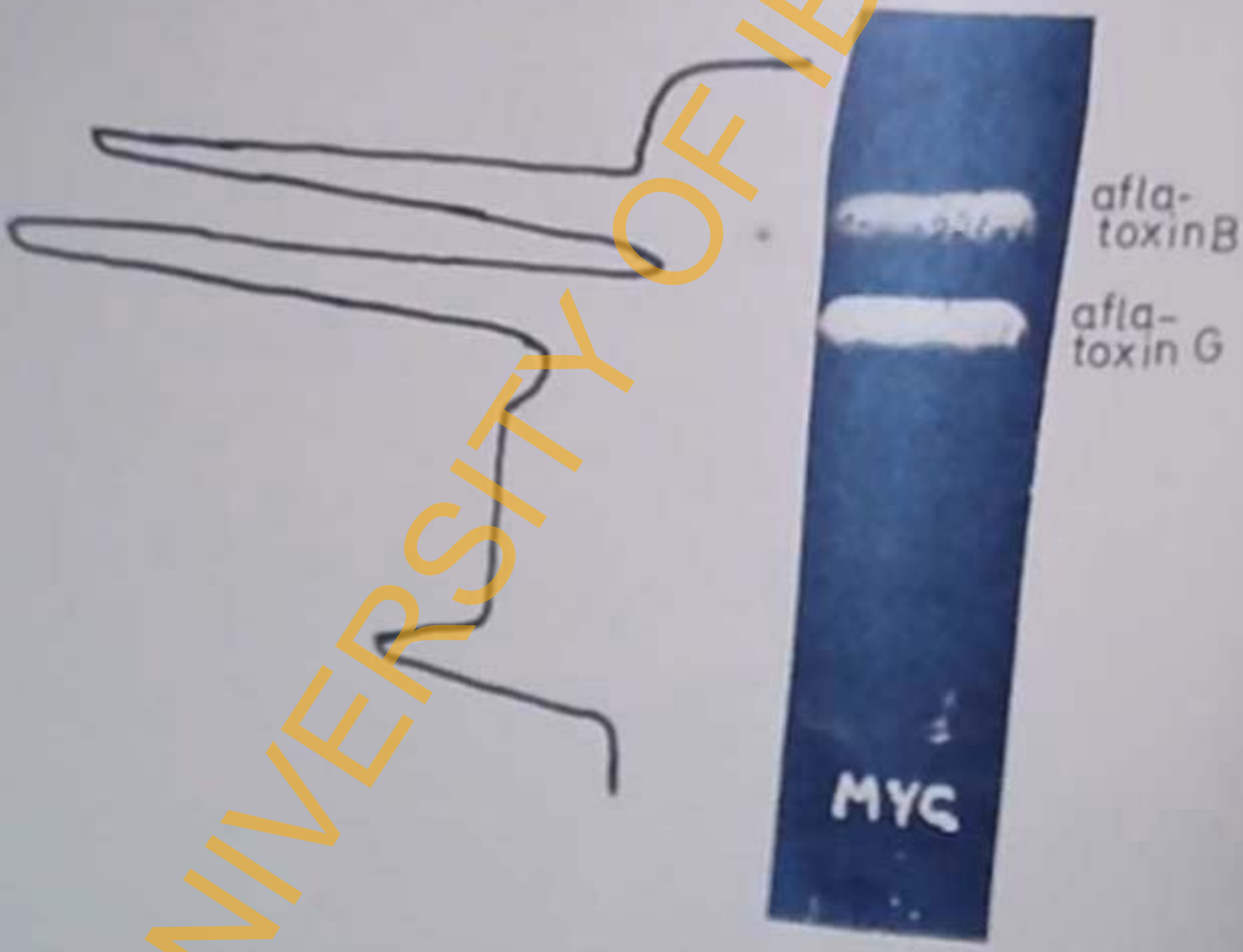

Fig 7

Fig. 7: Chloroform extract of culture of Aspergillus flavus containing aflatoxins B and G was chromatographed on thin-layer plate. The plate was photographed under ultraviolet light. The picture was then scanned on a densitometer (Chromoscan-Joyce-Leobls Co. Ltd).

Section III: Techniques Used For Measurement of Radioactivity.

(a) Liquid Scintillation Counting of Soft β Radiations.

A scintillation counter type 6012, supplied by the Isotope Development Limited, Berkshire, England was used. This equipment is capable of measuring low-energy beta active liquid samples of millimicrocurie quantities, when suspended in a suitable scintillator medium. It has two photomultiplier tubes connected to a two channel system.

The following precautions were observed during use:

(i) the electronic equipment was switched on at least 30 minutes before commencing operations;

(ii) the performance of the instrument was checked, before and after each set of

measurements, using a reference source and a background sample as controls;

(iii) the choice of the best working condition was made after preliminary experiments in which the reference source was counted at varying voltage imput and differing discriminating bias. An efficiency of 64.5 per cent was achieved at a voltage imput of 940 volts, in a single channel counting.

Use of End-Window Geiger Muller Counter.

The scaler (EKCO Type N530P) was turned on for 10 minutes before use.

A standard source of ^{14}C was placed in the counting chamber, which had a 2π geometry. The count rate of the sample at varying operating potential was recorded. The plateau of the curve obtained indicated a region where the counting rate was relatively

insensitive to voltage change. The voltage was set at 1250 volts, corresponding to the midpoint of the plateau.

The background count rate at this setting was recorded, for each operation. The time necessary to obtain 10,000 counts was recorded. An efficiency of 32 per cent was achieved, under the operating conditions.

Section IV: Operations on Experimental Animals.

(a) Animal Husbandry.

(i) Rats.

Rats were kept in a battery of metal cages of standard size 10" x 10" x 8" for each rat. Each cage was provided with a water bottle and a beaker containing the experimental diet. A wire mesh placed under the cage was used as a device for collecting faeces, whilst urine flowed on to a perspex tray which drained into a collecting beaker.

Fig. 8: Growth of rats on experimental diets. Each point in the figure represents the average weight of 7-male rats.

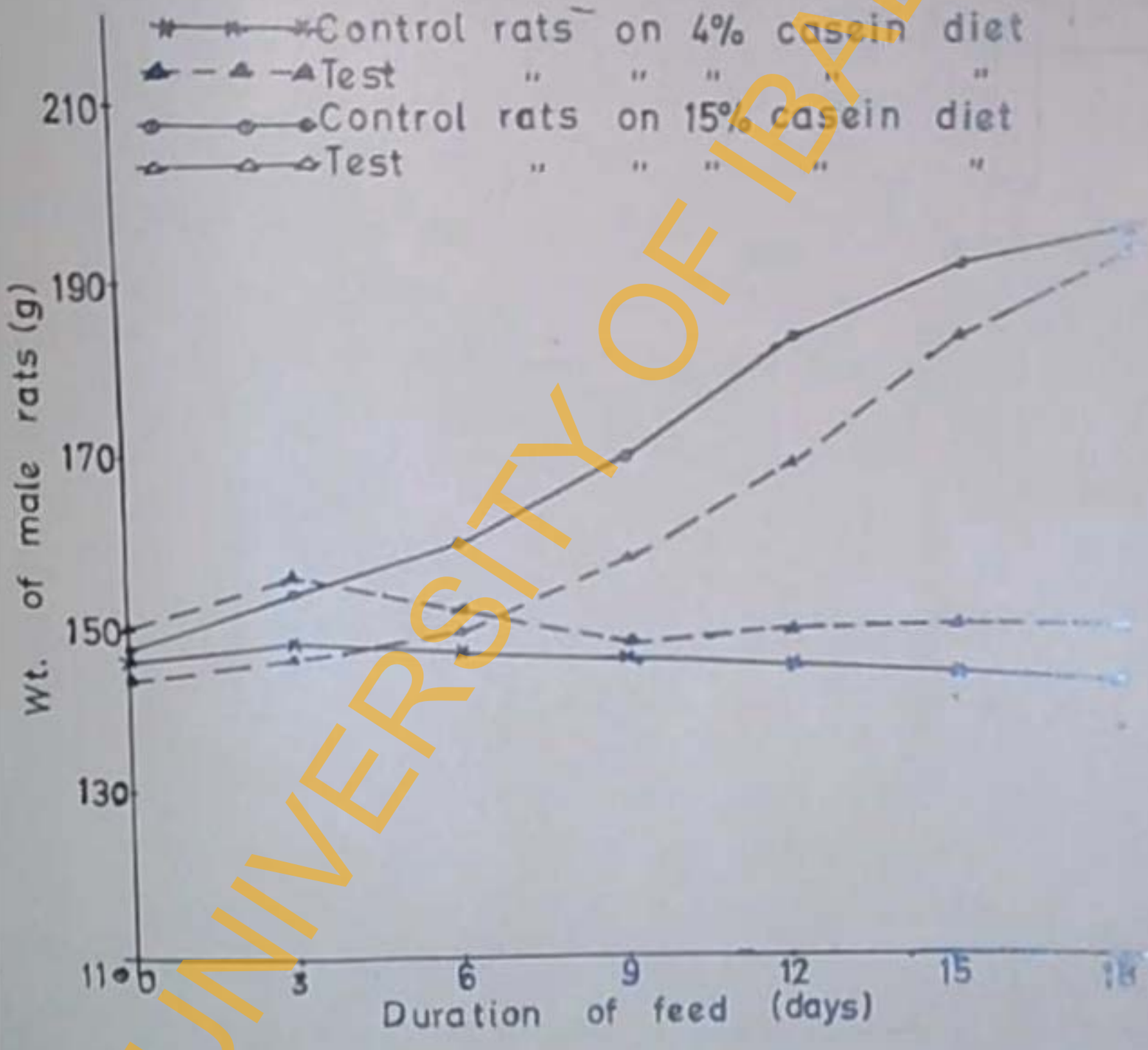

Fig. 8.

Rats kept under the above conditions developed normally on adequate diets. There was apparently no gain in weight in rats fed on 4 per cent casein diet, as shown in Fig. 8. A sub-lethal dose (5 µg aflatoxin B_1) in saline was injected intra-peritoneally into test rats twice weekly. The controls received saline alone. At this dosage the growth of rats on adequate diets was slightly impaired by administration of the toxin. Rats on low protein diets were similarly affected.

(ii) Rabbits.

A larger cage, but of similar design as for rats was provided for the rabbits.

Two rabbits, given 50mg per kg of 4-hydroxy coumarin suspended in water by stomach tube, were supplied with water ad libitum. Urine was collected during the following 48 hours from these animals. The ether extract of the urine contained 4-hydroxy-coumarin glucuronide ~~which~~ was ~~isolated~~ separated and purified according to the procedure of Mead, Smith and Williams (1958).

(b) Administration of Aflatoxin.

Aflatoxin B_1 was dissolved in normal saline (0.9% NaCl) at a concentration of 10 µg per ml. Each rat was weighed and a single dose of 50 µg aflatoxin B_1 per kg was administered intra-peritoneally. An equal volume of normal saline was given to the control rats. Rabbits were given intravenous injection in a single dose of 50 µg of aflatoxin B_1 per kg.

(c) Cannulation of the Bile Duct of the Experimental Animal Under Anaesthesia and Collection of Bile.

(i) Rats.

A rat weighing approximately 200 g was kept under light ether anaesthesia. A slit was made in the wall of the bile duct 1 cm from the junction with the duodenum, and a thin polythene catheter 20 cm long was inserted surgically according to the procedures of Boyland, Ramsay and Sim (1961), and Pryor and Slater (1967). A glass-saddle, designed by Van-Zyl

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(1958) was made from a pyrex tube (5 cm diameter) and strapped to the back of the rat. The open end of the biliary fistula was taken subdermally through the base connective tissue and passed out through the skin at the back of the neck into one of the chambers of the glass-container. Bile samples were withdrawn for analysis from this chamber periodically. After the operation, the animal was maintained on an experimental diet, and 0.85 per cent saline was given ad libitum instead of water. A preparation containing 10,000 i.u. penicillin was injected subcutaneously daily during the experimental period, which lasted two to three days.

(ii) Rabbits.

A rabbit, weighing 1.2 to 1.5 kg, was anaesthetised by intravenous injection of urethane (1.25 g/kg) and pentobarbitone sodium (12.5 mg/kg). To collect bile a glass cannula (with a narrow bore) was inserted through the fundus of the gall bladder and was placed as close as possible to the opening

of the cystic duct. The common bile duct was tied off. Bile samples were collected serially at regular intervals of time in glass test tubes.

(d) Stimulation of Diuresis in Mammals Under Anaesthesia.

(i) Rats.

A modification of the method of Child and Dodds (1966) was used.

Male rat approximately 200g was anaesthetised by intra-peritoneal injection of urethane solution (20 gm/kg). A cannula was inserted surgically into the trachea. The left external jugular vein was prepared for cannulation. A short midline incision was made in the lower abdomen and two urethral catheters were inserted for urine collection. A thin polythene cannula, connected to a reservoir containing 5 per cent (w/v) mannitol in saline (0.9 per cent, w/v, NaCl) was tied into the external jugular vein with an atraumatic suture. Thus an infusion of this solution was passed through the

vein at 0.2 ml per minute throughout the experiment to ensure moderate diuresis. Urine samples were collected at regular intervals.

(ii) Rabbits.

Rabbits, weighing 1.5 kg to 2 kg, were anaesthetized by intravenous injection of urethane (1.25g/kg) and pentobarbitone sodium (12.5mg/kg). The procedure used was similar to that outlined for the rats, but a higher rate of saline infusion, i.e. 0.75 ml per minute, was used.

Section V: Isolation of Metabolites of Aflatoxins B_1.

(a) Analysis of Bile Samples.

Bile samples obtained from experimental animals, after prior injection of labelled aflatoxin B_1, were analysed as follows :-

A known volume of the bile sample was transferred into a large excess of chloroform (at least five times the volume of the bile). The preparation was kept in this condition at 4°C until required for use.

The chloroform layer was carefully removed with a pasteur pipette and the aqueous layer was extracted twice with fresh chloroform solution. The chloroform extracts were pulled together and concentrated to a small volume (0.5 ml) on the vacuum rotary evaporator. This extract was transferred quantitatively unto a spot on an 'activated' chromatoplate of Merck's silica gel G. A hot air drier was employed during this process such that the diameter of each spot was not more than 1 cm. The prepared plate was chromatographed, using 2 per cent methanol in chloroform as solvent as described by de Iongh, Vles and Pelt (1964) and modified by Butler and Clifford (1965).

The aqueous layer was dried in a desiccator, in vacuo, over calcium chloride. The residue was agitated with 0.5 ml methanol at room temperature. The methanolic

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Fig. 9: Thin-layer chromatogram of rat bile samples collected at 10 minutes intervals, on silica-gel G.

solution was chromatographed on Merck's silica gel G thin layer plate using butanol, acetic acid, water (10 : 1: 1 v/v) as solvent. This system was essentially the same as that used by Frosch and Wagener (1967) for the quantitative determination of bile acids in serum.

For the identification of aflatoxin B_1 and its metabolites, the developed plates were viewed under ultraviolet light. The fluorescent spots on chromatograms which were coincident with radioactive peaks were removed from the plate with a 'zone extractor' (Osiyemi, 1964) prepared from a glass sintered tube, and eluted with methanol. The eluates were then chromatographed alongside standard solutions of aflatoxin B_1, M_1, and 4-hydroxy-coumarin glucuronide. The separation of metabolites of aflatoxin B_1 on the thin layer chromatogram is illustrated in Fig. 9.

The conjugated metabolites remained at the origin in the systems described above. In order to resolve this fraction from other minor fluorescent components, extracts from these fractions were analysed by

descending chromatographic technique on Whatman No. 1 paper (20cm x 20cm). The chromatogram was run overnight (13 hours) using butanol; acetic acid; water (10 : 1 : 1 v/v) as solvent. After drying briefly in air, the chromatogram was sprayed with naphthorescorcinol reagent for glucuronides as described by Williams, et al (1944) and modified by Elliot, Robertson and Williams (1966). Colour developed on heating the chromatogram in an oven at 110°C for 10 minutes.

(b) Analysis of Urine Samples.

Urine samples (4 ml to 5 ml) were collected into glass test tubes. The volume of each sample was reduced to 0.5 ml on the vacuum rotary evaporator. The concentrate was placed in a desiccator, and the last trace of water, removed. The residue was taken up in methanol (0.2 ml) and analysed by thin-layer chromatographic techniques.

(c) Methylation of De-Methyl Aflatoxin B_1.

A blue fluorescent spot (Rf. 0.60) was isolated close to that of aflatoxin B_1 (Rf. 0.65) on thin-layer

chromatogram of bile run in accordance with the method of Frosch and Wagener (1967). This metabolite was then separated from aflatoxin M_1 on a chromatogram, using the system described by Allcroft, Rogers and Lewis (1966); scraped off with silica gel G from the plate and eluted with methanol. The methanol extract was evaporated to dryness under reduced pressure, and diethyl ether was added to the residue.

A nitrosoderivative of p-toluene-N-methyl sulphonamide was heated with ethanolic potassium hydroxide on a water bath. Diazomethane, generated from this reaction, was passed into the ethereal solution. After two hours, the solvent was evaporated off and the residual solid was then dissolved in dry ether.

A portion of the methylated product obtained above was chromatographed on thin layers of kiesel gel G, using 2 per cent methanol in chloroform as solvent. Standard solutions of aflatoxin B_1 and aflatoxin M_1 were run on the same plate.

The Rf. value of the methylated product corresponded with that of aflatoxin B_1, giving a blue-violet fluorescence.

The ultraviolet and the infrared spectra of aflatoxin B, and that of the metabolites were recorded on the infracord (Perkin-Elmer, Limited, U.S.A.).

(d) Hydrolysis of Aflatoxin M_1 Glucuronide

(i) Enzyme Hydrolysis.

After the initial extraction of bile or urine samples with chloroform, the aqueous layer was taken to dryness under reduced pressure. The dry residue was dissolved in a known volume of water and the sample was adjusted to pH 4.5 with 2N acetic acid. The enzymic-hydrolytic procedure was similar to that used by Taylor and Scratcherd (1961) except that β-glucuronidase (2,000, Fishman Units per mg) supplied by Koch-Light Laboratory Ltd. was employed instead of an extract from limpet viscera. 0.5 ml of acetate buffer pH 4.5 containing the enzyme was added to the mixture followed by the addition of 0.1 ml of 0.2M KH_2PO_4. The mixture

was then incubated at 37°C for 8 hours. At the end of the period 5 ml of chloroform was added. The extraction was repeated twice. The chloroform extract was reduced to 0.5 ml on the vacuum rotary evaporator and chromatographed on thin-layer of Merck's silica-gel G using 2 per cent methanol as solvent.

(ii) Hot-Acid Hydrolysed Metabolites.

Bile or urine samples were acifidied and made 2N, by adding a few drops of 10N H_2SO_4. The tube was then heated in a boiling water bath for 30 minutes, cooled and extracted with ether. Ether was removed by playing a stream of hot air over the surface of the liquid. The residue was taken up in chloroform and chromatographed on thin-layers of Merck's silica-gel G, using 2 per cent methanol as solvent.

CHAPTER FOUR

EXPERIMENTS AND RESULTS.

Experiment I: Distribution of Aflatoxin B_1, and Its Metabolites in Rat Tissues After a Single Dose.

The aim of the experiments described in this thesis was to provide additional information on the metabolism of aflatoxin. This toxin had been reported to show toxicity to animals at very low concentrations. Hence sublethal dosages in the range of 10 to 50 µg per kg. wt. of the experimental animals were used. The availability of labelled aflatoxins facilitated detection of a fraction of a microgram of the metabolites.

In one of the preliminary experiments, the distribution of labelled aflatoxin in rat tissues after a single dose was investigated.

A male rat (200g) was given an intraperitoneal injection of [C^{14}]-aflatoxin B_1 (10 µg) in normal saline (0.9 per cent NaCl) in a single dose. The rat was sacrifised six hours later. The heart, intestine plus intestinal contents, kidneys; liver, muscle and stomach were removed and rinsed in saline. The tissues were minced separately in a waring-blendor and analysed according to the method of Butler and Clifford (1965). In this procedure, the tissues was extracted exhaustively in hot methanol. The extract was then partitioned between chloroform and aqueous methanol layers. The radioactivity present in each layer was determined by the liquid scintillation technique.

Result.

The result in table 6 shows that the bulk of the administered dose was found in the intestine plus faecal contents. The radioactive counts present in the liver, kidney, heart and muscle were also recorded. The high radioactivity in the intestinal content was significant. The liver and kidney also retained some

Table 6

Distribution of Aflatoxin and Its Metabolites in Rat.

Tissue	Counts/Sec.		
	Chloroform Extract	Methanol Extract	Total % of dose
Heart	0	13	0.2
Intestinal contents	431	1691	31.5
Kidney	208	42	3.7
Liver	119	240	5.3
Muscle	0	47	0.7

Total dose is equivalent to 6500 counts/sec.

of the toxin, and very little was present in the heart and muscle. The significance of this pattern of distribution was appreciated by Wogan (1966) when he suggested that the aflatoxin was probably secreted via the bile into the intestine. This inference was verified in our next experiment on the excretion of aflatoxin in the rats. It was also observed that a greater proportion of the radioactivity from rat tissues was present in the methanol layer except in the kidney which retained more of the chloroform extractable material. Earlier investigators (de Iongh, Vles and Pelt 1964; Butler and Clifford 1965; Allcroft, Rogers and Lewis 1966) reported only on the chloroform extract. Further investigations on the more polar aqueous methanolic extract was therefore contemplated.

Experiment II: Excretion of Aflatoxin B_1 and Its Metabolites in the Rat.

The aim of this experiment was to study the mode of excretion of aflatoxin in the rat. $[C^{14}]$-aflatoxin B_1 (10μ g) was injected intraperitoneally into a

Fig.10: Rate of excretion of aflatoxin in the bile of a rat after a single dose of $[C^{14}]$aflatoxin B_1 (Bassir and Osiyemi, 1967).

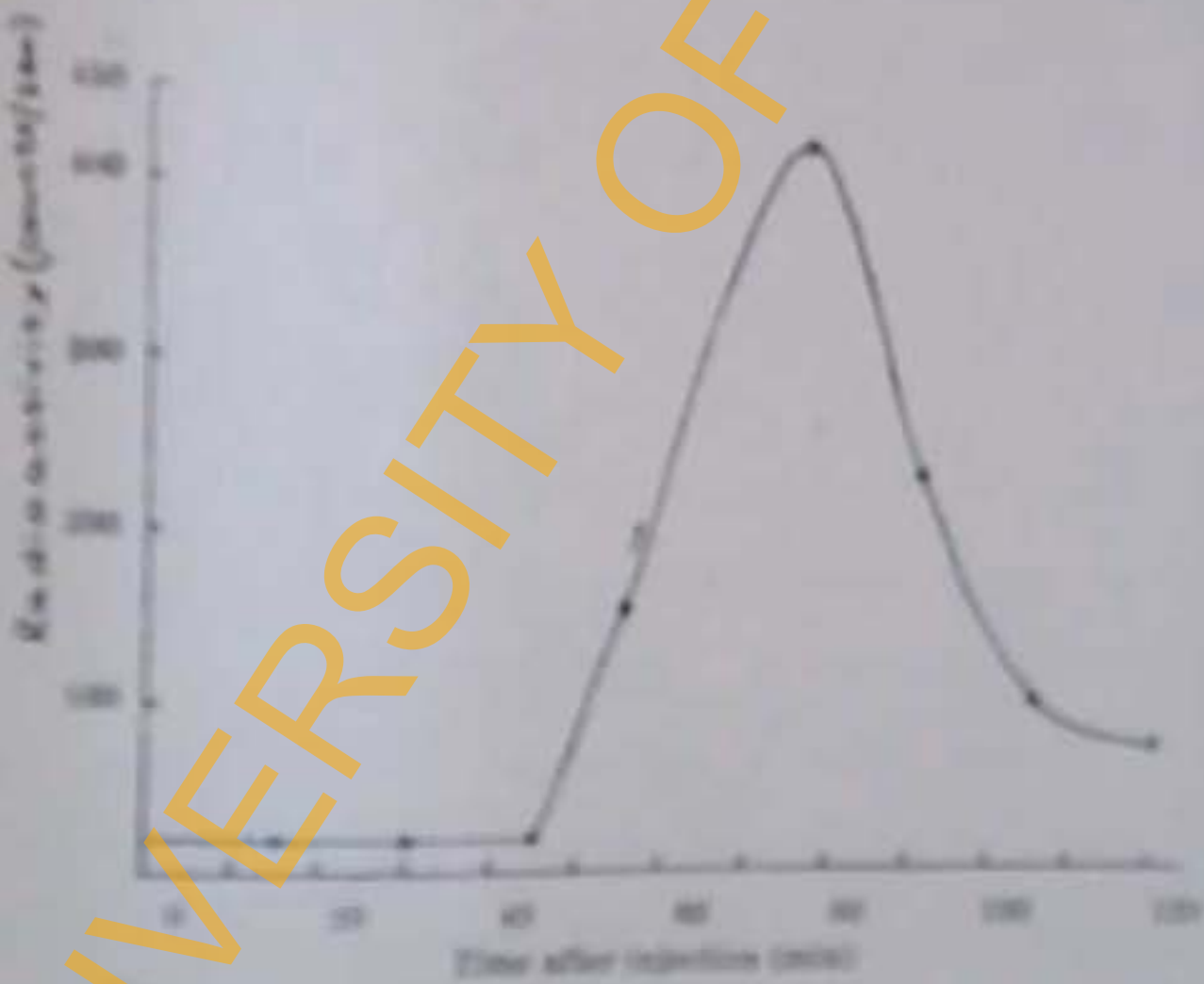


Fig 10

biliary cannulated rat (200g). The excretion of aflatoxin in the bile of the rat was investigated by the method of Hanahan, Daskalakis, Edward and Dauben (Jr.) (1953). This method had also been employed by Fischer, Millburn, Smith and Williams (1966) in their investigations on the nature of the biliary metabolites of [^{14}C]-stilbesterol in the rat.

(a) Bile

The rate of biliary excretion of aflatoxin is recorded in Fig.10. Following intraduodenal infusion of [^{14}C]-aflatoxin B_1 into the experimental animal, the portal blood samples were found to contain aflatoxin B_1 and its metabolites. These samples were analysed by thin-layer and paper chromatographic techniques, as described in chapter three, section v(a).

The rate of excretion of aflatoxin B_1 after a single dose in the rat has been reported by Bassir and Osiyemi (1967). This experiment confirmed an earlier observation by Falk, Thompson and Kotin (1965) that aflatoxin was rapidly excreted in the bile.

The presence of [C^{14}]-aflatoxin B_1 and its metabolites in the portal blood obtained from rat given intraduodenal infusion of [C^{14}]-aflatoxin B_1 in saline, indicated that aflatoxin B_1 or its metabolite was reabsorbed into the entero-hepatic circulation.

Analysis of the bile and faecal samples revealed that aflatoxin M_1 and its conjugates were the major metabolites in the bile. The faeces in the biliary cannulated rat was devoid of radioactivity. This observation is consistent with the view that metabolic products of aflatoxin might be excreted via the bile into the intestine, (Wogan, 1966).

(b) Urine

In order to evaluate the urinary excretion of aflatoxin in the rat, urethral catethers were implanted into a rat. Diuresis was stimulated by an infusion of 5 per cent mannitol in saline into the external jugular vein. 10 µ g of [C^{14}]-aflatoxin B_1 in saline was injected intraperitoneally into the rat. Urine samples were analysed by thin-layer and paper chromatographic methods.

Fig. 11: Cumulative excretion of [C^{14}] aflatoxin in the urine of the rat. The percentage of dose excreted after a given interval of time (T) in minutes was plotted against log T.

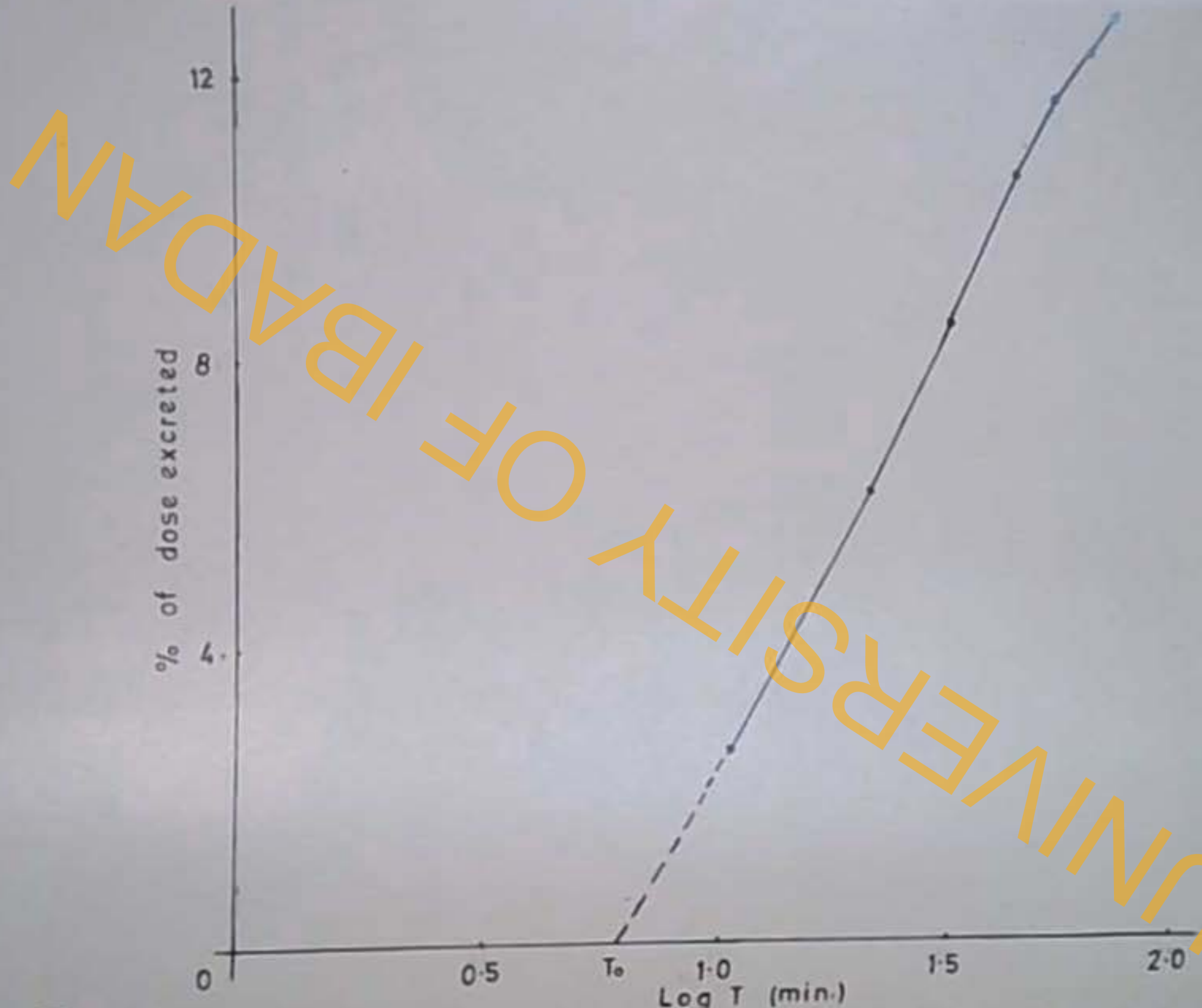


Fig. 11.

The result in Fig. 11 shows the cumulative excretion of aflatoxin in the urine of the rat. This pattern of excretion represents "the combined effect of the passage of a substance into (and out of) the intestitial fluid and the glomerular filtration" (Hough, Barnard and Bassir, 1955) after a single dose. The graphical representation shown in Fig. 11, suggesting that the excretion of aflatoxin was exponential, conforms to the equation of Stern (1955).

Experiment III Excretion of Aflatoxin B_1 and Its Metabolites in the Rabbit.

An adult male rabbit (1.5 kg) was given a single 50 µg dose of $[C^{14}]$aflatoxin B_1 in saline by intravenous injection. Another male rabbit (1.5 kg) was given the same dose of $[C^{14}]$aflatoxin B_1 intraperitoneally. The effect of the route of administration on the excretion of labelled aflatoxin was thus investigated.

Each rabbit was anaesthetized as described in chapter 3 and then given an intravenous infusion of 5 per cent mannitol in saline through the external jugular vein at the rate of 0.75 ml per minute. 3 to 4 ml of urine was collected in 5 minutes through implanted urethral catheters.

Bile was collected periodically from each animal via glass cannula inserted through the gall bladder into the cystic duct by the method of Child and Dodds (1966) which followed essentially the procedure of Hanahan, Daskalakis, Edwards and Daubon,Jr (1953).

Result

It can be seen from tables 7 and 8 that after intravenous injection, aflatoxin $[C^{14}]$, aflatoxin B_1 and/or its metabolites appeared in the urine and bile at an earlier period of time than when the toxin was injected intraperitoneally. It also appears that the main path of release of radioactivity from the labelled aflatoxin B_1 in the body was the bile.

TABLE 7: EXCRETION OF AFLATOXIN B_1 IN THE URINE OF RABBIT.

Time in Minutes	Intraperitoneal Injection			Intravenous Injection		
	Counts per sec.	Cumulative counts per sec.	Percentage dose	Counts per sec.	Cumulative counts per sec.	Percentage dose
0	0	0	0	0	0	0
10	0	0	0	98	98	1.4
20	25	25	0.4	33	131	1.9
30	52	77	1.1	27	158	2.3
40	78	153	2.4	18	176	2.6
50	53	206	3.1	14	190	2.8
60	34	240	3.8	10	200	3.0
70	19	259	4.0	8	208	3.2
80	11	270	4.2	6	214	3.3
90	5	275	4.3	3	217	3.3

TABLE 8: EXCRETION OF AFLATOXIN B_1 IN THE BILE OF RABBIT

Time in Minutes	Intraperitoneal Injection			Intravenous Injection		
	Counts per sec.	Cumulative counts per sec.	Percentage dose	Counts per sec.	Cumulative counts per sec.	Percentage dose
0	0	0	0	0	0	0
10	0	0	0	0	0	0
20	0	0	0	97	97	1.4
30	10	10	0.16	133	230	3.4
40	55	65	1.0	189	419	6.2
50	178	243	3.7	229	648	9.6
60	433	676	10.4	122	770	11.4
70	276	952	14.6	67	836	12.3
80	104	1056	16.2	35	872	12.9
90	87	1143	17.6	23	895	13.1

13.1 per cent of the dose administered and 17.6 per cent of the amount given intraperitoneally were recovered in the bile within 90 minutes. In none of these experiments was more than 5 per cent of the radioactivity found in the urine. On the basis of these results, it is apparent that the biliary excretion represents an important metabolic pathway of the metabolism of aflatoxin B_1.

It is known that a large proportion of a drug administered intraperitoneally is absorbed directly into portal blood which then flows through the liver before it reaches the systemic circulation (Garattini and Shore, 1966). Accordingly, if aflatoxin is metabolised in the liver as suggested by the experiments of Butler and Clifford (1966) and is rapidly excreted in the bile as demonstrated by Bassir and Osiyemi (1967), little of the administered dose will be found in the systemic circulation. In contrast, the toxin injected intravenously enters the systemic circulation directly and is distributed into both the intra- and extra-cellular fluids.

Experiment IV: Effects of Aflatoxin B_1 on the Liver of Rats on Low or High-Protein Diets

The liver is the largest organ in the animal body. It contains a system of enzymes which are concerned with the metabolism of foreign compounds. Williams (1967) has described those enzymes involved in the metabolism of natural substrates as 'Para-metabolic', whilst 'Xeno-metabolic' enzymes refer to the group of microsomal enzymes involved in the metabolism of foreign compounds. The influence of diet on the activities of these enzymes has been indicated by many investigators (Williams, 1938; McLean and Witschi, 1966; McLean and McLean, 1966).

There is a widely held belief that animals fed on a diet deficient in protein are especially susceptible to poisons that affect the liver (Newbern, Wogan and Hall 1966; Williams 1963). In this experiment the metabolism of acetate was investigated using liver tissues obtained from (a) rats poisoned by aflatoxin, (b) normally-fed healthy rats. 7 litters of weanling albino rats (males and females) were used. Each litter consisted of 6 rats,

making a total of 42 rats. These were divided into six groups as follows :- One rat from each litter, weighing about 50g, was transferred into a battery of cages such that each group contained 7 rats of about the same weight.

Two groups, composed of 14 rats were fed on a low-protein diet (4 per cent casein diet) and another pair on high-protein diet (25 per cent casein diet). The last two groups were fed on 15 per cent casein diet as a control.

A single dose of 5 μ g aflatoxin B_1 in 1 ml of saline (0.9 per cent NaCl) was administered intraperitoneally to half of the rats on each experimental diet. The other half was given equal volume of saline alone. This operation was repeated twice weekly for three weeks.

At the end of this period the rats were sacrifised. The liver was removed immediately afterwards and liver slices were prepared by a 'hand cutting' method described by Umbreit, Burris and Staufner (1964). These slices (weighing 57 to 70 mg wet-weight) were distributed into

Warburg flasks, containing Krebs-Ringer bicarbonate solutions (2 ml) and a solution of 10µC $[1\text{-}C^{14}]$-acetate (0.5ml) in the side arm. The solutions were mixed and then incubated in a water bath at 37°C for 90 minutes.

The $C^{14}O_2$ evolved in the process was absorbed into alkali (0.2ml of 5 per cent KOH) placed in the centre well. The amount of radioactivity incorporated into the acetone extractable liver fat was assessed by a procedure similar to that used by Barnes and Boothroyd (1961). The contents of the centre well were transferred quantitatively into excess $BaCl_2$ in a centrifuge tube. The $BaCO_3$ precipitated was counted at 'infinite thickness' in an end-window Geiger-Muller counter.

Result

The result in table 9a shows the relationship between the effects of Aflatoxin B_1 and diet on the incorporation of $[1\text{-}C^{14}]$acetate into rat-liver fat. Using the students 't' test, at a probability of 0.05, the value of 't' for six samples is 2.45. The result indicated that the effect of aflatoxin on rat on low-protein diet was significant

TABLE 9a

THE RELATIONSHIP BETWEEN THE EFFECTS OF AFLATOXIN AND DIET ON THE INCORPORATION OF [1-C^{14}]-ACETATE INTO RAT LIVER-FAT

Comparison of Treatments Given to Experimental Rats		No. of Sample	(Mean) Count/Sec./ mg Fat	Variance	't'	Probability
Low-Protein	+ Aflatoxin	6	112	22.7	2.7	< 0.05 Significant
	- Aflatoxin	6	105	9.3		
High-Protein	+ Aflatoxin	6	104	5.2	2.2	> 0.05 Not Significant
	- Aflatoxin	6	100	11.2		
Control	+ Aflatoxin	6	100	6.7	0.0	> 0.05 Not Significant
	- Aflatoxin	6	100	20.7		
Low-Protein Vs High-Protein	- Aflatoxin	6	105	9.3	2.5	< 0.05 Significant
	- Aflatoxin	6	100	11.2		
Low-Protein Vs Control	- Aflatoxin	6	105	9.3	2.6	< 0.05 Significant
	- Aflatoxin	6	100	20.7		
High-Protein Vs Control	- Aflatoxin	6	100	11.2	0.0	> 0.05 Not Significant
	- Aflatoxin	6	100	20.7		
Low-Protein Vs High-Protein	+ Aflatoxin	6	112	22.7	3.4	< 0.05 Significant
	+ Aflatoxin	6	104	5.2		
Low-Protein Vs Control	+ Aflatoxin	6	112	22.7	4.8	< 0.01 Highly Significant
	+ Aflatoxin	6	100	6.7		
High-Protein Vs Control	+ Aflatoxin	6	104	5.2	2.3	> 0.05 Not Significant
	+ Aflatoxin	6	100	6.7		

TABLE 9b

THE RELATIONSHIP BETWEEN THE EFFECTS OF AFLATOXIN AND DIET ON THE PRODUCTION OF CO_2 BY RAT LIVER SLICES.

Comparison of Treatments Given to Experimental Rats		No. of Sample	(Mean) Count/Sec./ mg Liver Wt.	Variance	't'	Probability
Low-Protein	+ Aflatoxin	6	15	5.7	6.2	< 0.001 Highly Significant
	- Aflatoxin	6	32	26.7		
High-Protein	+ Aflatoxin	7	41	23.7	0.7	> 0.05 Not Significant
	- Aflatoxin	7	43	22.0		
Control	+ Aflatoxin	7	35	11.9	1.8	> 0.05 Not Significant
	- Aflatoxin	7	39	14.0		
Low-Protein Vs High-Protein	- Aflatoxin	6	32	26.7	3.8	< 0.01 Significant
	- Aflatoxin	7	43	22		
Low-Protein Vs Control	- Aflatoxin	6	32	26.7	3.6	< 0.05 Significant
	- Aflatoxin	7	39	14.0		
High-Protein Vs Control	- Aflatoxin	7	43	22.0	1.7	> 0.05 Not Significant
	- Aflatoxin	7	39	14.0		
Low-Protein Vs High-Protein	+ Aflatoxin	6	15	5.7	19.8	< 0.001 Highly Significant
	+ Aflatoxin	7	41	23.7		
Low-Protein Vs Control	+ Aflatoxin	6	15	5.7	10.3	< 0.001 Highly Significant
	+ Aflatoxin	7	35	11.9		
High-Protein Vs Control	+ Aflatoxin	7	41	23.7	3.5	< 0.05 Probably Significant
	- Aflatoxin	7	35	11.9		

(t = 2.7), at 95 per cent confidence level. The 't' values for rats on high-protein and control diets are lower than this limit and are therefore not significant.

The influence of diet alone on the experimental rats was evaluated by comparing the 't' values obtained for the three different treatments either with aflatoxin or without. The results are recorded in table 9a. The effect of a low-protein diet was significant when compared with the controls or rats on a high-protein diet. There was however no significance in the differences between the controls and the rats on the high-protein diet.

The production of $C^{14}O_2$ by liver slices depends on the rate of oxidation of $[1\text{-}C^{14}]$acetate. The result presented in table 9b shows that this rate was depressed in rats on the low-protein diet by the administration of aflatoxin B_1 but not significantly affected in rats on the adequate diets.

The effect of diet, per se , on the rate of production of $C^{14}O_2$ was significantly different in the rats on low-protein diet when compared with those on control or high-protein diet. On the administration of aflatoxin, dietary influence was more marked the greater the differences in the dietary protein component.

Experiment V: Investigations on the Metabolism of Aflatoxin B_1 by Liver Tissues from Rats on Low- or High-Protein Diets.

In this experiment, the same number of experimental rats were fed on diets as described in experiment IV, but were not treated with aflatoxins. Within 15 minutes after sacrifice, liver tissues from each rat was homogenised in Krebs-phosphate buffer and then distributed among three Warburg flasks, containing 2 ml of medium. 100mg (wet weight) of tissue were placed in each flask. 10 µg [C^{14}] aflatoxin B_1 dissolved in the buffer (0.5ml) was put in the side arm. After mixing the contents of the flask, the preparation was placed in a water bath at 37°C and incubated for 2.5 hours with constant agitation as described by Craig (1943).

At the end of this period chloroform (5ml) was added to the mixture and then filtered through cotton. The residue was washed with chloroform. The combined chloroform extract of the filtrate and liver tissues, was concentrated to 0.5ml on a rotary evaporator and chromatographed on thin-layers

TABLE 10: METABOLISM OF AFLATOXIN B_1 BY LIVER TISSUES FROM RAT ON LOW OR HIGH PROTEIN-DIETS

Experiment	Wt. of liver tissue used (mg)	C^{14}-labelled Aflatoxin B_1 added to medium (µg)	Amount remaining in medium after incubation (µg)	%[C^{14}]-Aflatoxin remaining in medium after incubation	%[C^{14}]-Aflatoxin metabolised
Rats on Low-Protein Diet	100	10	7.6 ± 0.5	76	24
Rats on High-Protein Diet	100	10	0.8 ± 0.3	8	92
Control Rats.	100	10	1.1 ± 0.3	11	89

Each figure represents the average of nine determinations.

of silica gel G. The amount of [C^{14}]-aflatoxin B_1 remaining in the extract was estimated and recorded in table 10.

Result.

The result showed that about 90 per cent of the [C^{14}]-aflatoxin was metabolised by tissues from rats on control and high-protein diets. Less than one-third of this amount was metabolised by liver tissues from rats on low-protein diet. This represented a fall of 65 to 68 per cent in the ability of liver tissues from these rats to metabolise aflatoxin B_1 when compared with the controls and rats on high-protein diets respectively.

McLean and McLean (1966) reported a fall of 80 per cent in the activities of liver enzymes that demethylate pyramidon and hydroxylate benzopyrene in rats fed on a 3 per cent casein diet. A 50 per cent decrease was found in rats fed on 6 per cent casein diet.

The significance of our observation is that the toxicity of aflatoxin may be pronounced in the animal on inadequate diets. Slater (1966), however, has warned that results from in vitro experiments may not reflect the situation in the whole animal. For this reason, subsequent experiments were performed on biliary cannulated rats.

Experiment VI: The Effect of Dietary Protein on the Excretion of Aflatoxin B_1 and its Metabolites in the Bile of Rat.

There are references in the literature showing that molecular weight, polarity and metabolism of foreign compounds are factors which determine the excretion of foreign compounds in the mammals. Millburn, Smith and Williams (1967a), (1967b); Abou-El-Makarem, Millburn, Smith and Williams (1967a); (1967b).

The significance of the proportions of aflatoxin or its metabolites excreted through the bile in relation

Fig. 12: A biliary Cannulated Rat carrying a glass saddle for the collection of bile.

to other routes of excretion has, however, not been evaluated. In the next experiment, the excretion of aflatoxin B_1 and/or its metabolites in the bile and urine of rats fed on high or low-protein diets was investigated.

The bile duct of a rat on an experimental diet was cannulated, and bile was collected in a glass saddle as described by van Zyl (1958). A biliary cannulated rat under light ether anaesthesia is shown in Fig. 12. The rat was given an injection of 10 µg of $[C^{14}]$-aflatoxin B_1 in 1 ml saline intraperitoneally. The collected bile samples were withdrawn from the glass container at regular intervals. Urine and faecal materials were collected at the same time. The amount of radioactivity present in the samples were determined as described in chapter 3. Urine and faeces were analysed by the method of de Iongh (1964) and bile, chromatographed as described by Frosch and Wagener (1967). The ultraviolet and infrared spectra of metabolites of aflatoxin B_1 were recorded as described in chapter 3.

TABLE 11: EXCRETION OF AFLATOXIN B_1 AND ITS METABOLITES IN RATS

Time after administration of aflatoxin	25% Casein Diet (High-Protein)			4% Casein Diet (Low Protein)			15% Casein Diet (Controls)		
	Counts per sec.			Counts per sec.			Counts per sec.		
	Bile	Urine	Faeces	Bile	Urine	Faeces	Bile	Urine	Faeces
0 - 6 Hours	2380	236	-	400	258	-	2431	215	-
6 - 12 Hours	170	18	-	300	133	-	120	11	-
12 - 18 Hours	57	12	-	21	21	-	48	5	-
18 - 24 Hours	31	5	58	33	5	78	15	3	63
Total Counts	2538	271	55	1854	417	78	2614	234	62
Percentage of Dose	37.5	4.1	0.8	28.5	6.2	1.2	40.2	3.5	0.9

NOTE: Each figure in the table represents the mean of three determinations.

TABLE 12: METABOLITES OF AFLATOXIN B_1 PRESENT IN RAT BILE AND URINE SAMPLES

Aflatoxin B_1 and Metabolites	High-Protein Diet		Low-Protein Diet		Control	
	Percentage of Dose Excreted		Percentage of Dose Excreted		Percentage of Dose Excreted	
	Bile	Urine	Bile	Urine	Bile	Urine
Aflatoxin B_1	-	0.3	5.6	3.1	-	0.2
Aflatoxin M_1	7.5	1.8	11.4	1.6	11.5	3.0
De-methyl Aflatoxin M_1	5.6	-	1.4	-	7.1	-
Glucuronide	24.1	2.0	10.1	1.5	21.6	2.1

Each figure represents the average for five determinations.

Result

Fig. 10 shows the rate of excretion of aflatoxin in biliary cannulated rats.

The results in table 11 show that aflatoxin B_1 and its metabolites are excreted rapidly through the bile and that urine and faeces are minor routes for the disposal of the toxin. In the intact animal, the bile duct joins the pancreatic duct and empties into the duodenum. This may account for some of the toxin or its metabolites found in the gastrointestinal content by Shank and Wogan (1965). It was, however, demonstrated in experiment II that some of the aflatoxin B_1 excreted via the bile was reabsorbed into the entero-hepatic system. Results of the analysis of the bile and urine for metabolic products of aflatoxin B_1 are recorded in table 12. The ultraviolet and infrared spectra of aflatoxin B_1 and its metabolites are recorded in Figs. 13a, 13b, 14 and 15.

There were significant differences in the pattern of excretion of aflatoxin and its metabolites in the rat fed on low- and high-protein diets. Aflatoxin B_1 was absent from the bile of rat on high-protein diet, the major metabolite being a glucuronide conjugate of the toxin. Aflatoxin B_1 was excreted in the bile and urine of rats on low-protein diet Also, aflatoxin M_1, a toxic metabolite of aflatoxin B_1, was excreted in the bile of all the experimental animals. Recirculation of these potent toxins in the entero-hepatic system would expose the liver of a rat on an inadequate diet to greater damage. This could be of importance in the pathology of aflatoxin poisoning in the mammal.

The relationship of molecular weight of aflatoxin B_1 and its metabolites to the pattern of excretion of these compounds in the bile is discussed later.

Fig. 13a: Ultraviolet spectrum of Aflatoxin B_1 in Methanol recorded on the Unicam Sp.500 Spectrophotometer showing an absorption peak at 363mμ.

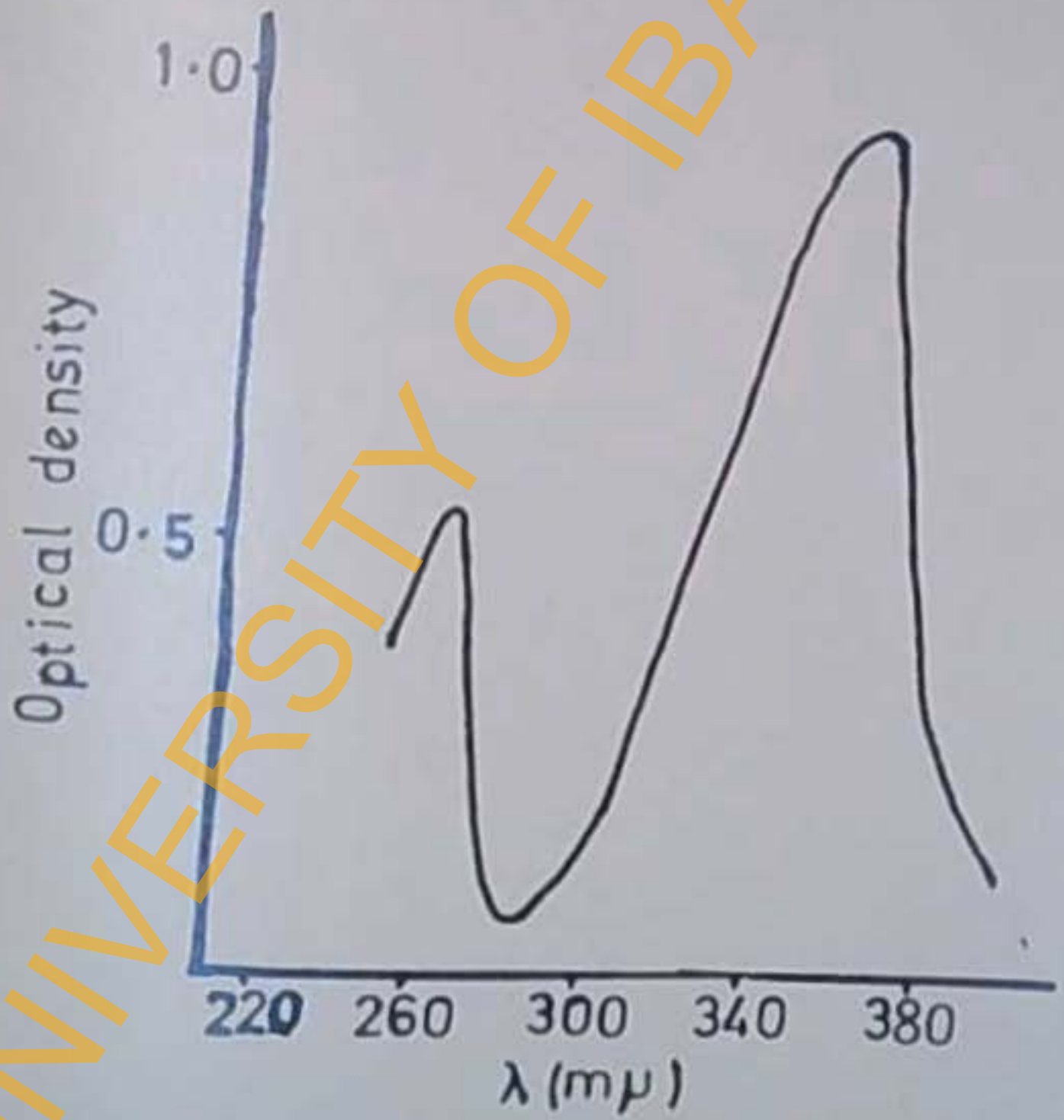


Fig. 13a.

Fig. 13b: Ultraviolet absorption of De-methyl Aflatoxin B_1 in methanol, showing absorption peaks at 310mμ and 350mμ.

Fig. 13b.

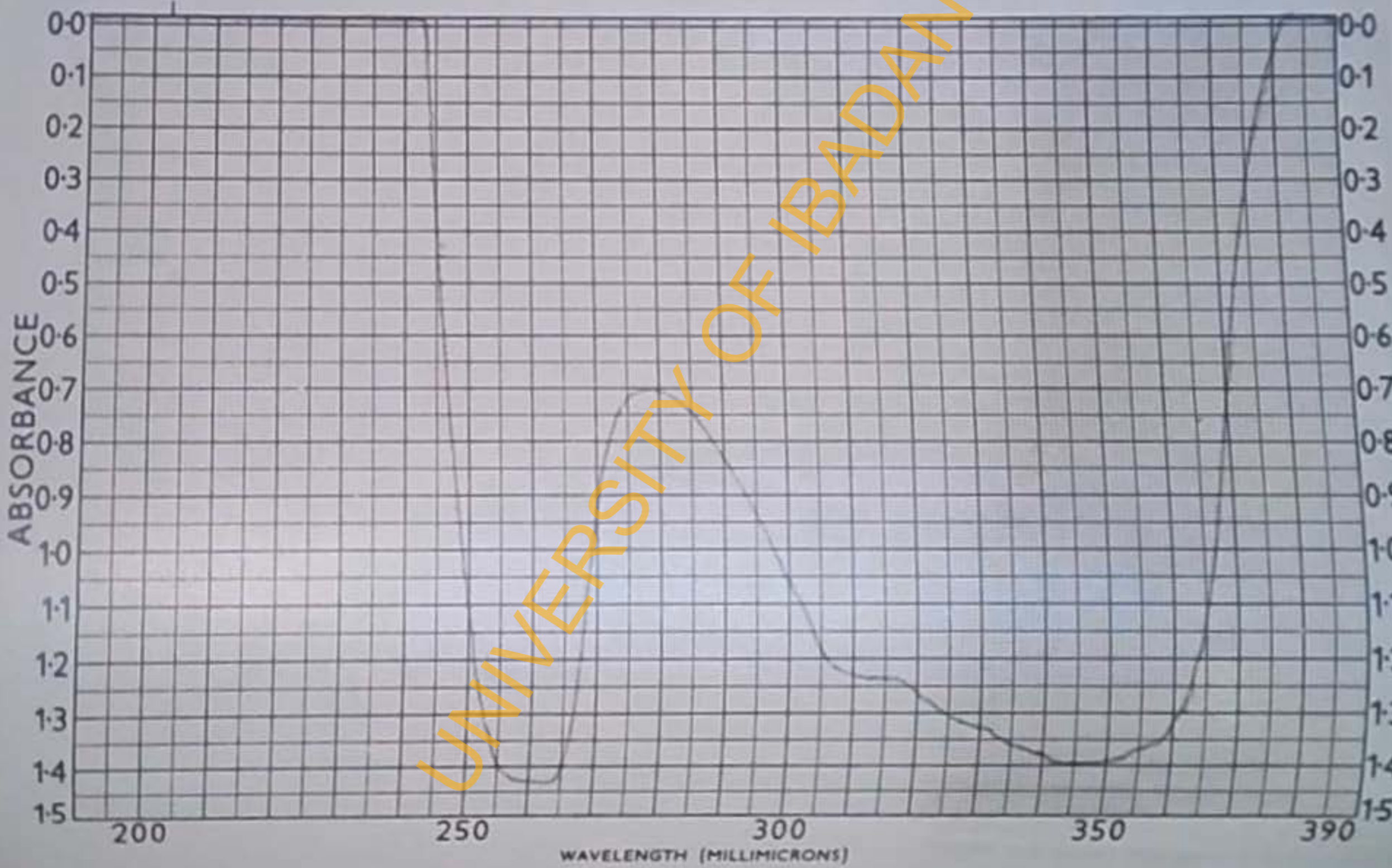


Fig. 14 Infra-red spectrum of Aflatoxin B_1 recorded on the Unicam SP.200 Spectrophotometer.

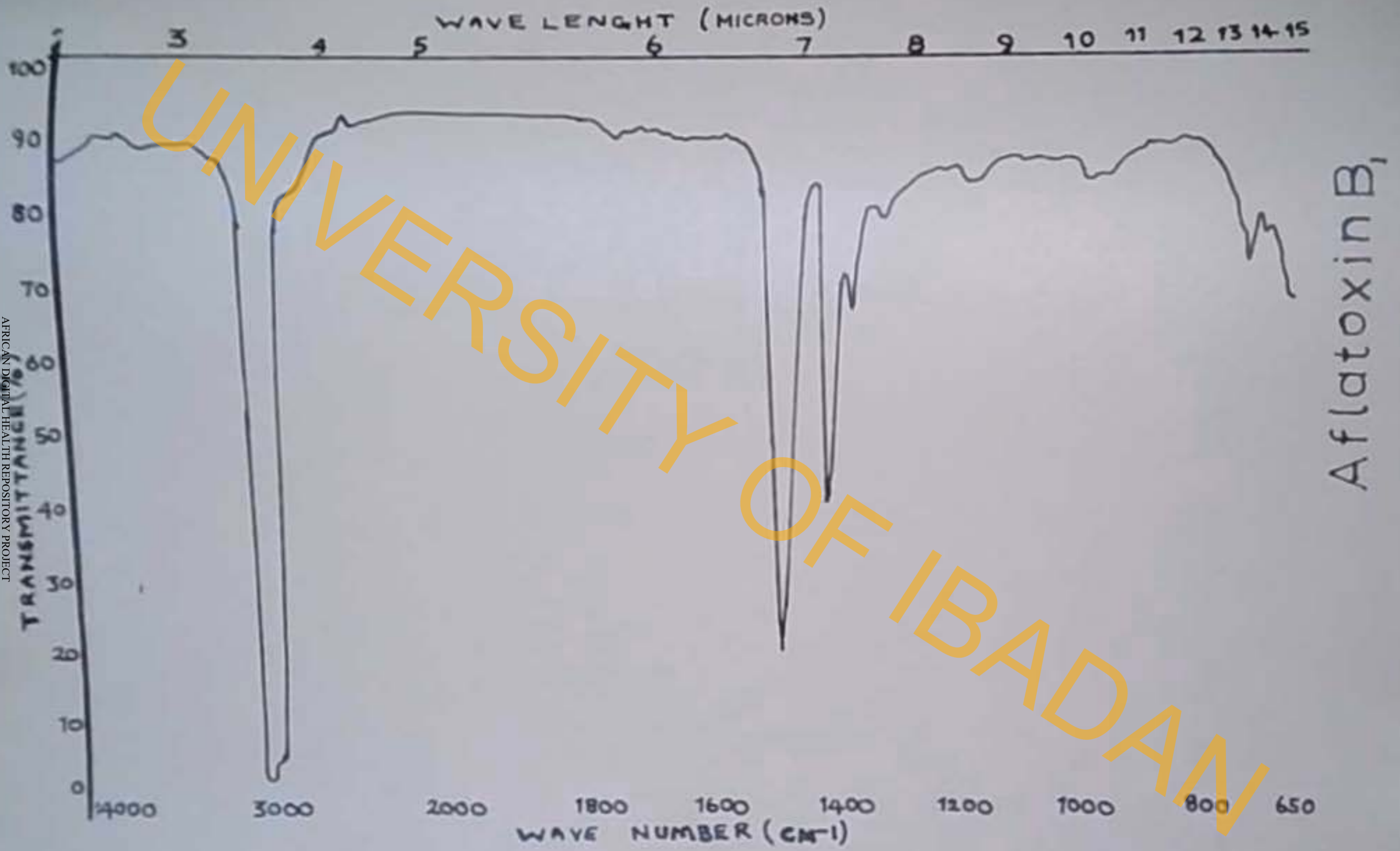
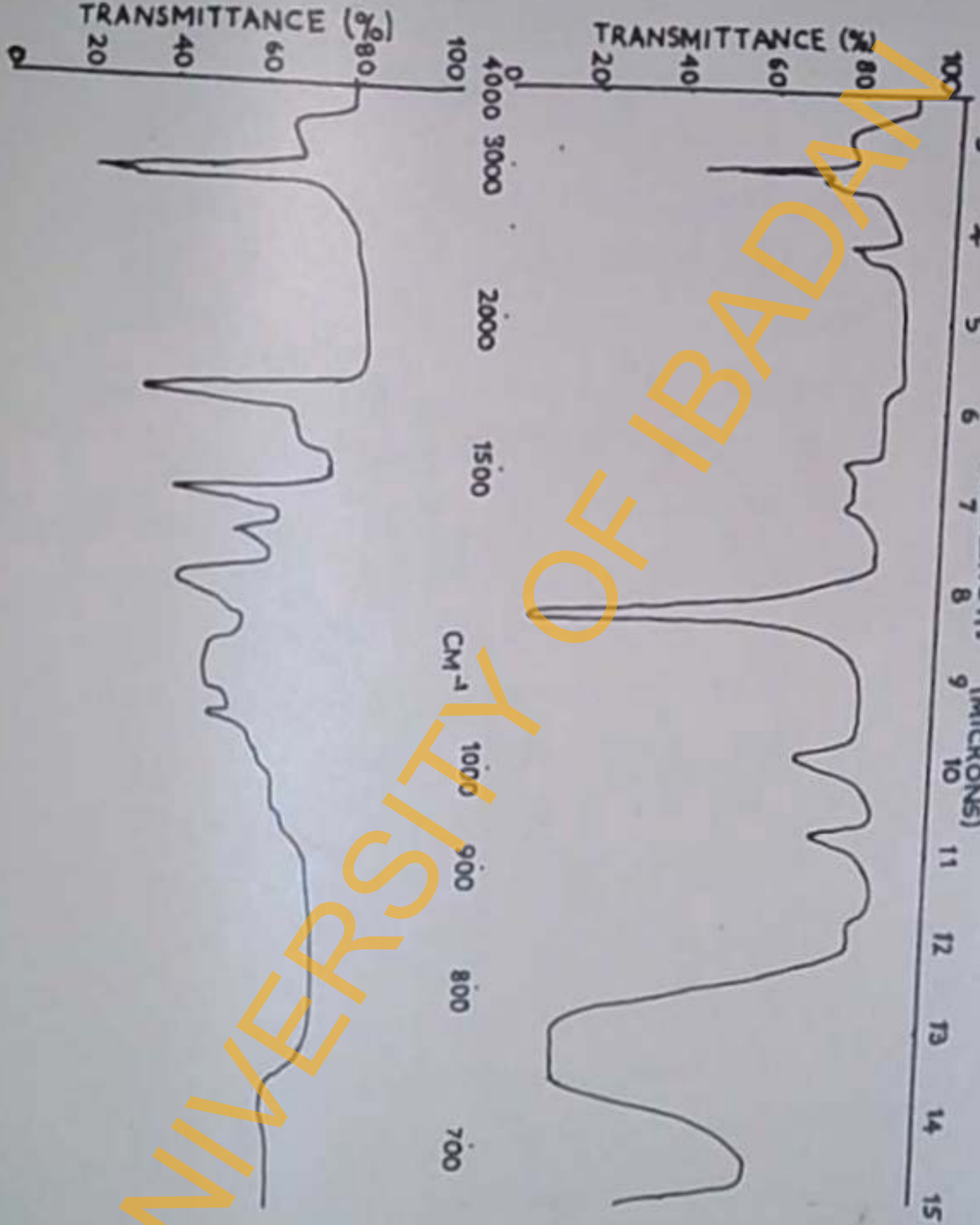


Fig. 14-

Fig. 15: Infra-cord recording, using NaCl crystals on Perkin-Elmer type spectrophotometer, showing infra-red patterns of two metabolites of aflatoxin B_1. Aflatoxin M_1 shows a maximum peak at 3475^{cm-1}, indicating the presence of -OH group (see table 1). The peaks in the 'finger print' region were diminished in demethyl aflatoxin B_1.

Fig. 15.



De-methyl aflatoxin B_1

Aflatoxin M_1

D I S C U S S I O N.

There are several reasons for studying the metabolism of aflatoxin in mammals. Firstly, the toxin has been found in human food as well as in animal feeds. Secondly, it is a potent carcinogen to young birds and mammals and concern has been aroused about the health hazard involved in the consumption of toxic diets. A third reason arises from the possibility that the transference of the hepatotoxic agent or its metabolites from the parent to the offspring, during intrauterine development or through the mother's milk may result in teratogenic effects on the embryo or gross malformation in the infant.

The possibility that the effect of marginally toxic feeds containing aflatoxin may be increased by rations not well balanced in energy and aminoacid has not been investigated. Recent reviews on the aetiology of infantile primary hepatoma indicate that diet as well as the presence of toxins in foods

may contribute to the high incidence of liver cancer in the underdeveloped parts of the world (Boyd, 1963). This view is strengthened by the observation that kwashiorkor, a disease of children due to protein deficiency, is widespread in the tropics and in certain areas of the world where chronic malnutrition abounds.

Robinson (1967) evaluated the incidence of infantile cirrhosis of the liver in India and attempted to correlate the consumption of peanut by mothers with the presence of liver cirrhosis in their infants. He found peanut-toffee in the possession of some of the mothers included in his survey, after they had denied eating it ! He therefore postulated that "since aflatoxin is excreted in the milk of cows and cirrhosis is due to this toxin, therefore if the infants are not exposed to this toxin from any other source, it has to be present in the milk of those mothers whose infants show clinical signs of liver cirrhosis".

It is however possible that aflatoxin may be found on proprietary food stuffs (Bassir, 1964). Recently, an infant food, (ARLAC) made from groundnut and milk powder was introduced into the market in Northern Nigeria. Adequate control must be exercised to prevent the growth of fungi and the production of toxins in these foods.

The work presented in this thesis is an attempt to explain some of the pathological effects of aflatoxin on the mammal. The toxin is known to be hepatotoxic, and may cause metabolic injury by altering the close relationships between the multienzyme systems in liver slices, homogenates and microsomes to which many authors have referred (Waterlow 1959, Williams 1963; McLean and McLean 1966, Williams 1967).

Glucuronide formation has been found to be a major route for the excretion of aflatoxin in the adult rat and rabbit. The amount of glucuronide formed in the rats fed on low protein diets was

lower than that found in animals fed on adequate diets. Williams (1963) has explained that any defect in the production of uridine diphosphate glucuronic acid (UDPGA) or in the activity of glucuronyl transferase could result in defective glucuronide synthesis.

The glucuronic acid utilized in glucuronide synthesis is derived from carbohydrates by a series of enzymically-controlled reactions. The level of glycogen in the liver of rats on low-protein diet is very low, and there is an accumulation of ketone bodies and liver fat. In these conditions, there is apparently a lowered capacity to conjugate drugs which are normally excreted as glucuronides.

In patients with congenital non-haemolytic jaundice, there is a fall in conjugation of bilirubin with glucuronic acid. It is believed that the defect is the result of reduced function of glucuronyl transferase. Glucuronide synthesis is also at a low level in young animals and in the

embryo, and here it appears that glucuronyl transferase and UDPGA are at a low level (Williams 1963). The decrease in toxicity of aflatoxin with age may, therefore, be a consequence of the development of this detoxication mechanism with age.

Waterlow (1959), has explained that there is a derangement of enzyme systems in animals on inadequate diet and that this lowers the resistance of these animals to toxic substances. He pointed out that a protein-deficient diet causes metabolic injury by altering the close relationships between the multi-enzyme systems. This derangement lowers the resistance of the cell to external stimuli. The induction of hepatoma and cancer in this circumstance is, therefore, a combination of bio-chemical and nutritional effects.

The composition of diet, and hence the state of the liver, may influence the metabolism of foreign compounds (Williams, 1938). This early statement emerged from observations that in the dog (and other animals) glucuronic acid for detoxication processess

could be derived from proteins, or synthesized from carbohydrates or aminoacids. Evidence is accumulating from recent studies that the activity of drug-metabolizing enzymes is depressed in animals on inadequate diets.

Seawright and McLean (1966) showed that young male rats fed on protein-free diet for one week are resistant to the lethal and hepatotoxic effects of carbon tetrachloride. These animals can be made sensitive again by injection of phenobarbitone in doses that induce synthesis of microsomal hydroxylating enzymes. This is in support of the hypothesis that carbon-tetrachloride is converted in the liver by the action of drug metabolizing enzymes into a molecule that is hepatotoxic to rats.

Madhavan and Gopalan (1965) demonstrated that rats fed on 4 per cent casein diet plus 50 µg of aflatoxin daily for two to three weeks develop the triad lesions of fatty liver, necrosis and biliary fibrosis, whilst the normal rat showed no defects. These authors therefore concluded

that aflatoxin does not exert its effect by inhibition of fibroblastic growth or fibrinogenesis, but by direct action on the liver cell.

Dickens and Jones (1963), Dickens, Jones, Waynforth et al (1966) had shown that a number of lactones and some other compounds containing a related chemical structure act as alkylating agents in reactions with cystein at ordinary temperatures and in neutral solutions. The striking exception in this series was aflatoxin, which reacts only slowly with the sulph-hydryl group of cystein. In other lactones, such as coumarin and hydroxycoumarin derivatives, opening of the lactone ring by hydrolysis resulted in loss of toxicity.

In our studies on the metabolism of aflatoxin in the mammal, the chemical structure of the compound and its metabolic fate were taken into consideration. In 1964, when we embarked on this investigation, the chemical structures of aflatoxin B_1 and G_1 had just been elucidated by Asao et al (1963). The metabolic pathways for the conversion of the toxin to non-toxic metabolites was not known. A toxic metabolite named 'milk toxin' was found in milk of

cows (de Iongh, Vles and Pelt, 1964). The structure of this substance was at this time an open question.

Our first endeavour was to develop a method for the production of large amounts of labelled aflatoxin that could be used in metabolic studies. This toxin was produced by Aspergillus flavus a common mould in Nigerian soils. A culture of this fungus in Ibadan produced large amounts of aflatoxin on Czapek-Dox medium. This mould had also been found growing luxuriantly on Nigerian stable diets (Bassir, 1964) and its presence in some of the traditional Nigerian foods such as ogi (fermented maize) and gari (a farinaceous grain made from cassava) had been regarded as a "problem of human nutrition in Nigeria", (Bassir, 1964). This fungus, as well as two other strains of Aspergillus, present in the microflora of local diets were investigated for the production of aflatoxin on synthetic media under varying experimental conditions used.

A better yield of aflatoxin was achieved in surface culture in contrast to the aerated culture.

The reduced redoxpotential of the medium (Visser, 1967c) under such conditions encouraged the accumulation of metabolic products by the fungus growing on Czapek-Dox medium. In these cultures, production of aflatoxin was encouraged during the growth phase. At the onset of sporulation in surface cultures, growth was minimal and there was a fall in the concentration of aflatoxin in the broth (see table 2). For these reasons, the cultures were harvested on the seventh day.

On addition of labelled isotope to a culture medium the labelled materials is channeled into a number of metabolic pathways. The utilization of isotopes such as $[1\text{-}C^{14}]$-acetate and $[2\text{-}C^{14}]$-acetate for the biogenesis of aflatoxin has been suggested by Adye and Mateles (1964). However, addition of 50 μC of $[1\text{-}C^{14}]$-acetate to the culture medium before starting incubation did not result in a high yield of labelled aflatoxin (Table 5). It was realised that half of the radioactivity was left in the medium on the third-day of incubation and, before the onset of the rapid growth phase of the fungus Aspergillus flavus. When $[2\text{-}C^{14}]$-acetate was used, and addition

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of the isotope to the culture was delayed for sixty hours after incubation had begun, aflatoxin of higher specific activity was obtained. A similar procedure has been described for the incorporation of C^{14}-labelled glucose into streptomycin (Hunter and Hockenhull (1955). It was observed that the incorporation of isotope was increased five times on delaying the addition of radioactive material until growth had proceeded for 60 hours. This suggested that in the first 48 hours of growth, the substrate was used largely for synthesis of mycelium and as a source of energy instead of being accumulated for aflatoxin synthesis.

The availability of C^{14}-labelled aflatoxin B_1 of high specific activity facilitated the identification and quantitative estimation of metabolic products of aflatoxin administered in a single dose to the rat. The use of isotopic derivatives in studies involving the identification of trace amounts of metabolic products has been employed by many investigators (Williams and Parke, 1953; Williams, Elliot and Parke, 1959; Eidunson and Gelier, 1963).

Shank and Wogan (1965) studied the distribution of C^{14}-labelled aflatoxin in the rat. Wogan (1966) reported that from 14 to 69.8 per cent of the radio-activity may be found in the intestinal content twenty-four hours after the administration of aflatoxin.

In our study of the effects of aflatoxin B_1 on the liver of rats (table 6) one-third of the dose was excreted via this route, six hours after administration of the C^{14}-labelled aflatoxin B_1 into a rat. When the bile duct of the experimental rat was cannulated, and the bile flow into the duodenum via the bile duct was thus prevented, 37.5 to 43.6 per cent of the administered dose was found in the bile content (table 11).

Williams(1963) suggested that an active drug is metabolised in the body by oxidation reduction, or hydrolysis. Drug activity is, therefore, terminated partly by the excretion of the active or inactive metabolite and partly by a second metabolic reaction. Among the coumarins and related compounds, hydroxylation is a common reaction. The hydroxylated product is then

conjugated with gluruconic acid. This reaction often leads to a considerable reduction in activity of the parent drug or its metabolites, which may be excreted in urine and/or bile. The excretory path way for aflatoxin might be similar to that enunciated above.

The results on table 11 suggest that aflatoxin B_1 and/or its metabolites were excreted via the bile duct into the intestine. The results in table 12 also show that 4 to 6 per cent of the dose was excreted in the urine. The significance of the mode of excretion of aflatoxin in relation to aflatoxin poisoning in rat on low-protein diet is discussed below.

Aflatoxin is a potent hepato-toxin. Metabolites of aflatoxin have been detected in the liver, half an hour after administration of the drug (Butler and Clifford, 1965). De Iongh et al (1963) reported the presence of metabolites of aflatoxin in milk. The 'milk toxin' (Mastri et al, 1967) was later found in urine by Holzapfel et al (1966). Evidence is presented in this thesis to show that the liver is a major site for the metabolism of aflatoxin in the rat. Table 10 shows the result of a comparative study on the

metabolism of aflatoxin by liver tissues obtained from rats on low-protein or on adequate diets. In this experiment, it is assumed that liver slices and homogenates, represent organised surviving tissue, the metabolism of which may reflect that of the whole organ. Laser (1942) has pointed out that this may not be so in all instances, and that results from in vitro experiments cannot be a substitute for evidence from the intact organism.

It was, therefore, considered necessary to compare the modes of excretion of aflatoxin in rats fed on low and high protein diets. Falk, Thompson and Kotin (1965) reported the appearance of fluorescent metabolites of aflatoxin in bile, after administration of the toxin to rat. The rate of excretion of aflatoxin B_1 in the bile after a single intra-peritoneal dose to male albino rats was measured and reported by Bassir and Oalyemi (1967) (see Fig.14) as "there seemed to be no published data on the quantitative pattern of biliary excretion of the toxin and the nature and partition of its metabolites in bile". These experiments have been extended to include a study of

the rate of excretion of the aflatoxin in the bile of rats under two different nutritional conditions. In table 19, it is shown that 37.5 per cent of the administered dose was excreted in a twenty-four-hour period by the rats on an adequate diet as compared with 28.5 per cent by those on low-protein diets. Analysis of the bile samples obtained from these rats (table 12) revealed that the rat on a high-protein diet excrete mainly conjugation products, but no free aflatoxin, and that the reverse was the case for the animals on low-protein diet. The activity of the drug metabolising enzymes is depressed in animals on inadequate diets (McLean and McLean, 1965). It is evident that the detoxication of aflatoxin was depressed in the rat on low-protein diet. Furthermore, since the rat has no gall bladder, the potent toxin excreted in the bile is passed into the duodenum and may be reabsorbed into the enterohepatic system. In such circumstance, the liver tissue is continuously exposed to the carcinogen. This may explain the observation by Madhavan and Gopalan (1965) that rats on low-protein diet are more susceptible to aflatoxin-

poisoning than those on adequate diets.

The following metabolites of aflatoxin B_1 have been identified:

(a) Aflatoxin M_1 is an oxidation product of aflatoxin B_1 (Holzapfel et al, 1966). This compound had been described as 'Milk-toxin' by De Iongh et al (1962). Variable amounts of this toxin were also found in the bile of rats on low-protein diet (17.4% of dose) and high protein diet (9.3% of dose).

(b) Aflatoxin M_1 glucuronide is a conjugate of aflatoxin M_1 and glucuronic acid. This conjugate is acid labile, which suggests that it is an ether glucuronide.

(c) De-methyl aflatoxin M_1 was isolated from rat bile.

Shank and Wogan (1965) fed methyl-C^{14}-labelled aflatoxin to rats and recorded a higher

Fig. 16: Shows the proposed pathways for the metabolism of Aflatoxin B_1 in the mammal. Aflatoxin M_1 glucuronide is the major metabolite in bile and urine. De-methyl aflatoxin was present only in the bile.

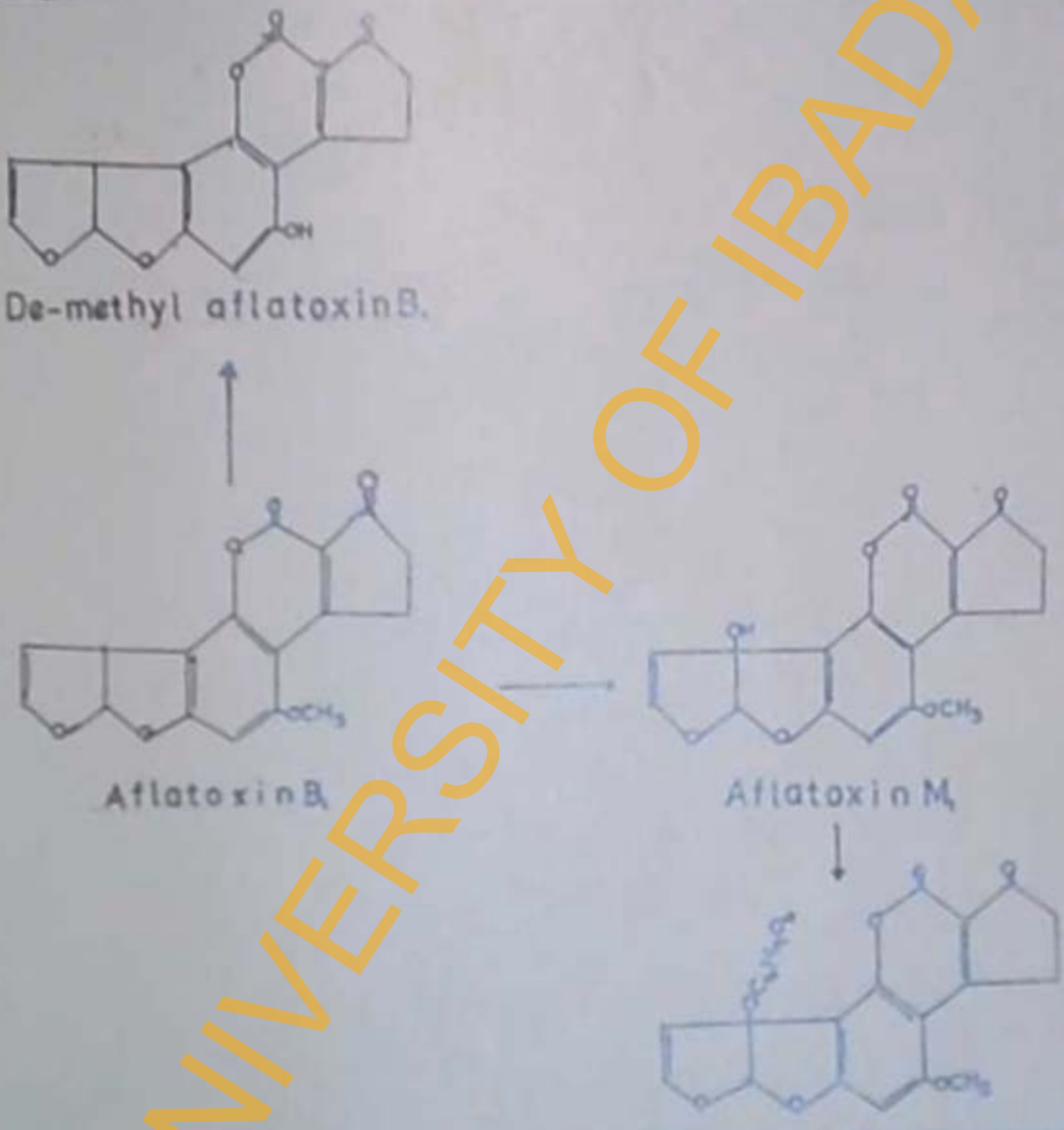

Fig. 16.

$C^{14}O_2$ in the respired air than when ring-labelled aflatoxin was used. The isolation of demethyl aflatoxin B_1 from bile of rats is therefore a further evidence that demethylation of aflatoxin B_1 probably takes place in the liver.

A proposed pathway for the metabolism of aflatoxin is illustrated in Fig. 16.

Abou-El-Makarem, Millburn, Smith and Williams (1967a) reported on the metabolism of some aromatic compounds of molecular weight less than 300 in the rat. The extent of biliary excretion of these compounds was low, being 0-10 per cent of the dose in 24 hours. It was concluded that simple benzene derivatives of molecular weight less than 300 are poorly excreted in the rat bile. Millburn, Smith and Williams (1967a) however, found that above this minimum value biliary excretion of foreign compounds depends on their molecular weights and the presence of a strongly polar anionic group.

The molecular weights of aflatoxin B_1, aflatoxin M_1 and aflatoxin M_1 glucuronide are 312, 328 and 505

respectively (calculated on formula basis). It is suggested that if the necessary molecular weight and polar groups can be acquired by metabolism, the biliary excretion of aflatoxin B_1 and its metabolites will be facilitated (See table 12). This may explain the difference in the rate of excretion of metabolites of $[c^{14}]$-aflatoxin B_1 by rats on adequate diet compared with those on low-protein diets. The rate of biliary excretion of $[c^{14}]$aflatoxin B_1 in rats on 25 per cent, and 15 per cent protein diet was significantly higher than that in rats of 4 per cent casein diet.

A clear species difference in the extent of biliary excretion was found between the rat, and rabbit. Aboo-El-Makarem, Millburn, Smith and Williams (1967b) described the rat as a "good excretor" of foreign compounds, and the rabbit a "poor excretor", (see tables 8 and 11). The reasons for this species differences in the excretion of aflatoxin has not been explained. Some of the reasons for species differences in drug metabolism, especially those of an enzymic nature, have been discussed by Williams (1967).

There are many well-known methods of interpreting data on the urinary excretion of drugs and their metabolites. Stern (1957), Haigh and Reiss (1950) found that the cumulative excretion of a drug is linearly related to the logarithm of the time. Hough, Barnard and Bassir (1955) explained that a discontinuity which occurs in the linear relationship in the case of inulin, (and similar substances) is the combined effect of the passage of inulin into (and out of) the intestitial fluid and glomerular filtration. For the low dosage of aflatoxin used in this investigation, the quantity of aflatoxin excreted showed an exponential growth curve with time; and the cumulative excretion of the drug is linearly related to the logarithm of time as indicated in Fig. 11. In this curve is defined by the equation

$$U = U_0 \exp. (- kt), \quad \text{(Haigh and Reiss, 1950)}$$

where U_0 = Original dose administered

U = Cumulative excretion of toxin

k = Rate constant for elimination of the drug

t = Time in seconds

then a straight line equation is obtained by putting the equation in its logarithmic form, i.e.

$$\text{Log } U = -\frac{kt}{2.303} + \text{Log } U_o$$

This graphical representation shows that the excretion of aflatoxin can be expressed by the Haigh and Reiss's equation.

SUMMARY OF RESULTS

A.

(i) Aspergillus flavus (75) was found to give good yield of aflatoxins on Czapek-Dox medium.

(ii) Incorporation of labelled isotopes into aflatoxin was enhanced by delaying the addition of labelled material for 60 hours after incubation at 27°C had commenced.

(iii) The highest specific activity of C^{14} - labelled aflatoxin B_1 achieved, when [2-C^{14}]-acetate was used as substrate, was 30m μ c/mM.

B.

After administration of a single dose of C^{14}-labelled aflatoxin B_1 to experimental animals intraperitoneally, the following observations were made.

(i) In the rat, 3.4 per cent of the original dose was excreted in urine and over 40 per cent

of the drug passed through the bile fluid in 6 hours. The large amounts of radio-activity found in the gastro intestinal tract was probably derived from the excretion of the toxin via the bile into the intestine.

(2) The rate of biliary excretion of aflatoxin B_1 in the rat was determined. In the rat given aflatoxin B_1 in a single intraperitoneal dose the peak level, was attained approximately 75 minutes after administration.

(3) Chromatograms of bile samples from experimental rats on thin-layer plates of silica gel G showed four fluorescent spots which corresponded to radioactive peaks on a scan of representative radio-chromatogram.

The following metabolites were identified:

(a) Aflatoxin M_1

(b) De-methyl aflatoxin B_1

(c) A glucuronide conjugate of aflatoxin M_1

At a dose of 50 µ g/kg weight for rat, on a normal diet, free aflatoxin B_1 was absent from bile samples. Bile samples obtained from rats on a low-protein diet, however, contained free aflatoxin. About 15% of the dose was excreted in this form.

(4) Analysis of urine sample gave two blue fluorescent metabolites. These were aflatoxin M_1 and a glucuronide conjugate. Free aflatoxin was present in urine samples obtained from rats on low-protein diets. Only traces or none of free aflatoxin B_1 was found in urine from rats on high-protein diet and the rabbit.

(5) Relative distribution of aflatoxin and its metabolites in different organs after a single dose (50 µ g/kg) was given intraperitoneally to albino rat was assessed. The toxin was present in the liver, kidney and intestinal contents.

(6) Liver slices or homogenates were prepared from rats fed on experimental diets and used to determine the rate of detoxication of aflatoxin in vitro. Liver samples from rats fed on high-protein diets were found to metabolise the drug faster than samples from animals on low-protein diet by a factor of three.

CONTRIBUTION TO KNOWLEDGE.

1. A new procedure for the incorporation of $[2\text{-}C^{14}]$-acetate into aflatoxin is described. The specific activity of the C^{14}-labelled aflatoxin produced was 30m μC per mM

2. Evidence is presented to show that the rate of detoxication of aflatoxin was depressed in the rat fed on a low-protein diet; and that the circulation of the toxin in the entero-hepatic system might be an important factor in aflatoxin poisoning.

3. A pathway for the metabolism of aflatoxin in the mammal is proposed.

4. A new metabolite of aflatoxin, de-methyl-aflatoxin B_1 has been isolated and identified.

5. It has been shown that free aflatoxin B_1 is excreted in the bile of the rat while feeding on a low-protein diet. Only conjugates of aflatoxin were found in the bile of rats fed on high-protein diets.

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