"SPECIES DIFFERENCES IN THE 'IN VITRO' METABOLISM OF AFLATOXIN B_1 AND PALMOTOXINS B_o AND G_o."

THESIS PRESENTED BY GODWIN ONYEMORO EMEROLE B.SC. (IBADAN)

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Investigation of the variation in total toxin, pH and mycelial weight, when Aspergillus flavus was grown on palm sap medium and yeast extraction medium. 102

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ABSTRACT

An initial experiment was carried out on the variation in the production of toxins with days of incubation by Aspergillus flavus Link grown on palm sap medium. This was compared with the production of toxins by the fungus grown under identical conditions on the yeast extract sucrose medium. The attendant variations in pH and weight of mycelial matt were recorded. Peak production of toxin on palm sap medium was observed on the 5th day of incubation while peak production on yeast-extract medium was on the sixth day.

Further investigation of some physical characteristics of palmotoxins Bo and Go have been carried out, Ultraviolet, Infra-red, Nuclear magnetic resonance and fluorescence spectra of palmotoxins Bo and Go have been obtained. When compared with known aflatoxins similar features are discernible.

The susceptibility of 20-day old rats to sub-lethal doses of palmotoxins Bo and Go were tested. Decrease in weight in relation to dosage; increase in the value of serum glutamic-oxaloacetic acid transaminase and serum alkaline phosphatase, when compared with control animals injected with carrier solvent, were observed in the case of palmotoxin Bo. There was, however, no remarkable difference in the above indices of toxicity for animals treated with palmotoxin Go. Animals treated with aflatoxin B_1 were also used for comparison.

The metabolism of aflatoxin B_1 and aflatoxin G_1 in

the rat *in vitro* have been compared. Two metabolites of aflatoxin B_1 believed to correspond to aflatoxins M_1 and B_{2a} respectively were observed on t.l.c. Similarly, two isolates from aflatoxin G_1 - probably the 'GM' and G_{2a} were also observed on thin layer plates. The rat liver microsomal fraction hydroxylated and demethylated aflatoxin B_1 at a higher rate than aflatoxin G_1.

The effect of variation of co-factors on the enzyme activity of the microsomal-plus-soluble fractions of rat liver with respect to the metabolism of palmotoxin Bo and palmotoxin Go were investigated. Effects of variation of the concentration of NADP and changes in the pH of incubation medium on the demethylation of palmotoxins Bo and Go were also investigated. Optimal conditions for the metabolism of palmotoxins Bo and Go were thus obtained.

Species variation in the '*in vitro*' metabolism of aflatoxin B_1 have been confirmed using eleven commonly available species. Using methods identical to those for the study of aflatoxin B_1 metabolism, the metabolism of palmotoxins Bo and Go have been investigated in the same animals, using both liver slices and microsomal-plus-soluble fractions of their livers. In general, a higher rate of metabolism was recorded for aflatoxin B_1 in all test animals than for palmotoxins Bo and Go. The trend in metabolite formation from aflatoxin B_1 and palmotoxin Bo appear similar. Evidence was obtained to show a great variation in the amount of isolates and the demethylation by test animals.

The effect of phenobarbitone treatment of animals on the enzyme activities has been studied. Pretreated animals showed a higher rate of hydroxylation and demethylation in all three toxins. Carbon monoxide inhibited the hydroxylation and demethylation of the toxins.

QUOTATION OF REFERENCES

The following systems of quotation of references were adopted in this thesis

(i) For a single author - the name of the author and the year of publication.

(ii) For two to four authors - the names of the authors and the year of publication.

(iii) For five or more authors - the name of the author first mentioned accompanied by et al. and the year of publication.

The System of Journal Abbreviation

The system of journal abbreviation used in the list of references in this thesis is that adopted in the World List of Scientific Periodical, 4th Ed. Butterworth, London, (1963, 1964, 1965)

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CHAPTER I

INTRODUCTION

MYCOTOXINS:

(a) Historical background:

Moulds have long been recognised as biochemically significant, primitive plants, capable of causing great economic losses. However, it was not widely recognised until recent years that certain common species may produce toxic metabolites besides those responsible for rendering food unacceptable. (Barger, 1931; Christensen, 1957).

Mycotoxins, therefore, belong to a group of mould metabolites produced mainly on foodstuffs and capable of causing illness or death when ingested by man or animals. Scott (1965) has shown the possible existence of fungal metabolites toxic to higher organisms. The various disease syndromes so induced are called mycotoxicoses.

Forgacs and Carll, (1962) studied the problem of mycotoxins with respect to the field of veterinary science but their findings did not appear to have been appreciated

then. The disastrous acute effects of mouldy feeds on turkey poults and ducklings and the attendant hepatic carcinoma in rats drew more attention to the biological importance of mycotoxins, (Carll, Forgacs, and Herring (1954); Forgacs, Carll, Herring, and Mahlandt (1954); Burnside et al, (1957); Blount, 1961). Attention has been paid mainly to diseases in poultry, swine and cattle arising from mycotoxins isolated from mouldy corn diets. Successful studies on stachybotryotoxicoses, a disease primarily affecting horses, cattle and man resulted in the organisation of a laboratory in Russia in 1940 for the study of toxic and pathogenic fungi (Sarkisov, 1947). Work on the influence of fungal-contaminated rice and studies on the carcinogenic effect of actinomycin by some Japanese workers, gave further emphasis on the role of fungal metabolites in cancer.

(b) Mycotoxins from food borne fungi:

Several fungal metabolites now known to have deleterious effects on both man and animals, have been isolated since it became clear that this group of substances constitute a formidable threat to human existence. These substances

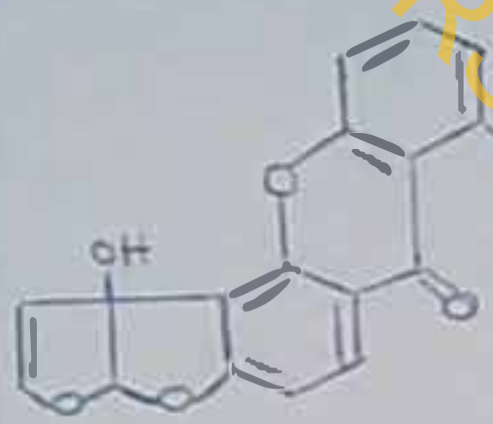
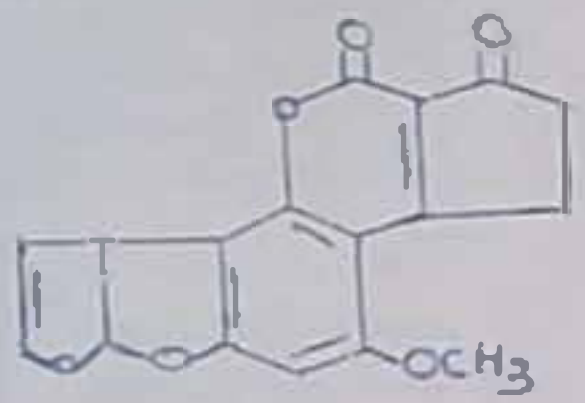
are believed to arise as secondary metabolites of fungi and the scheme below has been proposed by Bentley and Campbell (1968) as possible pathways for the formation of the fungal metabolites (SCHEME I).

The accompanying table summarises some of the known mycotoxins, their origin and possible effects, together with the relevant references.

SCHEME I

Secondary metabolism of fungi

TABLE I

MYCOTOXIN	STRUCTURE	ORIGIN	MAIN TOXIC EFFECTS	REFERENCES
ASPERTOXIN		ASPERGILLUS FLAVUS	Hepatotoxic	RODRICKS J.V., et al (1968) (a&b)
AFLATOXIN B_1		ASPERGILLUS FLAVUS	Hepatotoxic	Smith and Hibernan (1962); Allcroft and Carnaghan (1962)

I Contd Page 5

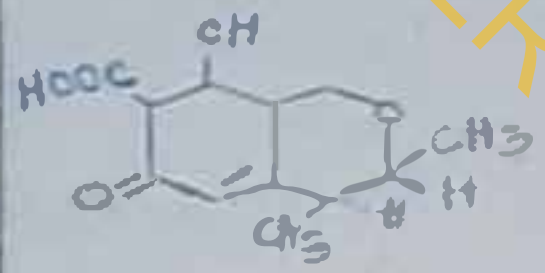
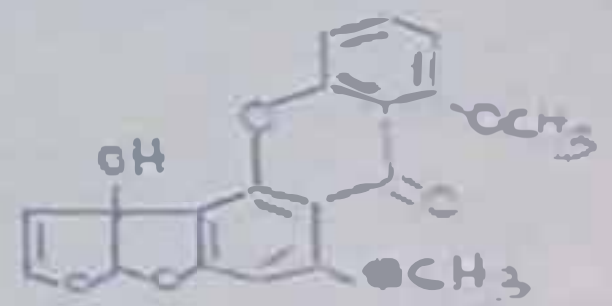
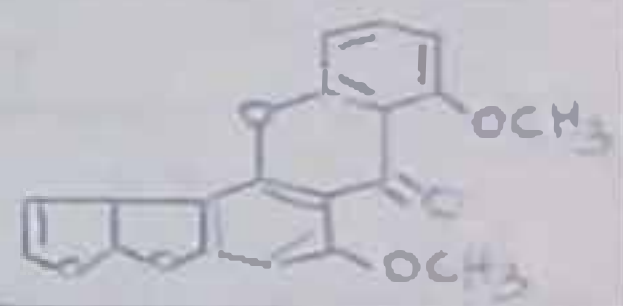
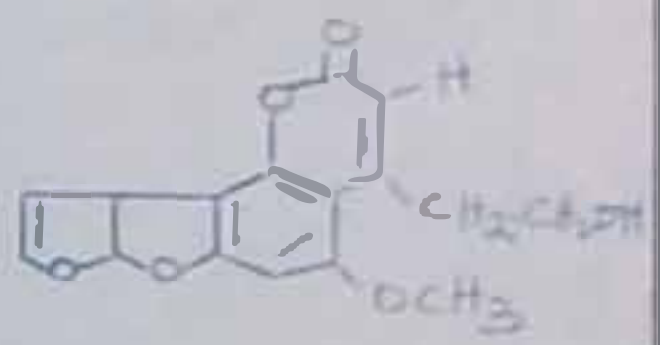
MYCOTOXIN	STRUCTURE	ORIGIN	MAIN TOXIC EFFECT	REFERENCE
CITRININ		PENICILLIUM CITRINUM	ANTIBIOTIC	PEREL (1966)
STERIGMATOCYSTIN		ASPERGILLUS VERSICOLOR	Hepatotoxic	Davis, Kirtsidy and Roberts (1968); Dalleck, Roberts and Underwood (1968)
DIHYDROSTERIGMATOCYSTIN		ASPERGILLUS FLAVUS	Hepatotoxic	BUCHANAN and FORGACS (1968)
AFLATOXIN		ASPERGILLUS FLAVUS	Hepatotoxic	Hartcroft and [illegible]

Table I Page 6

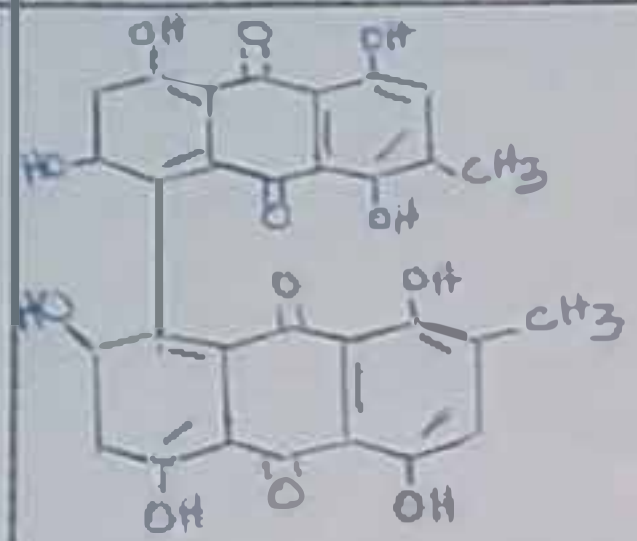
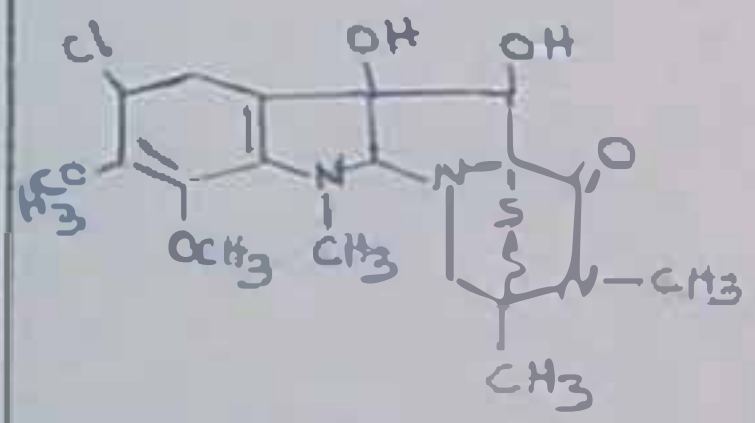
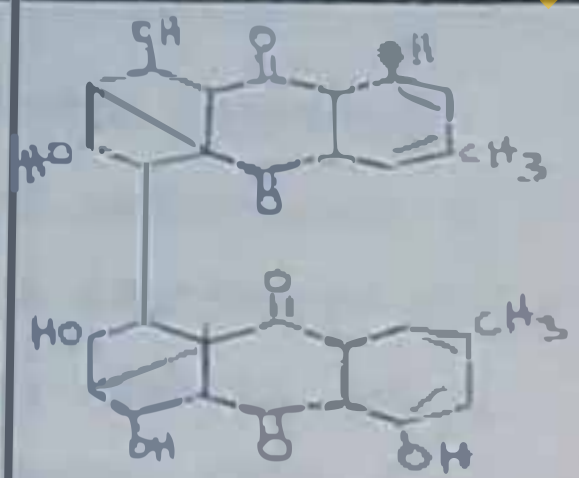
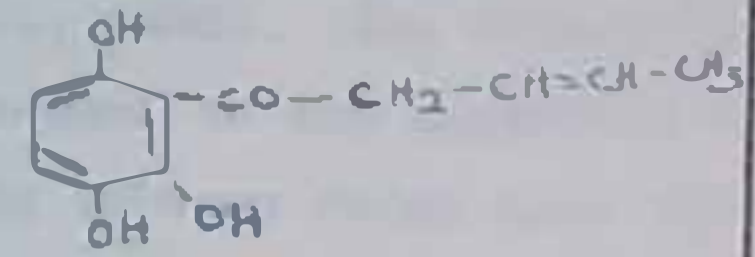
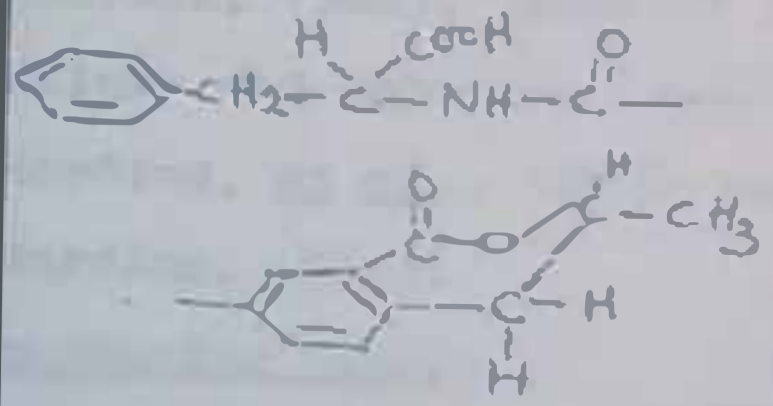
DTOXIN	STRUCTURE	ORIGIN	MAIN TOXIC EFFECTS	REFERENCES
ULIN		PENICILLIUM URTICAE	ANTIBIOTIC	WOODWARD AND SINCE 1950
OSTRIN		PENICILLIUM ISLANDICUM	TUMORIGENIC AGENT	Shibata and Kitagawa, 1958 and 1960.
RIDESMIN		PITHOMYCES CHARTARUM	FACIAL ECZEMA OF SHEEP AND CATTLE	Diddens et al 1962 Steyn and White 1959

Table 1 page 7

MYCOTOXIN	STRUCTURE	ORIGIN	MAIN TOXIC EFFECTS	REFERENCES
ULOSIN		PENICILLIUM RUGULOSUM PENICILLIUM BRUNNEUM PENICILLIUM TARDUM	Fatty degeneration Cetrolobular necrosis chronic hepatomas	Shibata and Kitagawa 1956 Kinosita and Shibata 1965
TOXIN		Mold isolates from rice	Non-specific	Limeira 1962
BATOXIN A		ASPERGILLUS OCHRACEUS	Hepatotoxic	Van der Merwe et al (1965)

THE AFLATOXINS:

(a) History:

The aflatoxins represent a group of fluorescent secondary fungal metabolites discovered as contaminants in animal feeds (Smith and McKernon, 1962; Allcroft and Carnaghan, 1962; Nesbitt, et al., 1962; Broadbent, Cornelius and Shone, 1963; Coomes, et al, 1964; and Coomes, et al., 1965). The discovery of these toxins had been sequel to the observation of toxicity syndromes in young turkeys which were fed with mouldy peanuts; (Blount, 1961). Toxicity syndromes of domestic animals ultimately attributed to aflatoxins had earlier been recognized in England by 1960 and 1961 (Stevens, et al. 1960; Swarbrick, 1960). Similar incidents were reported in ducklings and chickens (Asplin and Carnaghan, 1961); Swine, (Harding, et al., 1963); and Calves, (Loosemore and Harding, 1961).

(b) Aflatoxin-producing fungi:

Sargeant, et al. (1961) associated toxicity of feeds, with heavy mould infestation of feedstuffs and demonstrated that the toxic agents were produced by certain strains of *Aspergillus flavus* isolated from such meals. It has

been shown (Hilner and Geddes 1954), that this fungus is a common one distributed throughout the world. It has also been established that other species of fungus can produce aflatoxins (Codner, Sargeant and Yeo, 1963; Hodges, et al. 1964; Murakami, Ohaki and Tatsse, 1966; Kulik and Holaday, 1967; Basappa, et al. 1967).

Prominent among these fungi are Aspergillus parasiticus; A. Parasiticus var. globosus; A. oryzae; Penicillium tuberculum; other species of both Aspergillus and Penicillium which have been shown to produce aflatoxins include A. niger; A. wentii; A. ruber; P. variable and P. frequentans. Penicillium citrinum has been shown to produce aflatoxin B_1 almost exclusively (Kulik and Holaday, 1967).

Scott, van Walbeek and Forgacs (1967) have shown that Aspergillus ostianus produces aflatoxins. van Walbeek, Scott and Thatcher (1968) have also demonstrated the production of aflatoxins by Aspergillus ochraceus and Aspergillus rhizopus. However, the amount of toxin produced and the composition, have been shown to vary with the strains, (Codner, Sargeant and Yeo, 1963; Hesseltine, et al. 1966; Diener and Davis, 1965; Boller and Schroeder 1966; Purchase, Steyn and Pretorious, 1968; Joffe, 1969).

(C) Media for aflatoxin Production:

I. Natural Media:

Differential production of aflatoxins with respect to the substrate on which the respective fungi grow, has been demonstrated, (Wayne, et al. 1966; Stubblefield, et al. 1967). Hesseltine, et al. (1966) demonstrated the production of aflatoxins by three strains of Aspergillus flavus when grown on six major agricultural commodities - sorghum, peanuts, soybeans and rice, with varying amounts of toxin recorded for each substrate. These some strains produced quantities of aflatoxin B_1, ranging from 77 - 288 ug/gm on whole oats, 35 - 593 ug/gm on groats and 0.15 to 4.2 ug/gm on oat Hulls; (Stubblefield, et al. 1967). Wayne, et al. (1966) found that sterilized whole cotton seed and kernels supported the production of aflatoxin, equivnlent to that produced on wheat but twice the levele on peanut. Numerous natural substrates have been shown to support the growth of Aspergillus flavus with the consequent production of aflatoxins. These include walnuts, poppy seeds, coconut, apple juice, and potato products; (Frank, 1966). Bassir and Adekunle (1972)

have reported the production of aflatoxin B_1 in many Nigerian fruits. These include pawpaw, sweet orange, banana, plantains and a host of others. Borker, et al. (1966); Kraybill and Shimkin, (1964); and Hesseltine, et al. (1966), have demonstrated weak aflatoxin production on soyabeans or soya proteins. It is thus seen, that the growth of Aspergillus flavus and the attendant production of the aflatoxins spreads through a variety of natural substrates.

II. Nutrient Media:

Production of aflatoxin from liquid medium in comparable quantities with natural substrates has not been possible. Wildman, Stoloff and Jacobs (1967) have suggested that this may be due to large surface areas exposed to air in natural substrates and to high nutrient to moisture ratios. Small quantities of aflatoxin have been obtained from a modified Czapeks dox medium containing zinc sulphate in addition, (Nesbitt, et al. 1962; Armbrecht, et al. 1963). Some other investigators have used this medium with the addition of yeast-extract to improve the yield, (Wallbridge, 1963; and Spensely, 1963). The glucose-ammonium nitrate medium of Brian, et al.

(1961) has been employed by Van der Zijden, et al. (1962); de Iongh, et al.(1962)and Lafont (1963), for the production of aflatoxins.

Matsles and Adye (1965); Ciegler, et al. (1966) and Hayes, Davis and Diener, (1966), have reported improved aflatoxin yields when the fungus is grown as a submerged culture. Values ranging from 30 - 500 mg/litre have been obtained. Large quantities of aflatoxins have been produced from Czapeks Dox medium in which corn-steep liquor was added (Codner, Sargeant and Yeo, 1963, and Schroeder (1966). Davis and Diener (1967) have also produced high yields of aflatoxin from yeast extract-sucrose medium. The medium, made up of 20% sucrose and 2% yeast extract in water, has been extensively used for the quantitative production of the aflatoxins (Enafo 1970; Uwaifo, 1971).

III. Factors affecting aflatoxin production in liquid media:

Several factors affect the production of aflatoxin in liquid medium. These include the method of culturing; carbon and nitrogen source; pH; temperature; duration of incubation and finally, the sterilization of the liquid media.

(a) Method of Culture:

Codner, Sargeant and Yeo (1963) have shown varied yields of aflatoxin using rotary shake flasks as compared to production by the same strain of fungus when incubated in three or twenty-litre aerated fermentors. Matoles and Adye (1967), produced up to 90 mg of aflatoxin per litre of medium in shaken flasks but only 63 mg/litre in submerged culture using glucose-ammonium nitrate medium and the same strain of fungus. The same culture produced up to 63 mg/100 ml of medium when grown as stationary culture in litre flasks and using 2% yeast extract sucrose medium (Davis, Diener and Eldridge, 1966). Similar trends have been reported by Hayes Davis and Diener (1966) and Ciegler, et al. (1966).

(b) Effect of pH:

The pH dependence of the production of aflatoxins appears to be interrelated to the mode of culture, composition of the medium and probably the strain of the fungus. Matoles and Adye (1965), using submerged cultures and the glucose-ammonium nitrate medium, found that pH decreased from slightly above 4.0 to 2.1 - 2.3. Davis, Diener and Eldridge (1966) using stationary culture,

have shown that initial pH has little or no effect on aflatoxin yield from yeast extract-sucrose medium, except that Aspergillus flavus did not grow well at an initial pH of less than 4. Regardless of the pH, they found that the final pH of the medium was approximately 4.

(c) Temperature and duration of incubation:

Temperature and duration of incubation, not only affect total aflatoxin production but also the ratio of the different components in the crude extract. Diener and Davis (1966) observed that the greatest yields of aflatoxin were obtained in 15 days at 20°C but only 11 days at 30°C. At higher temperatures, however, final yields were lower than maximum yields when the fermentation is extended to 21 days. Schindler, Palmer and Eisenberg, (1967) reported maximum aflatoxin production at 24°C whereas maximum growth occurred at 29°C or 35°C depending on the Aspergillus flavus isolate. They also found a difference in the ratio of aflatoxin B_1 and G_1 with respect to temperature.

Sterilization of the liquid medium may affect the production of aflatoxins. Steam sterilization under pressure has been known to cause undesirable changes in

the composition of nutrient solutions. Toxic materials may be produced particularly when high concentrations of carbohydrate are autoclaved together with organic nitrogen compounds. Davis and Diener (1968) found that A. parasiticus did not grow in a medium containing 20% ribose (or xylose) and 2% yeast extract, when autoclaved together. However, when the two components were autoclaved separately and mixed prior to innoculation, growth was achieved.

(d) Effect of Carbon and Nitrogen Sources:

The influence of carbon source on the production of aflatoxins has been extensively studied (de Iongh, Vles and de Vogel, 1965; Mateles and Adyo, 1965; Davis, Diener and Eldridge, 1966 and Davis and Diener (1967). From available data, sucrose, glucose, fructose, xylose, ribose and glycerol were the most potent carbon sources for aflatoxin production. Similarly, nitrogen sources could also affect the production of aflatoxin in nutrient culture. Eldridge (1965), Mateles and Adye (1965); Schroeder (1966) and Davis, Diener and Eldridge (1967) have shown that ammonium sulphate and potassium nitrate appear to be the best inorganic nitrogen sources for aflatoxin production. Highest yields, however, have

been produced in media containing corn steep liquor, a mixture of casamino acids, yeast extract or peptone as nitrogen sources. It must be mentioned also that zinc has been found to have a remarkable effect on aflatoxin production. Armbrecht, et al. (1963); Nesbitt, et al. (1962); Matoles and Adye (1965); Lee, Tormsley and Walden (1966) and Davis, Diener and Eldridge (1967), have described the significant role of zinc in aflatoxin production. However, the mechanism of this stimulation is yet unknown.

(d) Isolation of Aflatoxin:

The aflatoxins are intensely fluorescent when exposed to long-wave Ultra-Violet light. This singular physical characteristic has been very useful in the identification, isolation and determination of the substances in extremely low concentrations.

(a) Extraction of aflatoxins:

Sargeant, et al. (1961) first demonstrated that a toxic principle in a mouldy meal could be removed by exhaustive extraction with methanol and further resolved by paper chromatography as a single spot exhibiting bluish fluorescence under Ultraviolet illumination. Subsequently, the isolation of the aflatoxins involved

the extraction of the substances using either the Soxhlet extractor or the liquid - liquid extractor and purification of the samples using chromatogrnphic teohniques mainly. Coomes and Saunders (1963) utilized a 6-hour Soxblet extraction with methanol while Broadbent and Shone (1963) recommended a 4-hour extraction. Variants of this basic exhaustive methanol extraction system have been utilized by Genest and Smith (1963); Trager, Stoloff and Campbell (1964); Tropical Products Report 1965; Coomes, et al. (1965) and Nabney and Nesbitt (1965). Most of these methods involve a 1.5% - 2% loss due to the destruction of aflatoxin B_1. de Iongh, et al. (1964), however, suggested a dual soxhlet extraction with methanol for one-hour and chloroform for two-hours in order to obtain a more quantitative extraction at a shorter time. These methods have been employed for obtaining the toxins from various natural products.

Aflatoxins produced in liquid oultures are almost quantitatively removed by partitioning into chloroform (Adye and Moteles, 1964). This has been efficiently achieved by using a Kollimnn liquid - liquid extrnctor (Emafo, 1970). Other methods involving the use of

mechanical blendors have been proposed (Nesheim, 1964; Pons and Goldblatt, 1965; Pons, et al. 1966; Stoloff, Graph and Rich, 1966). All these investigators used polar solvent systems such that lipid materials were excluded from the extract.

(b) Purification of extracts:

Several methods have been employed for the preliminary purification of crude aflatoxin extracts prior to chromatography. Coomes and Sander (1963), de Iongh, et al. (1964) have employed a partitioning procedure between methanol: water and petroleum ether in a separating funnel, for removing interferring lipids, carbohydrates and pigments. Broadbent, Cornelius and Shone (1963); Geneat and Saith (1963); Coomes, et al. (1965) and workers at the Tropical Products Institute (1965) have partitioned the crude extract between methanol, water and chloroform for initial purification. Pons and Goldblatt (1965), however, removed interferring gossypol pigments in primary aqueous acetone extracts of cotton seed materials as insoluble lead derivatives by treating the extracts with lead acetate. Further partitioning with chloroform,

separated the aflatoxins from residual lead salts and pigments.

(c) Resolution of crude extracts:

Crude aflatoxin extracts have been effectively resolved using chromatographic techniques. Most prominent among these are the column, paper and thin layer chromatographic methods.

I. Column chromatography:

The first chromatographic purification of partially purified primary extract was carried out by Coomes and Sanders (1963) using a column of neutral alumina. This procedure resolved aflatoxin B_1 and B_2. Pons, et al. (1966) used a column of silica gel G (Merck). Interferring pigments were removed by first eluting with diethyl ether and finally eluting the column with chloroform: methanol (97:3 v/v). Eppley (1966) adapted the above method of Pons, et al. (1966) to the purification of wet chloroform extracts of peanut products using sequential elution with hexane and diethyl ether to remove pigments and lipids while aflatoxins were eluted with chloroform: methanol (97:3 v/v). Holaday (1968) had also suggested the use of silica gel

millicolumn for a sensitive semi-quantitative procedure for aflatoxin detection in peanuts. Partition chromatography on a cellulose column is the basis of the procedure used by Stoloff, Graph and Rich (1966). Pigments were eluted with hexane and aflatoxins with hexane: chloroform (1:1 v/v).

II. Paper chromatography:

Paper chromatography has not been found so adequate for the fractionation of crude aflatoxins. It has, therefore, been sparingly employed for the purpose of aflatoxin separation. It was, however, introduced together with column chromatography (Sargeant, et al. 1961). 5% Acetic acid in butanol used as developer did not resolve the aflatoxins into more than one component on a Whatman No. 1 paper. Coomes and Sanders (1963) introduced the use of a benzene: toluene: cyclohexane: ethanol: water solvent (3:3:3:5:8 v/v). Resolution to B_1 and B_2 was not achieved. van der Merwe, Fourie and Scott (1963) and Davies, Eldridge and Diener (1966) have introduced the use of benzene and water, respectively, as developers in paper chromatography but none of these has proved efficient for the resolution of crude aflatoxins into pure

compounds.

III. Thin layer chromatography:

Thin layer chromatography has been found most useful for both preparative and quantitative aflatoxin research. Several modifications of the same basic method have been employed. Broadbent, Cornelius and Shone (1963) used plates coated with neutral alumina and a chloroform: methanol (98:5:1.5 v/v) solvent system. Though this did not resolve aflatoxin B, and B, there was about a 30-fold increase in sensitivity over the paper method of Coomes and Sander. de Iongh, et al. (1964) introduced the use of silica gel G (Kiesel gel G) plates for the resolution of aflatoxins. They used a chloroform: methanol (98:2 v/v) solvent system and reported about 6-fold increase in sensitivity over alumina plates. Coomes, et al. (1965); Nabney and Nesbitt (1965); Tropical Products Institute (1965); Engebrecht, Ayres and Sinnhuber, (1965); Eppley (1966) and Pons, et al. (1966) have employed various procedures involving kieselgel G of 300-500 u thickness and various combinations of the chloroform: methanol solvent systems. Trager, Stoloff and Campbell (1964) introduced the use of silica gel G-HR for improved

resolution of aflatoxins B_1, B_2, G_1 and G_2 using the chloroform - methanol (95:5 v/v) solvent system. The use of silica gel G-HR and chloroform - methanol mixtures in lined and equilibrated chambers were incorporated in procedures reported by Nesheim (1964); Robertson, et al. (1965); Pons and Goldblatt (1965) and Pons, et al. (1968).

Pons, et al. (1968) and Pons (1968), introduced the use of adsorbsil-1-silica gel plates for improved fluorodensitometric measurements, using chloroform: methanol and propanol (850:125:25). Wiley (1966) recommended a two-stage development on thin layer plates coated with silica gel - cellulose mixture and employing methyl acetate for first development and chloroform: methanol (98:2 v/v) for the second development. It is claimed that this procedure gives better separation from interferring fluorescent materials in extracts of agricultural commodities. Peterson and Ciegler (1967) have introduced the use of two dimensional thin layer chromatography using acetone: chloroform (9:1 v/v) followed by development in ethyl acetate: isopropanol: water (10:2:1 v/v). This procedure is known to remove impurities from plant extracts and for better resolution of aflatoxin

B_2 from G_1. Nocheim (1968) has shown, however, that variations in developing solvent, commercial silica gels calcium sulphate binders, gel thickness, humidity and vapour phase composition, may influence the separation of aflatoxins on thin layer plates.

(E) Estimation of Aflatoxins:

Assay of the aflatoxins can be divided into two broad outlines:

(a) physico-chemical methods (Nesheim 1967) and

(b) biological assay methods.

(a) Physico-chemical methods:

The physico-chemical assay method is based on the intense fluorescence characteristics of the aflatoxins under ultra-violet illumination. This method of assay mainly falls into three groups - the visual method, Spectrophotometric and fluorodensitometric methods

. Visual Method:

This method had depended, until recently, on the visual comparison of fluorescence intensities of unknown extracts with standard samples, chromatographed under identical conditions (de Iongh, 1963; Nesheim, 1964; Robertson, et al. 1965; Pons, et al. 1966; Stoloff, Graph and Rich, 1966 and Hopley, 1968). An improved method based

on the same principle has been the serial dilution method. Samples are diluted serially until there is no more fluorescence when run on thin layer plates (Coomes, et al. 1964; Coomes, et al. 1965). Carnaghan, Hartley and O'Kelly (1963) have shown that when run on Kieselgel G plates, the smallest weight of aflatoxin B_2 and G_1 giving an observable fluorescence is 0.0003 ug and for B_1, 0.0004 ug. Holzapfel, Steyn and Purchase (1966) have shown that the fluorescence of aflatoxin M_1 is three times as intense as the fluorescence of aflatoxin B_1 and this finding has been employed in computing aflatoxin M_1 concentrations in test samples. However, the variations in fluorescence due to solvent effects, silica gel sample and the presence of fluorescent interferring materials, have been a major set back to the use of fluorescence assay of aflatoxins (Roberts, Pons and Goldblatt, 1967; Ashworth, McKinney and McMeans, 1967; Rodericks, Cushmac and Stoloff, 1967; Wiseman, Jacobson and Harmeyer, 1967; Crowther, 1963 and Schroeder, 1963).

ii. Fluorodensitometric Method:

The use of the fluorodensitometer for aflatoxin assay was introduced by Pons and Goldblatt (1965), to reduce inaccuracies arising from the visual methods. This

method involved the comparison of fluorescent peaks traced out by a fluorodensitometer with that of a known standard. Ayres and Sinnhuber (1966), using a recording densitometer equipped for fluorescence emission measurements; found that the logarithmic relationship between emitted fluorescence energy and concentration was linear over a concentration range of 2.5 to 15 x 10^{-4} ug of aflatoxin B_1 per spot. Pons, Robertson and Goldblatt (1966) have also reported linear relationship between emitted fluorescence as measured by peak areas and concentration over a concentration range of at least 2 x 10^{-4} to 105 x 10^{-4} ug per spot of the four respective aflatoxins. Extensive use of this method has been employed in view of the precision attainable with fluorodensitometric measurements (Beckwith and Stoloff, 1968; Pons, et al. 1966, Fleischer and Falk, 1969).

VI. Spectrophotometric Method:

The first effort to improve the accuracy and precision of aflatoxin measurements by the use of objective methods was reported by Nabney and Nesbitt (1965). This method is based on the Ultraviolet absorption of aflatoxins at 363 m u after purification by thin layer chromatography. The concentration was given

by the formula:

$$\text{Concentration} = \frac{D \times M \times 10^6}{E \times 200 \times 2} \text{ ug per 5 ml}$$

where D = Corrected optical density at 363m u

M = Molecular weight of the aflatoxin

E = Molar extinction coefficient.

This method is applicable to all the four aflatoxins.

(b) Biological Assay:

Biological assays have been used where substances are known to produce measurable effects relative to the dose administered. It has been employed both for confirmatory test for aflatoxin toxicity and for its estimation (Brown, Wildman and Eppley, 1968 and Brown, 1969). Several organisms have been used but a few of these are discussed here because of their known reproducibility.

I. Duckling Assay:

Duckling assay has been n most widely used and accepted procedure for aflatoxin assay in various materials. Lethal effects of the toxin and degenerative changes in the liver of day-old ducklings have been utilized in this assay (Sargeant, O'Kelly, Carnaghan, 1961; Smith and McKernan, 1962; Carnaghan, Hartley and

O'Kelly, 1963; Sargeant, Carnaghan and Allcraft, 1963; Armbrecht and Fitzhugh, 1964; Newberne, Wogan, Carlton and Abdel-Kader, 1964). An LD50 of 0.37 mg aflatoxin B, per kg of body weight of the duckling has been reported. Sensitivity to aflatoxin injury and the immediate induction of bile duct proliferation are two contributory factors for the widespread use of the day-old duckling.

II. Chick Embryo:

The chick embryo technique has been found to be simple, reproducible and sensitive. Platt, Stewart and Gupta (1962) reported that 5-day old chick embryo was sensitive to aflatoxin injury and as little as 0.3 ug of crude preparations caused death in two days. The development of the chick embryo technique as n bio-assay method was reported by Verett, Marliac and Mclaughlin (1964) and by Herbst, Ikams and Jayaraman (1968). They used fertile white leg horn eggs, injecting them with the toxin either through the yolk sac or the air cell routes. It was, however, found that the air cell route was more sensitive (Verett, Marliac and Mclaughlin, 1964).

Mortality, growth retardation in surviving embryos and gross pathological findings were used as index of assay and evaluation has been based mainly on mortality

at 21 days. An LD_{50} for aflatoxin B_1 of 0.025 ug per egg has been reported for the air cell route and twice as high for the yolk sac route. Gabliks, et al. (1965) established the LD_{50} for ten day old chick embryos as 1.0 - 2.5 ug per egg when administered through the chorio-allantoic sac. Under similar conditions, the duck embryo was found to be four to five times as sensitive.

111. Fish:

The larvae and embryos of zebra fish Brachydanio rerio (Abedi and McKinley, 1965) and the larvae of Uperodon species, 15mm in length (Arseculeratine, et al, 1969) have also been used for assaying aflatoxins. The larvae of the zebra fish, however, show greater susceptibility to aflatoxin toxicity than the embryos.

1V. Cell Cultures:

Tissue cell cultures are known to be susceptible to aflatoxin toxicity. Calf kidney culture (Juhasz and Greczi, 1964); He La and Chang liver cultures, duck and chick embryo cell cultures, have been recommended for assaying aflatoxins (Gabliks, 1965, Gabliks, et al. 1965). Toxicity is measured by the destruction of the cells, which is, preceded by an inhibition of growth. Daniel (1965) introduced the use of the 1.1 fibroblast cell

to be sufficient to kill all the cells within 48 hours. The LD_{50} is given as 0.062 ± 0.004 µg/ml of culture medium.

V. Micro-organisms:

Aflatoxins have been shown to possess antibiotic properties (Burmeister and Hesseltine, 1966; Arai, Ito and Koyama, 1967; Tennissen and Robertson, 1967). Aflatoxin B_1 suppresses the growth of Aspergillus awamorii; Penicillium chrysogenum; Penicillium duclauxi and some strains of Aspergillus flavus (Lillehoj, Ciegler and Hall, 1967). In addition, it induces aberrant forms of Flavobacterium auratiacum in media containing the toxin in a concentration below the fungistatic levels. Assays of aflatoxin B_1 utilizing micro-organisms sensitive to its toxicity, have, therefore, been postulated (Clements, 1968; Herbst, Ikawa and Jayaraman 1968). Bacillus megaterium is usually used as the test organism and paper disc method employed. A concentration of 0.1 ug aflatoxin B_1 per disc inhibits Bacillus megaterium spores significantly (Clements, 1968, Herbst, Ikawa and Jayaraman, 1968). The assay method is convenient to carry out as a clear zone of inhibition is observed after 15 - 18 hours of incubation at 35°C - 37°C.

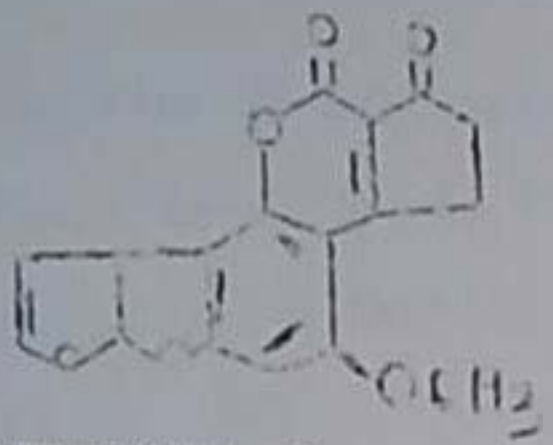
(F) Chemistry of the Aflatoxins:

The aflatoxins constitute a group of highly substituted coumarins which have been isolated from mould cultures of Aspergillus flavus. They are separable into single compounds by thin layer chromatography and can be differentiated by their Rf. values and fluorescent characteristics in Ultraviolet light. Eight aflatoxins have been characterized and labelled B_1, B_2, G_1, G_2, M_1, M_2, B_{2a}, and G_{2a} based on both their colour under Ultraviolet light and their Rf. values.

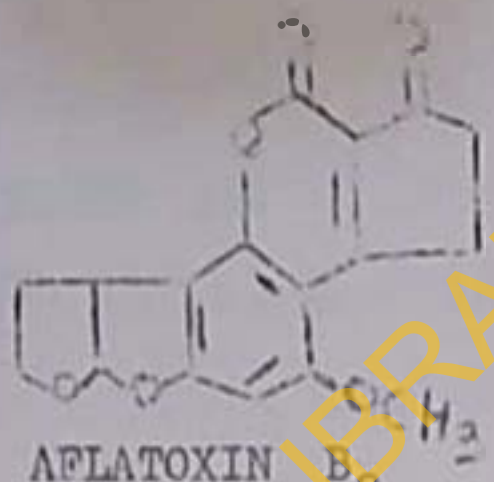
In view of the availability of only small quantities of these toxins, structural elucidation has proceeded with great reliance on the interpretation of Ultraviolet, infra-red, nuclear magnetic resonance and mass spectra (Asao, et al. 1965). Their chemical nature has been extensively reviewed by Wogan (1966). Aflatoxin B_1 has been shown to be a highly unsaturated compound, consisting of only carbon, hydrogen and oxygen (Nesbitt, et al. 1962; van der Zijden, et al. 1962). Aflatoxin B_1 and G_1, possess a vinyl ether system each (Hartley, Nesbitt and O'Kelly, 1963; van der Merwe, Fourier and Scott, 1963). A carbonyl group and a methoxy group are present in each of Aflatoxins B_1, B_2, G_1 and G_2 (de Iongh, et al. 1962;

van der Merwe, Fourier and Scott, 1963). The terminal lactone ring in aflatoxin G is substituted with a pentanone ring in aflatoxin B (Carnaghan, Hartley and O'Kelly, 1963).

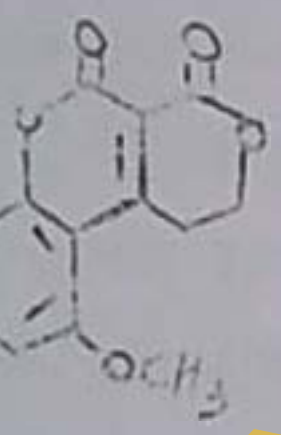
STRUCTURE OF THE AFLATOXINS



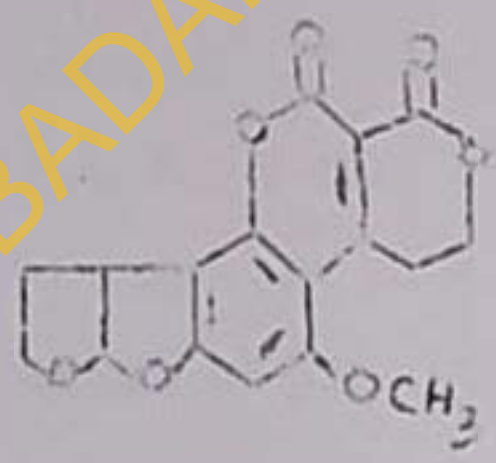
AFLATOXIN B_1



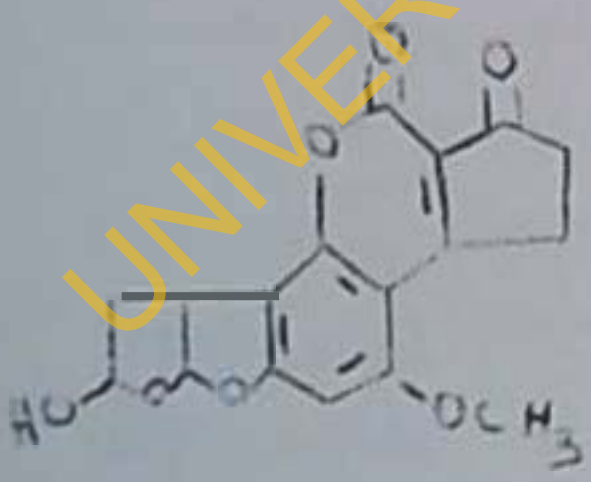
AFLATOXIN B_2



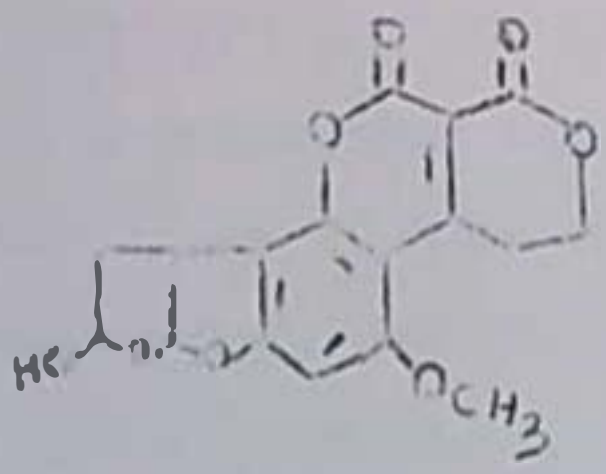
AFLATOXIN G_1



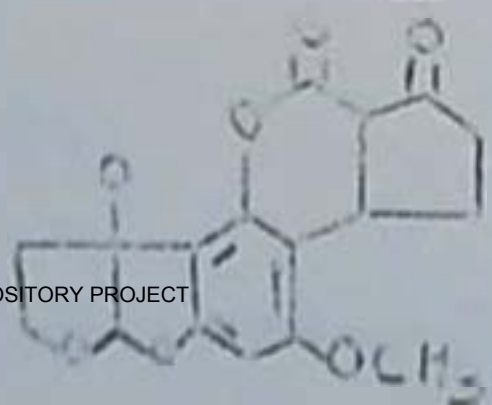
AFLATOXIN G_2



AFLATOXIN B_{2a}



AFLATOXIN G_{2a}



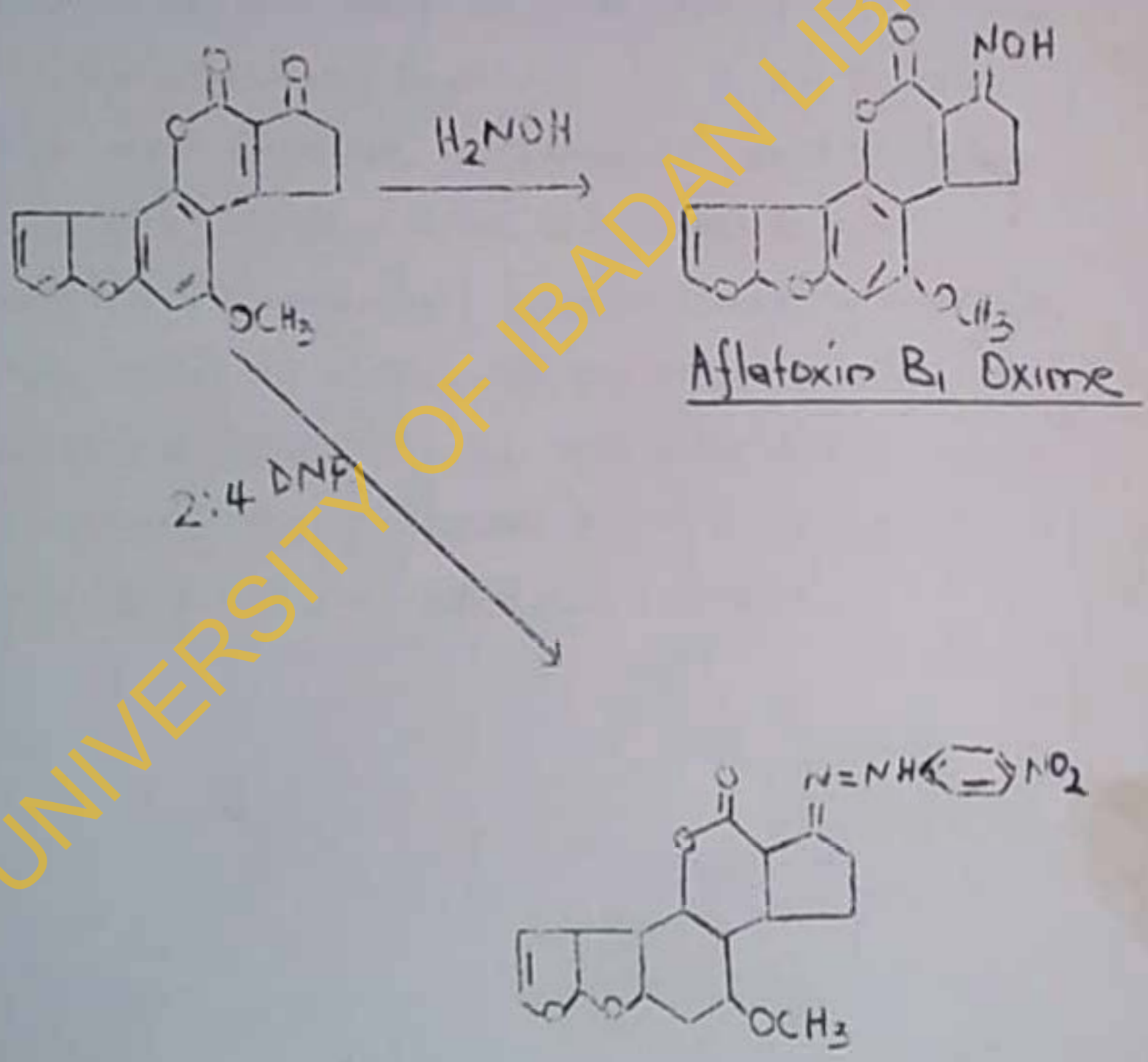
The ultraviolet, infra-red and mass spectral characteristics of the aflatoxins are similar (de Iongh, et al. 1962; Nesbitt, et al. 1962; and von der Zijden, et al. 1962). The interpretation of these physical characteristics has been used in the elucidation of the strucuture (Asao, et al. 1963 and 1965). X-ray cryatallography has also been employed in the structural analysis of the aflatoxins (Cheung and Singh, 1964). Chemical synthesis from phloroglucinol has also been used in the confirmation of the structure of aflatoxin B_1 (Buchi, et al. 1966). The structure of aflatoxin M_1 has been confirmed by chemical synthesis (Buchi and Weinreb, 1969). Catalytic introduction of one molecule of hydrogen to one molecule of either aflatoxin B_1 or aflatoxin G_1 yields aflatoxin B_2 and G_2 respectively (Chang, et al. 1963, Van Dorp, et al. 1963; van der Merwe, Fourier and Scott, 1963). Aflatoxin B_2 was recently synthesized chemically from a benzofuran (Roberts, et al. 1968).

(B) Properties of the aflatoxins:

1. Chemical Properties:

The carbonyl group of the cyclopentenone ring of

aflatoxin B_1 takes part in ketonic reactions. Thus it reats with 2:4 dinitrophenyl hydrazine and hydroxylamine to form phenyl hydrazones and oximes (Crisan and Orefig, 1967)



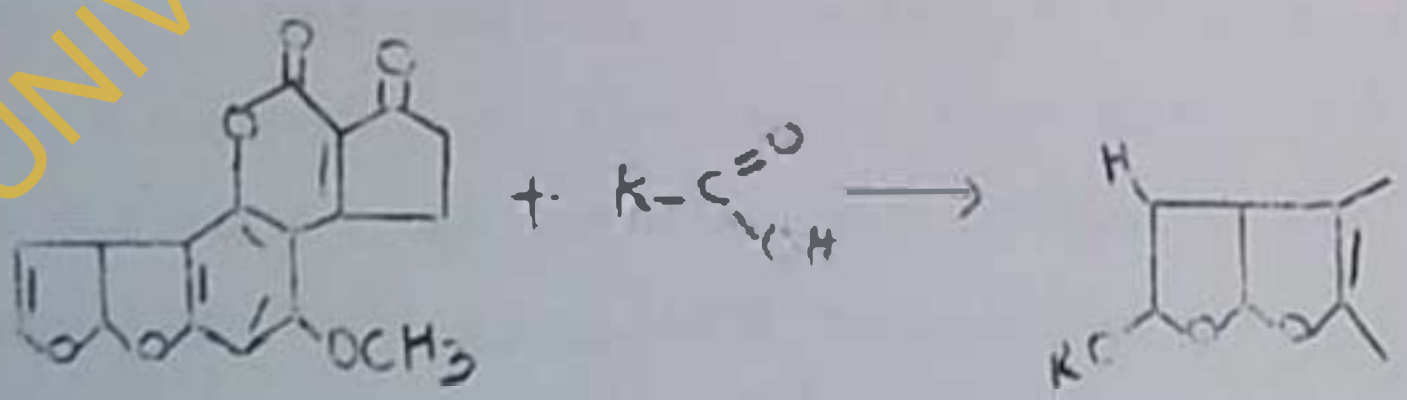
Aflatoxin B_1 Oxime

Aflatoxin B_1 dinitrophenyl hydrazine

This reaction has been employed in the detection of aflatoxin B_1 and B_2 on thin layer plates (Crisan, 1968).

A deep yellow to orange colour is regarded as being positive for aflatoxin B_1 and B_2. Aflatoxin G_1 and G_2 which lack this ketogenic group, do not take part in the reaction and thus cannot be identified by this method.

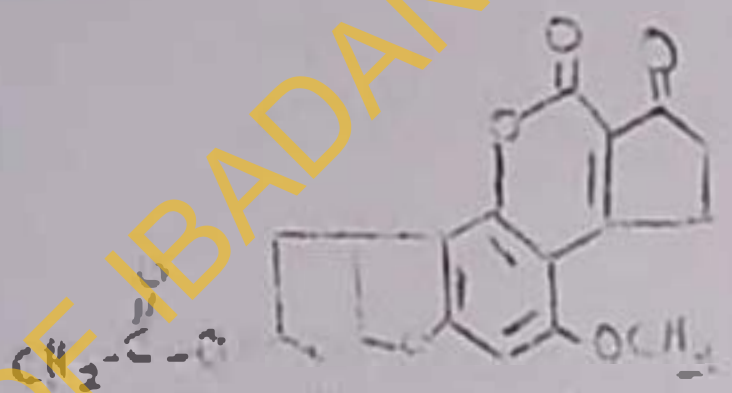
The aflatoxins, notably B_1, B_2, G_1 and G_2 react with acetic anhydride, trifluoroacetic acid or formic acid, with thionyl chloride as catalyst to form derivatives of the parent compound (Andrellos and Reid, 1964; Crisan and Grefig, 1967 and Stoloff, 1967). It has been suggested that the vinyl ether double bond of the bifuran ring of aflatoxin B_1 and G_1 is the site at which these acids are added unto the toxins.



Aflatoxin B_1 and G_1 form acetyl derivatives of B_{2a} and G_{2a} when they react with glacial acetic acid in the

presence of pyridine and thionyl chloride as catalyst. They also react with acetic anhydride to form the acetyl derivatives (Dutton and Heathcote, 1968).

B_1 Excess glacial acetic acid + thionyl chloride



2-acetoxy-3-hydro-aflatoxin B_1

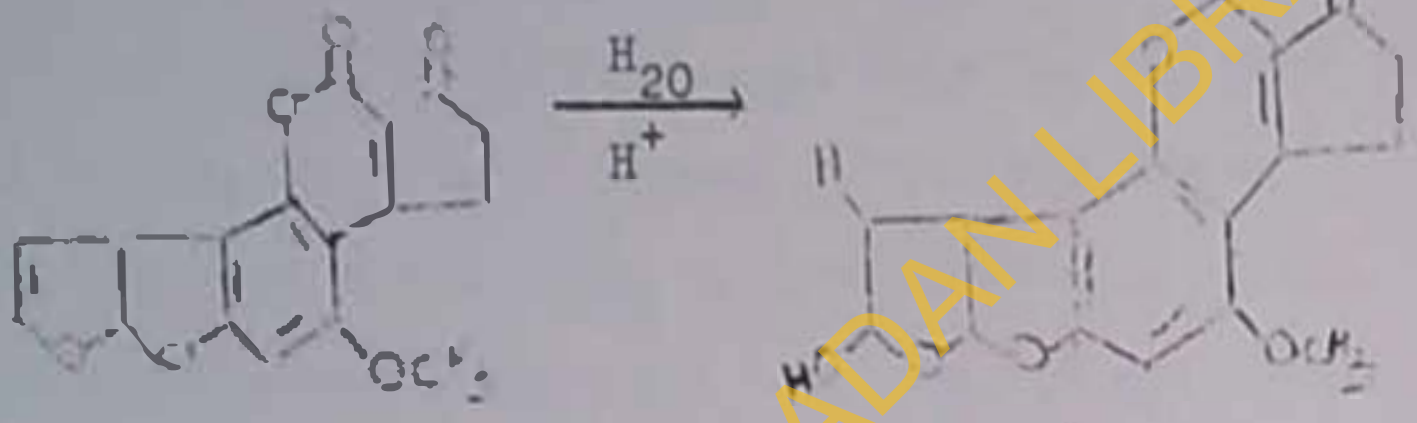
G_1 Excess glacial acetic acid + thionyl chloride

2-acetoxy-3-hydro-aflatoxin G_1

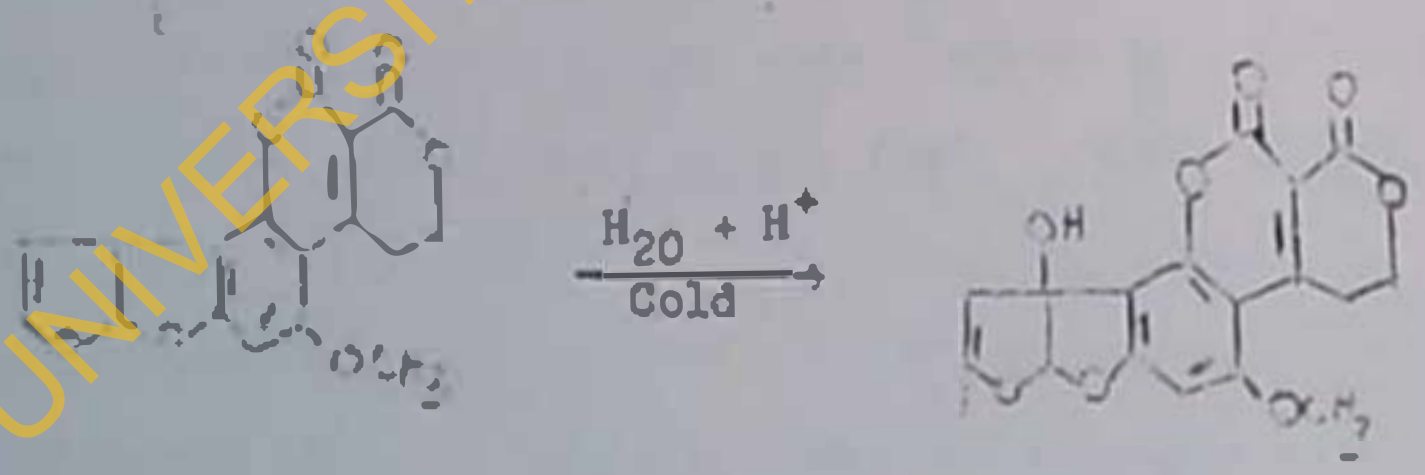
Under mild acid conditions aflatoxin B_1 is transformed into a hemiacetal; the formation of which is regarded as confirmatory test for aflatoxin B_1 (Andrellos and Reid, 1964; Buchi, et al. 1966; Pohland, Cushmac and Andrelloc, 1968; Ciegler and Peterson, 1968). This is an acid catalyzed addition of water across the olefinic linkage of the bifuran ring.

Dutton and Heathcote (1968) have also shown similar

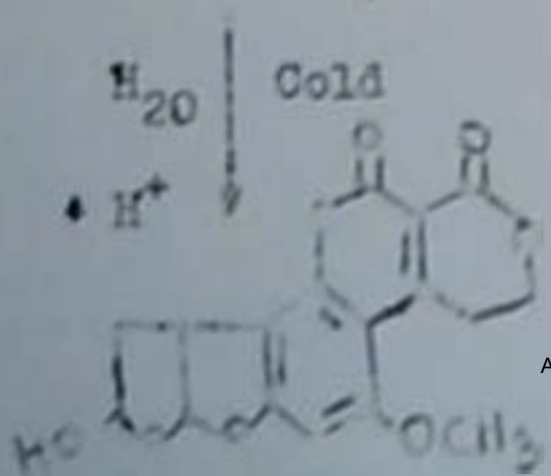
reactions with aflatoxin G_1 in the cold and under mild acid conditions. Addition at carbon 4 yields aflatoxin 'GM' and at the 2-position to give aflatoxin G_{2a}.



Aflatoxin B_1 — Aflatoxin B_{2a}



Aflatoxin G_1 — Aflatoxin 'GM'



The olefinic double bond of aflatoxins B_1 and G_1 appear to take part in addition reactions with ozone (Dwarakanath, et al. 1968), benzyl peroxide, osmium tetroxide and potassium - iodine complex (Trager and Stoloff, 1966).

Oxidation of aflatoxin B_1 results in the formation of succinic acid while ozonolysis and subsequent oxidation of the products of ozonolysis with silver oxide gives laevulinic acid (van Dorp, et al. 1963).

11. Physical Properties:

The physical properties of the aflatoxins have been extensively studied. A summary of these features is presented in the accompanying Table (11) (Asao, et al. 1963; Chang, et al. 1963; Carnaghan, Hartley and O'Kelly, 1963; Asao, et al. 1965; Robertson, et al. 1965; Holzapfel, Steyn and Purchase, 1966; Robertson, Pons and Goldblatt, 1967; Dutton and Heathcote, 1968; Goldblatt, 1969; and Fishbein and Falk, (1970).

TABLE II:

Fluorescence excitation and emission maxima of aflatoxins

Measurement conditions	Excitation maxima (nm)	Fluorescence emission maxima (nm)			
		B_1	B_2	G_1	G_2
Methanol[a]	365	425	425	450	450
Methanol[b]	365	430	430	450	450
Ethanol	365	430	430	450	450
Chloroform	365	413	413	430	430
Acetonitrile	360	415	412	440	437
Silica gel Solid State[c]	368-369	432	427	455	450

(a) Carnaghan, Hartley and O'Kelly (1963)

(b) Robertson, et al. (1965).

(c) Roberts and Pons (1968).

TABLE III contd.

AFLATOXIN	MOLECULAR FORMULAR	MOLECULAR WEIGHT	MELTING POINT	$(\alpha)_D^{230}$	U. V. characteristics IN methanol λ max.nm (E)	Infra-red characteristics max ν ($CHCl_3$)
B_1	$C_{17}H_{12}O_6$	312	268-269	-558	197 (28,900) 223 (24,650) 265 (13,400) 362 (21,800)	1780 intense 1684 weak 1633 1598 1582
B_2	$C_{17}H_{14}O_6$	314	286-289	-430	200 (20,300) 222 (17,000) 265 (12,200) 362 (23,800)	1760 1625 1685 1600
G_1	$C_{17}H_{12}O_7$	328	244-246	-556	201 (28,050) 216 (27,100) 265 (10,000) 360 (18,900) additional maxima also at: 242 (9,800) 257 (9,200)	1780 1684 1624 1597
G_2	$C_{17}H_{14}O_7$	330	237-240	-473	200 (27,300) 217 (26,000) 244 (9,800) 257 (9,200) 363 (20,900)	1780 1684 1624 1597

TABLE III contd.

AFLATOXIN	MOLECULAR FORMULAE	MOLECULAR WEIGHT	MELTING POINT	$(\alpha)_D^{23^\circ}$	U. V. characteristics in methanol/λ_{max}.(nm) (E)	Infra red characteristi ν ($CHCl_3$)
B_2^a	$C_{17}H_{14}O_7$	330	240	-	228 (17,600) 256 (10,200) 363 (20,400)	3620 1625 1780 1600 1685
G_2^a	$C_{17}H_{14}O_8$	346	190	-	220 (18,900) 242 (10,900) 262 (8,700) 345 (18,200)	3620 1624 1785 1684 1587
B_1	$C_{17}H_{12}O_7$	328	268	-280	228 (23,100) 265 (11,000) 357 (19,800) (in [illegible])	3425 1760 1690
G_1	$C_{17}H_{14}O_7$	330	287	-	221 (20,000) 264 (10,900) 357 (21,000)	3425 1780 1690

(H) Biochemical effects of aflatoxins:

The observation of the death of turkeys fed with mould contaminated groundnut in England in 1960, was later traced to the aflatoxins (Allcroft and Carnaghan, 1963; Van der Zijden, 1962). The aflatoxins have now been associated with several disease syndromes generally referred to as aflatoxicoses. These include:

1. Hepatotoxicity:

The aflatoxins are known to be hepatotoxic, inducing carcinogenesis in liver cells (Lancaster, Jenkins and Philp, 1961; Smith and McKernan, 1962; Barnes and Butler, 1964; Butler, 1964; Wogan, 1966; Carnaghan, 1967 and Schoental, 1970). Ingestion of aflatoxins over a long period induces hepatomas to mammals, birds and fish (Schoental, 1961; Salmon and Newberne, 1963; Carnaghan, 1965; Newberne, 1967; Wogan and Newberne, 1967; Rogers and Newberne, 1969). Hepatomas of parenchymal cells and of bile duct epithelial origin have been observed in trouts (Ashley, Halver and Wogan, 1964; Ashley, et al. 1965; Solomon, Jensen and Tanner, 1965).

In addition to hepatic tumors, the aflatoxins also induce tumours in subcutaneous tissues of the rat and mice especially during subcutaneous administration

(Dickens and Jones, 1963 and 1965).

11. Mutagenic and teratogenic effects:

Aflatoxins are very potent mutagens being capable of causing extensive genetic damage (Legator, Zuffante and Harp, 1965; Lilly, 1965; Gelboin, et al. 1966; Clifford and Rees, 1966; Clifford, Rees and Stephens, 1967; Dolimpio, Jacobson and Legator, 1968; Epstein and Shafner, 1968). The effect is achieved through the interaction of the aflatoxins with nucleic acids and the subsequent interference with gene transcription.

As a teratogen, aflatoxin B_1 shows a marked species specificity. Foetal malformations in the golden hamster have been reported at a dosage of 4 mg/kg body weight. Death and resorption of foetus have also been reported within the 13th and 15th day of pregnancy (Elis and Dipaolo, 1967). Chick and duck embryos are susceptible to the effect of sublethal doses of aflatoxin B_1. Malformations and stunted growth have been reported (Verett, Marliac and Malaughlin, 1964; Gabliks, et al. 1965; and Shiboko, et al. 1968). Species like swine, dog and mouse have no demonstrable susceptibility to the teratogenic effect of aflatoxin B_1 besides the induction of foetal resorption (Newberne, Russo and Wogan, 1966;

Hintz, et al. 1967; Dipaolo, Elio and Erwin, 1967 and Elio and Dipaolo, 1967).

III. Miscellaneous effects:

Besides, these afore-mentioned effects, aflatoxins have been shown to cause several other biochemical changes in many animal species. Increase in serum alkaline phosphatase in sheep, rats and monkeys (Allcroft, 1965; Clifford and Rees, 1967a; Rao and Ghering, 1970), and increase in serum levels of isocitrate, malate and glutamate dehydrogenase have been known to occur (Clifford and Rees, 1967b; Rao and Ghering, 1970). Brown and Abrams (1965) reported increase in serum levels of lactic dehydrogenase, aldolase and glutamic-oxaloacetic acid and glutamic-pyruvic transaminase in chickens. Platonow (1965) also found increase in the level of urocanase in the serum of chickens treated with aflatoxins. Datta and Gajan (1965) have also demonstrated alterations in plasma proteins of ducklings poisoned with aflatoxin. Decrease in drug metabolizing enzymes in rat liver damaged by aflatoxin B_1 have been reported by Clifford and Rees (1966), though Schabort and Steyn, (1969) claimed aflatoxin B_1 could

induce its own metabolism in the rat.

Marked biochemical alterations thus appear to be consequent on aflatoxin poisoning of several species. Nucleic acid and protein synthesis are altered and alteration of gene transcription is attendant on aflatoxin poisoning (Neabitt, et al. 1962; Clifford and Rees, 1966; Sporn, et al. 1966; Clifford, Rees and Stevens, 1967; Clifford and Rees, 1967b; Black and Jirgensons, 1967 and Chang, et al. 1963). Based upon these accumulated data and the present theories of protein synthesis, and the role of DNA transcription on this process, interaction of aflatoxins with DNA has been postulated as the initial and critical feature of aflatoxicoses.

THE PALMOTOXINS

Aspergillus flavus appears to synthesize a complex array of substances in addition to the aflatoxins. Flavocol and Aspergillic acid, have been isolated from culture filtrates of Aspergillus flavus grown on a medium containing casein hydrolysate and sodium chloride (Dunn, Newbold and Spring, 1949). Extensive review of reports on these toxins other than aflatoxins produced by Aspergillus flavus has been given by Wilson (1966).

These include oxalates, kojic acid, Aspergillic acid, flavoool and some other substances grouped by the author as endotoxins and tremorgenic substances. Recently, Rodericks, et al. (1968a) has added to this, a substance called aspertoxin. The structure of the substance was given simultaneously by Roderioks, et al. (1968b) and Weiss, et al. (1968).

And yet more recently, two new fluorescent metabolites of Aspergillus flavus have been isolated from the fungal culture when grown on the palm sap medium. These substances have been labelled Palmotoxin Bo and Palmotoxin Go (Bassir and Adekunle, 1968, Adekunle, 1969).

(A) The palm sap medium:

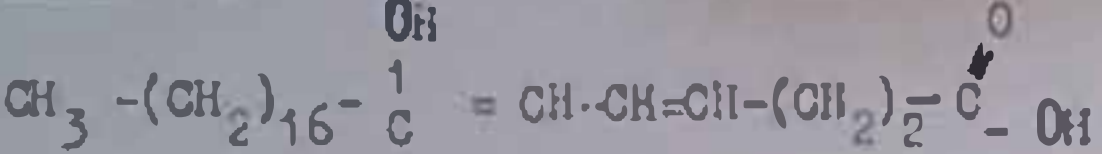
The sap collected from the oil palm tree (Elaeis guineensis) contains fermentable sugars notably sucrose (Bassir, 1962). Recent work by Faparusi (1966), Adekunle (1969) shows that it contains fructose, glucose, sucrose, maltose, raffinose and glucuronic acid. The relative abundance of each constituent varies according to the hybrid variety of the oil palm and to the period of collection. However, sucrose is known to be the predominant sugar in palm sap (Faparusi, 1966). In addition

to sugars, these authors have found vitamins, amino acids and trace elements. The predominant vitamins are vitamin B_1, B_2, B_6 and vitamin C. Iron, zinc, sodium, sulfur and cobalt are among the trace elements found to be present in palm sap. Adekunle (1969) has established that fresh palm sap supports the growth of Aspergillus flavus with the resultant production of aflatoxins and other metabolites in comparable quantities with most other natural media.

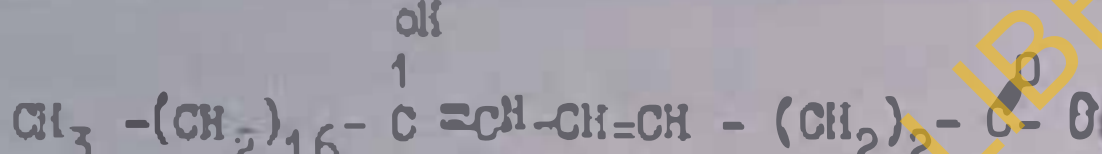
(B) Nature of the Palmotoxins:

Little is yet known about the palmotoxins. The substance referred to as palmotoxin Bo and Go, fluoresce blue and green respectively on thin layer plates illuminated with ultraviolet light. They have Rf values 0.2 and 0.13 respectively on Kieselgel G plates of 0.25 mm thickness and using chloroform: methanol (50:2 v/v) as developer (Adekunle, 1968). They are precipitated from their concentrated chloroform solution by n-hexane and petroleum ether - a manner similar to the aflatoxins (Asao, et al. 1965). The palmotoxins are also reported to contain carbon, hydrogen and oxygen only. The structures as proposed by Adekunle,(1969) are thus:

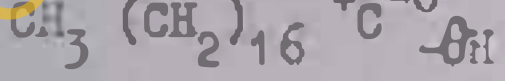
PALMOTOXIN Bo



PALMOTOXIN Co



It is proposed that they could be spatial isomers of each other and do undergo oxidative ozonolysis yielding fractions believed to correspond to

 in both cases.

I. PHYSICAL PROPERTIES (ADEKUNLE, 1969)

TABLE IV:

Toxin	Melting Point	Molecular Formula	Mol. Weight	ν_{max}	λ_{max}	Iodine Number
Bo	52-53	$C_{24}H_{42}O_3$	380	3500 1620 1665 720	217	15.9 ±0.35
Go	51-52	$C_{24}H_{42}O_3$	380	3400 1620 1665 720	217	15.45±0.42

(C) Biological effects of the palmotoxins:

Very few data have been accumulated on the biological effect of the palmotoxins. Most of the studies have also been limited to avian embryos.

They are believed to be of comparable toxicity as the aflatoxins (Bassir and Adekunle, 1968 and 1969). Their LD_{50} using 6-day old white rock chick and domestic pigeon embryos have been given as 0.333 ± 0.024 for palmotoxin Bo, 4.220 ± 0.083 for palmotoxin Go as compared to 0.38 ± 0.01 for aflatoxin B_1 in pigeon embryo. With chick embryo the LD_{50}s are given as 0.434 ± 0.070 for aflatoxin B_1, 0.323 for palmotoxin Bo and 4.683 ± 0.076 for palmotoxin Go.

The palmotoxins are teratogenic to chick embryo. Palmotoxin Bo has been observed to be as teratogenic as aflatoxin B_1 while palmotoxin Go is, however, less so. Malformations associated with palmotoxin poisoning include malformations of the beak; incomplete ossification of the skull; limb defects, and stunted growth (Bassir and Adekunle, 1970a). These toxins are also reported to induce liver lesions in chick embryo, in a manner similar to aflatoxin treated embryos.

Hepatoxicity is characterized by fatty infilteration of the liver, and peripheral necrosis of the liver lobules. The severity of these effects in aflatoxin B_1 and palmotoxin Bo treated embryos are similar and less so for palmotoxin Go treated embryos (Bassir and Adekunle, 1970b).

METABOLISM OF DRUGS AND FOREIGN COMPOUNDS:

Living organisms biotransform compounds foreign to their metabolic network. Generally, this has become necessary as a means of dealing with the compounds preparatory to their excretion. Extensive reviews on this process have been given (Brodie, Gillette and Ladu, 1958; and Gillette, 1966, and Vocell, 1971). As a result of these transformations, the pharmacological activities of the compounds are remarkably altered and in some cases, the compounds are denotivated (Williams, 1969; Gillette, 1965 and Uehlcke, 1969).

The reactions involved in this process are mediated by enzyme located in the endoplasmic reticulum of most organs but notably the liver and generally referred to as the mixed function oxidases or mono-oxygenases in view of their heterogenous character (Mason, 1957a and

1957b; Conney and Burns, 1962; Hayaishi, 1962 and Mason, et al. 1965). These enzymes are found bound to the membranous component of the microsomes and are released on rupture of the particles (Siekevitz and Palade, 1956; Fouts, 1961; Ernster, Siekevitz and Palade, 1962; Dallner, 1963). They are unique in activity and have been shown to catalyze a large variety of oxidative and reductive processes in the body. These include aromatic hydroxylation; side chain oxidation; deamination; N-, S- and O- dealkylation; sulfoxidation; N- oxidation and epoxidation (Axelrod, 1955; Brodie, et al. 1955; Brodie, 1956; Brodie, Gillette and Ladu, 1958; Gillette, 1959; and Williams, 1959).

A. Mechanism of metabolism of foreign compounds:

In broad outlines, the metabolism of foreign compounds in the body, involve two distinct processes:

(a) an initial oxidative or reductive process and

(b) a synthetic process in which the compound is conjugated with amino acids, glucuronic acid, sulphate and a host of other compounds (Williams, 1959 and 1969).

Schematically, this has been represented as follows:

Drug or foreign compound —(oxidation)→ oxidized drug or foreign compound —(conjugation)→ conjugated product

I. Oxidative Process:

Oxidative enzymes in various animal tissues require reducing agents mainly pyridine nucleotides - NADPH and NADH (Mueller and Miller, 1953; Gillette, 1966; Nilsson and Johnson, 1963; Gram, Rogers and Fouts, 1967; Gigon, Gram and Gillette, 1968; Holtzman, et al. 1968). In addition, they require atmospheric oxygen (Mason, 1957; Posner, Mitoma and Udenfriend, 1961; Hayano, 1962; Baker and Chaykin, 1962).

The oxidation of drugs or foreign compounds is a coupled process involving cytochrome P450 mainly and NADPH - Cytochrome C reductase. Cytochrome P450 was first detected by G.R. Williams and later described by Klingenberg (1958), Garfinkel (1958) and Omura and Sato (1962). The extent of participation of cytochrome P450 in microsomal oxidative processes has been widely studied (Dallner, 1963; Cooper, et al. 1965; Gillette,

1966; Gram, et al. 1967; Holtzman, et al. 1968; Gigon, et al. 1968; Hilderbrandt, Remmer and Esterbrook, 1968; Sladek and Monnering, 1966; and Alvares, et al. 1967).

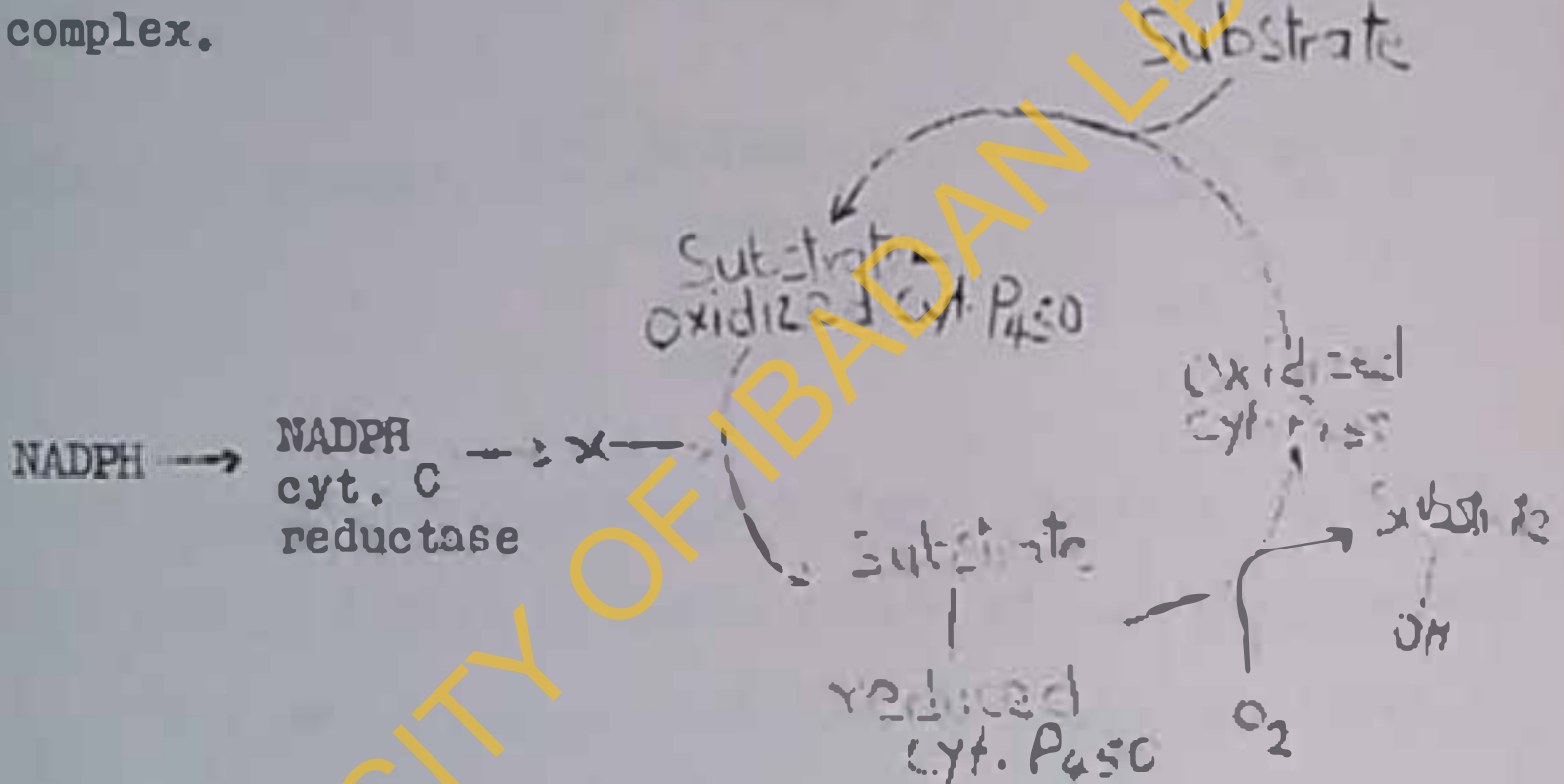
According to the mixed oxygenase theory of Brodie, Gillette and LaDu (1958) and Gillette, 1966, NADPH reduces a component in the microsome (Cytochrome P450) which reacts with oxygen to form an 'active oxygen' intermediate. The active oxygen is then transferred to the drug substrate.

1. $NADPH + A + H^+ \longrightarrow AH_2 + NADP^+$
2. $AH_2 + O_2 \longrightarrow$ 'active oxygen'
3. Active oxygen + drug $\rightarrow$ oxidized drug + A + H_2O

A is the microsomal component.

The cyclic process postulated by Holtzman, et al. (1968), Gillette (1969), envisages the formation of a cytochrome P450 - substrate complex, which is then reduced by NADPH, and mediated by NADPH - cytochrome - C-reductase and the eventual oxidation of the complex by molecular oxygen. The finding that various substrates caused changes in the visible absorption spectrum of

liver microsomes, even in the absence of NADPH (Remmer, et al, 1966) also suggested that substrates formed complexes with the oxidized form of cytochrome P450 and that the rate limiting step of the reaction was the reduction of the substrate - cytochrome P450 complex.



Oxidation of foreign compounds may involve a more complex mechanism but the participation of cytochrome P450 and pyridine nucleotides in most of the reactions, is well established.

II. Synthetic or Conjugation Process:

The second phase of the enzymic reactions, involve the synthesis of conjugated products. Two possible mechanisms have been proposed for this. The first mechanism may involve the activation of the conjugating

Agent and the transference of this, to the substrate (Dutton and Storey, 1954; Smith and Hilln, 1954; Strominger, et al. 1954; Isselbacher and Axelrod, 1955; Dutton, 1955 and Brown, Zuelzer and Burnett, 1956). This mechanism can be represented thus:

Conjugating agent —ATP→ activated nucleotide conjugating agent —transferase enzyme→ conjugated drug

This process has been demonstrated by Axelrod, Inscoe and Tomkins (1957) in the formation of some glucuronides.

The second postulate envisages a situation in which the foreign compound is first activated and then, forged to the conjugating agent thus:

Foreign compound —ATP→ activated nucleotide foreign compound —Conjugating agent→ Conjugated product

Williams(1959) has demonstrated this mechanism in the formation of hippuric acid.

Benzoic acid ---> Benzoyl-CoA $\xrightarrow[\text{N-acylase}]{\text{glycine}}$ Hippuric acid.

(B) Factors influencing the metabolism of drugs and foreign Compounds:

I. Species:

The pattern of metabolism of foreign compounds has been shown to vary from species to species. This species variations in the metabolism of compounds have long been recognized and Parke, (1968) has shown that the variations might be in the quantity of substances metabolised and in the products of such metabolism. Thus, it is now known that different species respond differently to an administered drug or foreign compound and this has been found most useful in chemotherapy (Brodie, et al. 1952 and Williams,1959).

Brodie, et al. 1953; Burns, et al. 1953 and 1955, have demonstrated species differences in the metabolism of mepridine; phenylbutazone, ethyl biscoumarin acetate and pentobarbital. Caldwell, Dring and Williams (1971) have shown that the main metabolic reaction of methamphetamine in the rat is aromatic hydroxylation

but N- demethylation in the guinea pig. Bridges, et al. (1969) have demonstrated extensive species differences in the metabolism of some methoxy-6-sulphanilamido-pyrimidines. Quin, Axelrod and Brodie (1958) studied the rates of metabolism of hexobarbital, antipyrine and aniline in the mouse, rat, guinea pig, rabbit, dog and man. They showed that in all cases the mice had a higher rate of metabolism than the others and also established an inverse relationship between the activity of the enzyme system and the duration of drug action. Species variations in the metabolism of a lot of substances have also been recorded, Murphy and Dubois (1957) have recorded species differences in the handling of an anticholine esterase agent formed in the liver from the dimethoxy ester of benzotriazine diphosphoric acid.

In a similar manner, it has been reported that differences in strain affect the metabolism of drugs (Jay, 1955; Quin, Axelrod and Brodie, 1958; Jori, Pescador and Puglisi, 1971).

II. Age:

Studies in the metabolism of compounds have revealed

that certain compounds which are metabolized by adult animals, cannot be handled by new born animals (Jondorf, Maickel and Brodie (1958), Fouts and Adamson (1959). It is thus postulated that the oxidative enzymes might be lacking in new born animals. Thus Weatherall (1960) found that phenobarbital was more toxic to new born than adult rabbits. Lathe, Claireaux and Norman (1958); Brown, Zuelzer and Burnett (1958); Schmid, et al. (1959), Inscoe and Axelrod (1960) have shown that the foetus and the new born have low activity for the hepatic enzyme - glucuronyl transferase which is required for the conjugation of bilirubin and many other drugs to form the glucuronides. It is thus established that the age of animals does affect remarkably, their ability to biotransform drugs and foreign compounds.

III. Influence of other compounds:

The presence of other compounds influences the activity of drug metabolizing enzymes. Some of the compounds notably, barbiturates and some polycyclic hydrocarbons enhance liver microsomal drug metabolizing enzyme activities (Brown, Miller and Miller,

1954; Conney, Miller and Miller, 1956; Miller, et al. 1958).

Conney and Burns, (1959 and 1960), Conney et al. (1960) and Remmer (1969) have shown that pretreatment of rats with phenobarbitone, barbital or amino-pyrine, increased the activity of liver microsomal enzymes that metabolize hexobnrbitone, zoxozolamine; phenyl butazone amino pyrine, amino azodyes and many other compounds.

Similarly, certain compounds are also known to inhibit the activity of the microsomal enzymes. Notable in this group is β-diethyl amino ethyl diphenyl propyl acetate (SKF 525A) (Axelrod, Udenfriend and Brodie, 1954; Cooper, Axelrod and Brodie, 1954; Brodie, 1956; Fouts and Brodie, 1955). Several other compounds have also been characterised for their effective inhibition of drug metnbolizing enzyme activities (Fouts and Brodie, 1955 and 1956; Christensen and Wissing, 1972).

IV. Nutritional Status:

The nutritional state of animals affect their drug metabolizing ability. Dixon (1960) has shown that starvation depresses drug metabolism both in vitro and

in vivo. The effects of some particular diets on drug metabolism have been demonstrated by Brown, Miller and Miller (1954); Reif, *et al*. (1954). It has further been observed that vitamin C deficiency could affect drug metabolism extensively (Axelrod, Udenfriend and Brodie, 1954; Conney, *et al*. 1961; Udenfriend, *et al*. 1954; Dalgliesh, 1955). Wills (1972) demonstrated that vitamin K and naphthoquinone had effects on lipid peroxidation and oxidative metabolism by rat liver microsomes. These compounds stimulate the rate of oxidation of NADPH so that less is available for oxidative metabolism of lipids and other compounds like amino pyrine.

Becking (1972) has reported that rats fed on iron deficient diets for 18-days showed a marked increase in the metabolism (*in vitro*) of aniline and aminopyrine. Microsomal cytochrome C reductase activity was also increased during iron deficiency.

V. Other Factors:

Several other factors are now known to affect the metabolism of drugs and foreign compounds. These include sex and routes of administration of the substance.

Quin, Axelrod and Brodie (1958) have observed sex differences in the metabolism of hexobarbital. Hexobarbital was metabolised more rapidly in male rats than in female rats. Murphy and Dubois (1958); Inscoe and Axelrod (1960) have also demonstrated similar effects using dimethoxy-ester of benzotriazine diphosphoric acid, and o-aminophenol respectively. These effects have also been traced to the sex hormones.

The discovery that gut bacteria could metabolize certain compounds, (Draser, Renwick and Williams, 1971; Gingell, Bridges and Williams, 1971; Williams, 1970 and 1971), led to the belief that compounds administered orally may have altered pattern of metabolism from those administered either intraperitoneally or subcutaneously. Differences in metabolism of compounds arising from the route of administration might, therefore, be accounted for partly by the influence of gut flora or by poor absorption from the gastro intestinal tract (Dollery, Davies and Conolly, 1971).

METABOLISM OF AFLATOXINS

The metabolism of aflatoxins, attracted attention following the appearance of aflatoxins or their toxic

metabolites, in human food products from animals fed on toxic diets. A potential food contamination problem in this regard was illustrated by the discovery of toxic metabolites in milk from dairy cattle fed contaminated rations under experimental conditions (Allcroft and Carnaghan, 1963). This milk isolate was found to be toxic to ducklings, being capable of causing liver lesions or death in a pattern similar to authentic aflatoxin samples (Holzapfel, Steyn and Purchase, 1966; Purchase, 1967). de Iongh; Vleo and Van Pelt, (1964) demonstrated the presence of this milk 'factor' in the milk of cows fed highly contaminated peanuts and also showed its presence in the milk of lactating rats fed chromatographically pure aflatoxin B_1, hence it was concluded that the milk factor was, in fact, a metabolic product of aflatoxin B_1 and has been given the trivial name aflatoxin M_1. Allcroft, et al. (1966); Rabney, Burbage, Allcroft and Lewis, (1967) have also identified aflatoxin M_1 in the kidney, urine, faeces, milk and liver extracts of sheep previously dosed with aflatoxin B_1. Van der Linde, Frens and van Esch studied further the appearance of aflatoxin metabolites in cows

milk. They showed that the toxic metnbolite appeared in milk 12 - 14 houra after dosing with aflatoxin B_1. The quantity of aflatoxin M_1 in the milk of cows is now known to bear a linear relationship with the aflatoxin B_1 ingested (Allcroft and Roberts, 1968; Maori, Garcia and Page, 1969).

A factor, with properties similar to aflatoxin M_1 has been identified in the livers of rats dosed with purified aflatoxin B_1 (Butler and Clifford, 1965), thus suggesting the conversion of the parent compound to the metabolite in the liver tissue. Bassir and Osiyemi (1967); Osiyemi (1968); Emafo (1970) have also shown that the liver is the primary organ responsible for the biotransformation of aflatoxin B_1.

Holzapfel, Steyn and Purchase (1966), Maori, et al. (1967) have established the chemical nature of aflatoxin M_1. They have shown that it is 4-hydroxy aflatoxin B_1 and a hydroxylating enzyme capable of converting aflatoxin B_1 to 4-hydroxy aflatoxin B_1 has been reported by Schabort and Steyn (1969) to be present in the rat liver. They have also shown that a minor product, the 2-hydroxy aflatoxin B_1, is also produced

by the rat liver enzymes.

In addition to hydroxylation, aflatoxin B_1 is also metabolized by the cleavage of the methoxy group giving rise to formaldehyde and a phenolic moiety (Schank and Wogan, 1965; Wogan, Edwards and Schank, 1967; Ooiycmi, 1968; Bassir and Emafo, 1970). The formaldehyde produced is finally converted to carbon dioxide. The fate of the phenolic moiety was unknown until Dalezois, Wogan and Weinreb (1971) showed that it was largely conjugated in the rhesus monkey, as a glucuronide and hence referred to it as aflatoxin Pi.

The metabolism of aflatoxin B_1 has been reported to be largely species dependent especially in the rate of metabolism (Portman, R. Plowman, R.M. and Campbell, T.C. (1968), Bacoir and Emafo, 1970; Steyn, Pitout and Purchase, 1971). The mouse for instance, appears to produce other substances in addition to aflatoxin M_1 (Steyn, Pitout and Purchase, 1971).

However, two major metabolic conversions of aflatoxin B_1, have been established - ring hydroxylation giving rise to monohydroxylated products and

o-demethylation which also constitutes another degradative pathway (Holzapfel, Steyn and Purchase, 1966; Allcroft, et al. 1966; Nasri, et al. 1967; and Nabney, et al. 1967).

Reports on the metabolism of other aflatoxins are very scanty. It is believed, however, that they are capable of undergoing similar degradations as aflatoxin B_1. Thus, aflatoxin B_2 yields aflatoxin M_2 - a 4-hydroxylated product. Aflatoxin G_1 is hydroxylated at the 4- and 2- position to give rise to the mono-hydroxylated products referred to as aflatoxin "GM" and G_{2a} respectively (Dutton and Heathcote, 1968; Schabort and Steyn, 1969). Allcroft, et al. (1966) have observed however, that aflatoxin B_1 was metabolized in the sheep to a larger extent than aflatoxin G_1.

I. Excretion:

Aflatoxin B_1 and metabolites are excreted through the bile (Basoir and Osiyemi, 1967) and in the faeces and urine mainly (Falk, Thompson and Kotin 1963; Wogan, Edwards and Shank, 1967). In the rabbit and rat, aflatoxin B_1 and the metabolites are excreted as

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the glucuronides (Osiyemi, 1968; Bassir and Osiyemi, 1969).

METABOLISM OF THE PALMOTOXINS

Nothing is as yet known about the metabolism of the palmotoxins. In the present work, 'in - vitro' techniques, as applied in the study of aflatoxin B_1, have been used to study the fate of the compounds in several animal species.

CHAPTER II

MATERIALS

I. Aspergillus flavus (Link) ex fries U.I. 81 stock:

The Aspergillus flavus U.I. 81 culture was obtained from the Department of Botany, University of Ibadon, Ibadan. This was part of the original aflatoxigenic isolate of Dr. S.O. Alasoodura of the same Department. Stock cultures were obtained by sub-culturing the original supply on sterile agar slants of the Yeast extract-sucrose medium (Davis, Diener and Eldridge, 1966). Cultures were grown at 25 ± 1 °C until a uniform growth of mycelia, showing green sporulation appeared. Stock cultures were preserved by sub-culturing at two weekly intervals.

II. Composition of medium for sub-culturing Aspergillus flavus ex fries (U.I. 81) (Davis, Diener and Eldridge, 1966):

Yeast extract (Difco)	20g.
Sucrose (bacteriological grade)	200g.

Bacto-Agar 15g.

Distilled water 1,000ml

The pH was adjusted to 7.0. The solution was heated to melt the agar, cooled and distributed into McCartney bottles in 10 ml portions. The bottles were sterilized at 15 lb/sq. in. for 30 minutes and allowed to cool while in a slanting position. The medium was then innoculated with spores of Aspergillus flavus using a platinum loop.

III. Culture medium for quantitative production of aflatoxin:

The above medium of Davis, Diener and Eldridge (1966) was retained except that bacto-agar was omitted.

Yeast extract (Difco) 20g.

Sucrose (bacteriological grade) 200g.

Distilled water 1000 ml.

The pH was also adjusted to 7.0 and the solution distributed into 250 ml. Erlenmeyer flasks in 50 ml aliquots. Cultures were grown for 6 to 8 days.

IV. Medium for production of Palmotoxins:

Palm sap from the oil palm (Elaeis guineensis) were collected with dry-heat sterilized flasks embedded

in an ice-salt mixture in a large thermos flask. The sap was collected from the top of the oil palm, overnight by placing the flask in position (Faparusi, 1966, Adekunle, 1969).

At collection, the unfermented sap had a pH of 6.0 to 7.0. It was then distributed into 250 ml Erlenmeyer flasks, sterilized at 15 lbs/sq. in. for 30 minutes and innoculated with Aspergillus flavus spores. Cultures were incubated at 30°C for 5 days.

V. Thin layer plates coated with silica gel G:

60g of silica gel G (chromalay brand supplied by May and Baker, Degenham, England) or 50g kieael gel G (E. Merck Darmstadt, Germany), where applicable, were shaken with 100 ml of water for 2 - 3 minutes and used to coat 6 glass plates, 20 cm x 20 cm x 0.3 cm, arranged on a Shandon "Unoplan Leveller". The spreader was so adjusted as to give a uniform thickness of 0.5 mm and used in coating the plates.

Plates were activated overnight at 110°C in an air oven before use. All plates used were prepared by the above procedure.

VI. Animals used for "in vitro" studies:

All through this work, male animals were used. The lizard and toad were used as caught from the surroundings. The sheep, goat and duck were bought from the local market and maintained on their respective normal diets of green grass for goat and sheep and insects and ants for the duck, until they were in use. All chickens used were obtained from the Faculty of Agriculture, Teaching and Research Farm, University of Ibadan. All other animals were obtained from the Pre-clinical Animal House, Faculty of Medicine, University of Ibadan, Ibadan. Litter mates were used as much as possible. Animals used were:

Rat (*Rattus* sp. wistar strain)	95-110g wt.
Albino mouse (*Musculus* sp.)	30-35g wt.
Guinea pig (*Cavia porcellus* sp.)	300-350g wt.
Rabbit (*Oryctolagus cuniculus*)	1.8-2.0 kg. wt.
Dog (Domestic) (*Canis* sp.)	5-5.5 kg. wt.
Sheep (Local dwarf breed)(*Ovis* sp.)	10-10.5 kg. wt.
Goat (Local dwarf breed) (*Capra hircus*)	12-12.5 kg. wt.

Duck (Anas sp.)	1.8-2.2 kg wt.
White rock cock (Gallus sp.)	2-2.2 kg wt.
Toad (Bufo regularis)	50-60g wt.
Rainbow Lizard (Agama agama)	70-80g wt.

VII. Composition of the incubation medium for Liver Slices (McEWAN, 1956):

Sodium chloride	7.6g.
Glucose	2.0g.
Sucrose	4.5g.
Sodium bicarbonate	2.1g.
Potassium chloride	0.42g.
Calcium chloride	0.42g.
Sodium dihydrogen phosphate	0.14g.

Distilled water to make up solution to 1 litre.

The calcium chloride was usually added at a latter stage, when the other components have been appreciably diluted with the maximum possible quantity of water, to avoid the precipitation of calcium phosphate. Fresh solutions were prepared each day.

VIII. Silver Nitrate reagent (Trevelyn, Proctor and Harrison, 1950).

Silver nitrate (saturated solution in water)

0.1 ml. Acetone to make a solution of 20.0 ml. Distilled water was added dropwise until the precipitated silver-nitrate redissolved.

IX. Ninhydrin reagent (Baesir, 1963):

Ninhydrin	0.2g.
Acetone to make a solution of 100 ml.	

X. Potassium Rhodizonate reagent:(Schneider and Lewbert, 1956):

Potassium rhodizonate	12.0 mg
Distilled water	15.0 ml
Concentrated ammonium hydroxide solution (Sp gr. 0.91)	10.0 ml.
Absolute alcohol	25.0 ml.

XI. Double Strength Nash Reagent (Cochin and Axelrod, 1959):

Ammonium acetate	150g.
Redistilled acetyl acetone	2.0 ml.
Distilled water to make solution up to 500 ml.	

XII. Naphthoresorcinol Spray reagent (Bridges, Kibby and Williams, 1965):

1% v/v naphthoresoroinol in acetone	20.0 ml.

10% v/v phosphoric acid to make a

solution of 25ml.

XIII. Aqueous naphthoresorcinol Solution:

(Fishman and Green, 1955).

Naphthoresorcinol 0.4g.

Distilled water 100ml.

The naphthoresorcinol was pulverized with a mortar and pestle, shaken with water in a glass stoppered, amber-coloured measuring cylinder for 10 minutes and then filtered. The filtrate was stored away from light.

XIV. Reagents for alkaline phosphatase determination:

(Bessey, Lowry and Brock, 1946).

(a) 0.05M Glycine Buffer:

Glycine 3.75g.

Magnesium chloride 47.5g.

Dissolved in about 800ml, distilled water, added 85 ml 1N sodium hydroxide and made up the solution to one litre.

(b) P-nitrophenyl phosphate substrate:

P-nitrophenyl phosphate.

Sodium salt to make 0.2% solution in 0.001 Normal hydrochloric acid. Adjusted pH. to-6.5 - 8.0.

These reagents were supplied in the BDH 'Biochemica

test combinations' for alkaline phosphatase determination (TCP, Cat. No. 15987 TAAF).

XV. Reagents for glutamate oxaloacetic acid transaminase determination: (Reitman and Frankel, 1957).

(a) Standard Pyruvate.

Sodium pyruvate 22.0 mg.

0.1M phosphate buffer,

pH 7.4 to make up to 100 ml.

(b) SGOT Substrate.

α-Ketoglutaric acid 29.2 mg.

dl-Aspartic acid 2.66 g.

These were dissolved in 1N sodium hydroxide and adjusted to pH 7.4. It was then made up to 100 ml with 0.1M phosphate buffer pH 7.4. This gave a solution of 2 mM α-ketoglutaric acid and 200 mM dl-aspartic acid per litre (Reitman and Frankel, 1957).

(c) 2:4 dinitrophenyl hydrazine reagent:

2:4 dinitrophenyl hydrazine 19.8 mg.

It was made up to 100 ml with 1N HCl.

(d) Aniline citrate reagent:

5g. citric acid (analytical grade) were dissolved in 5 ml water and 5 ml of Aniline (analytical grade) were added.

XVI. Phenobarbitone Solution:

Phenobarbitone sodium salt 2.5g.

The salt was warmed with a little of 2N sodium hydroxide until the substance dissolved completely. The solution was made up to 100 ml with 0.9% saline.

XVII. Carbon monoxide:

Carbon monoxide was generated from sodium formate (BDH Chemicals) and concentrated sulphuric acid (SP. gr. 1.88).

XVIII. Iodine Number determination (Baesir, 1963):

(a) Dame Iodine:

8.2 ml of pyridine and 6 ml. concentrated sulphuric acid were added to 20 ml glacial acetic acid cooled with ice. To this was added a solution of 2.6 ml bromine dissolved in 20 ml. acetic acid. The mixture was diluted to 1 litre with glacial acetic acid and stored in the dark.

(b) 10% Potassium iodide solution:

10g. potassium iodide was dissolved in 100 ml of distilled water.

(c) N/40 Sodium thiosulphate solution:

6.6g of sodium thiosulphate was dissolved in distilled water and made up to 1 litre.

(d) 1% Starch Solution:

1g. of starch was stirred with 4 ml. of distilled water. The suspension was poured into 96 ml. of boiling distilled water. The resulting solution was cooled and made up to 100 ml.

CHAPTER III

METHODS

I. Preparation of Pure samples of aflatoxin B_1 and G_1:

Following a preliminary experiment, yeast-extract-sucrose medium inoculated, with Aspergillus flavus spores, was incubated for 6 days at 30°C. The medium was harvested by filtering off the mycelial matt. One litre of the liquid broth was extracted for 24 hours with chloroform in a Holliman's quickfit, liquid - liquid extractor (Holliman, 1952). This method of Trager, Stoloff and Campbell, (1964) and Nabney and Nesbitt (1965), was retained for all quantitative extraction of the medium. The chloroform extracts were concentrated in a rotary film evaporator. Precautions were taken to avoid direct exposure to light, as Emafo (1970) had shown that the aflatoxins were photosensitive and degenerate rapidly when exposed to light.

Primary Purification:

Crude chloroform concentrates were filtered through

a double layer of Whatman No. 1 filter paper, over anhydrous sodium sulphate. The extracts were then stored overnight at about 0°C, when some impurities separated out. The supernatant chloroform solution, was then stored in amber-coloured bottles at a temperature below 4°C.

Chromatographic Separation:

A modified thin layer procedure as reported by de Iongh, et al. (1962 and 1964) was employed in the chromatography of crude toxins. Kiesel gel G (E. Merck, Dermstadt, Germany) and silica gel G ('Chromalay', May and Baker, Degenham, England) were used in coating the plates used in the experiments. Crude extracts were run on coated thin layer plates activated overnight at 110°C, in an air-oven. The plates were developed with chloroform: methanol: formic acid (95:5:1) in unlined and unequilibrated Baird and Tatlock (London) Ltd., multi-sheet chromatographic tank (Smith and McKernan, 1962). About 16 plates were run at a time using about 400 ml. of the solvent mixture (Plate I).

The plates were removed from the tank when the

Plate I. Thin layer chromatogram for the isolation of aflatoxins B and G.

solvent front was about 5 cm. to the top and stored in a dark chamber to dry. On viewing the plates in Ultraviolet light, fluorescent bands corresponding to aflatoxin B_1 and G_1 were marked out and scraped off. The toxins were eluted from the silica gel with 2% methanol in chloroform (v/v). The eluate was concentrated in a rotary film evaporator and re-chromatographed using 10% acetone in chloroform (v/v) as the developing solvent. The aflatoxin B_1 and G_1 were recovered from the silica gel scraping, by eluting with 2% methanol in chloroform (v/v).

The solutions were taken to dryness in a rotary film evaporator and the residue redissolved in a minimum amount of chloroform. The toxins were reprecipitated from chloroform by dropwise addition of either n-hexane or petroleum ether (Shotwell, et al. 1966). The solutions were left in a deep freezer at about - 10°C. Crystals formed by this method were dried under vacuum, in a dessicator.

Spectrophotometric Analysis:

To ascertain the purity of the samples so obtained, spectral analysis - mainly ultraviolet and infra-red spectra were used.

Ultraviolet Spectrum:

The aflatoxin B_1 sample was dissolved in methanol (analytical grade) and the ultraviolet spectrum (Fig I) obtained in a Perkin Elmer Ultraviolet-visible Spectrophotometer 137UV. The spectrum of aflatoxin G_1 was obtained both in methanol (Fig 2) and in water (Fig 3).

Infra-red Spectral Analysis:

The infra-red spectra of both aflatoxin B_1 and G_1 were obtained in nujol using a Perkin Elmer sodium chloride Spectrophotometer 137 (Fig 4 and 5).

Table 5 is a summary of the Ultraviolet and infra-red characteristics obtained.

II. Quantitative estimation of aflatoxin B_1 and G_1:

(a) Spectrophotometric Method:

The Spectrophotometric method of Nabney and Nesbitt (1965), was used in the estimation of the aflatoxin B_1 and G_1 used in this work. Chloroform solutions of the toxin were gradually added to distilled water and the chloroform blown off with Nitrogen gas (supplied in cylinders by the Industrial Gases,(Nigeria) Ltd.). The optical densities of the aqueous solutions were obtained

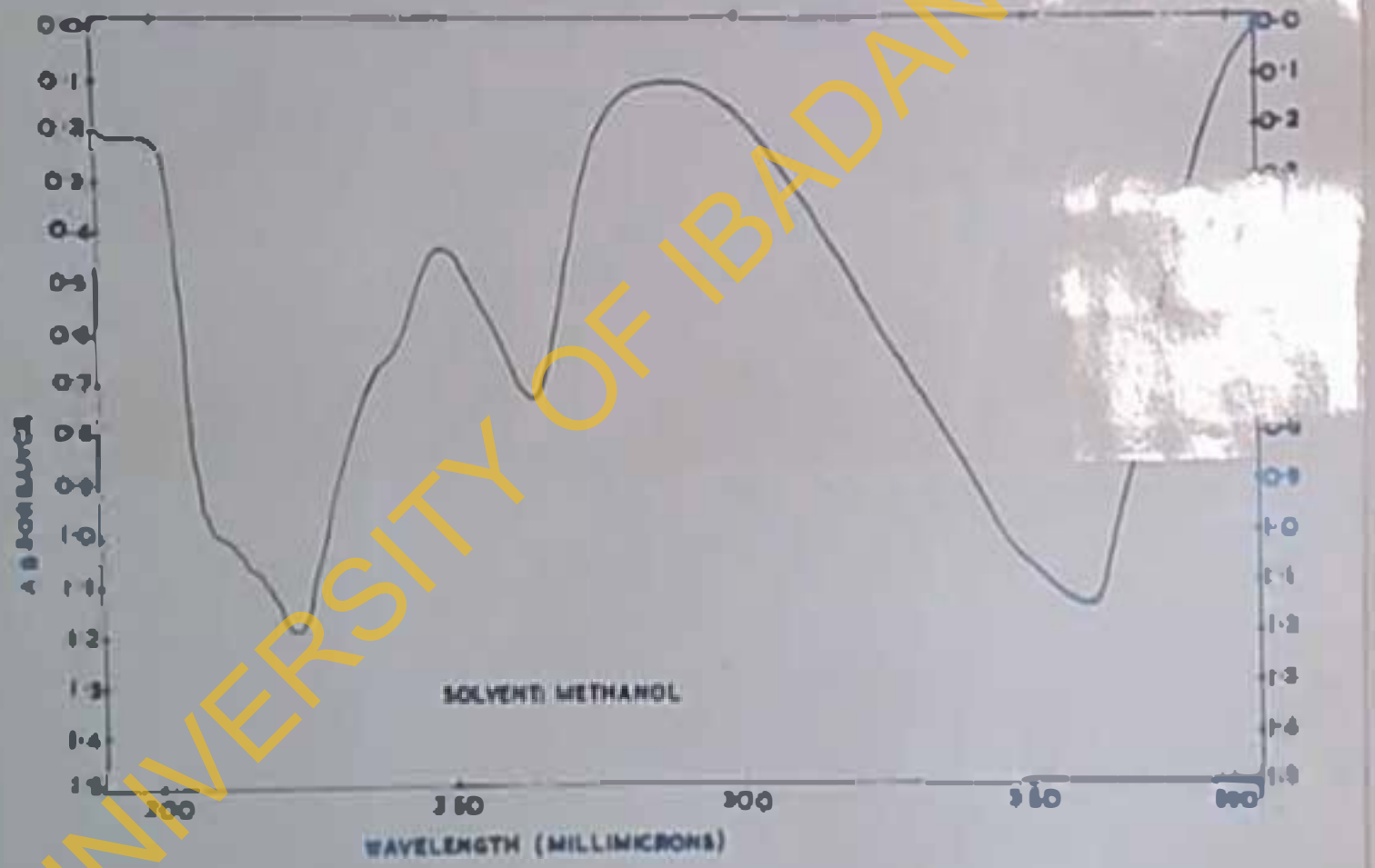

Fig. I: ULTRAVIOLET SPECTRUM OF AFLATOXIN B1.

Fig AFLATOXIN G1 (SOLVENT METHANOL)

Fig. 2

Fig. AFLATOXIN G1 (SOLVENT: WATER)

Fig. 3

B1 & V

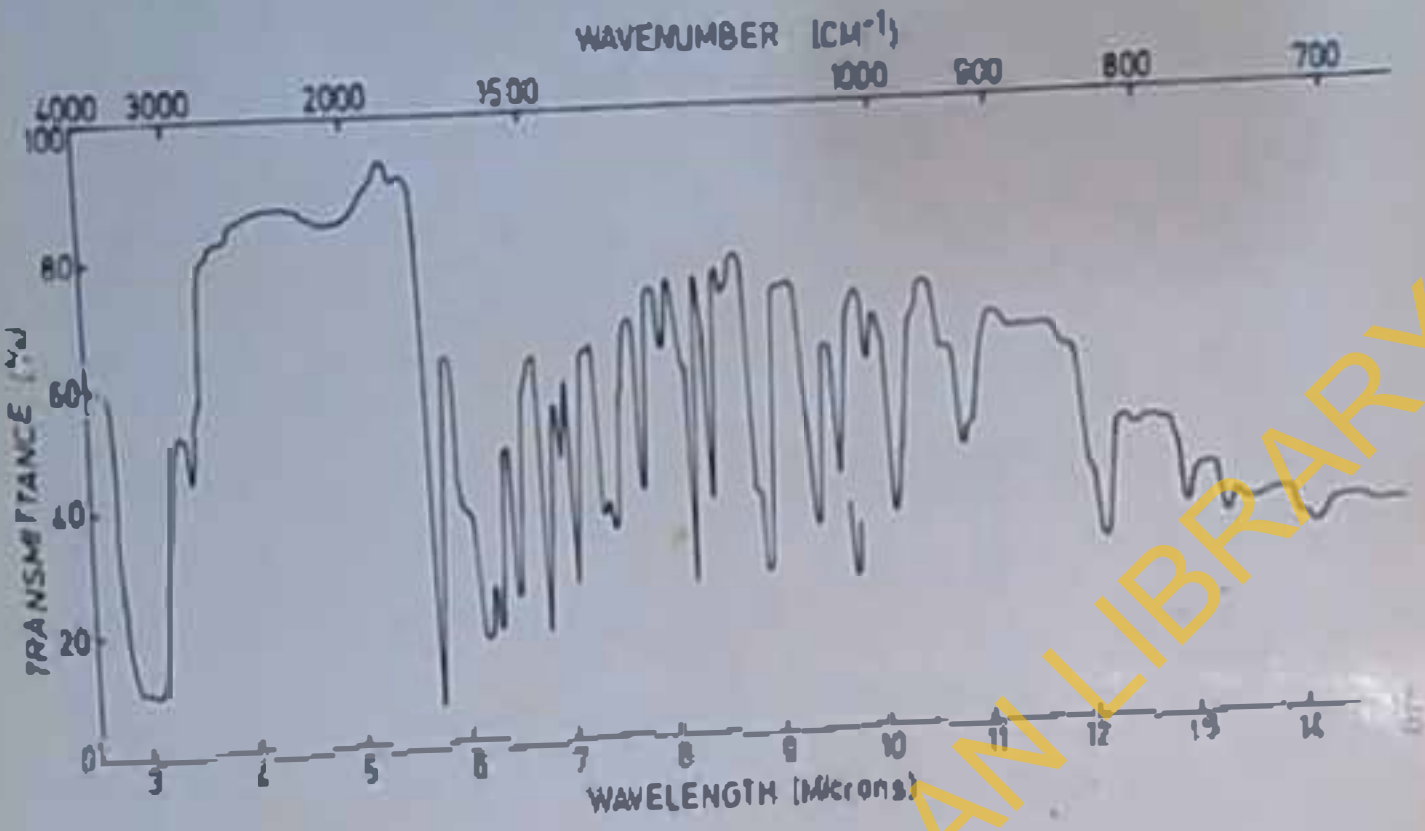

Fig. AFLATOXIN B1

Fig. 4. INFRARED SPECTRUM

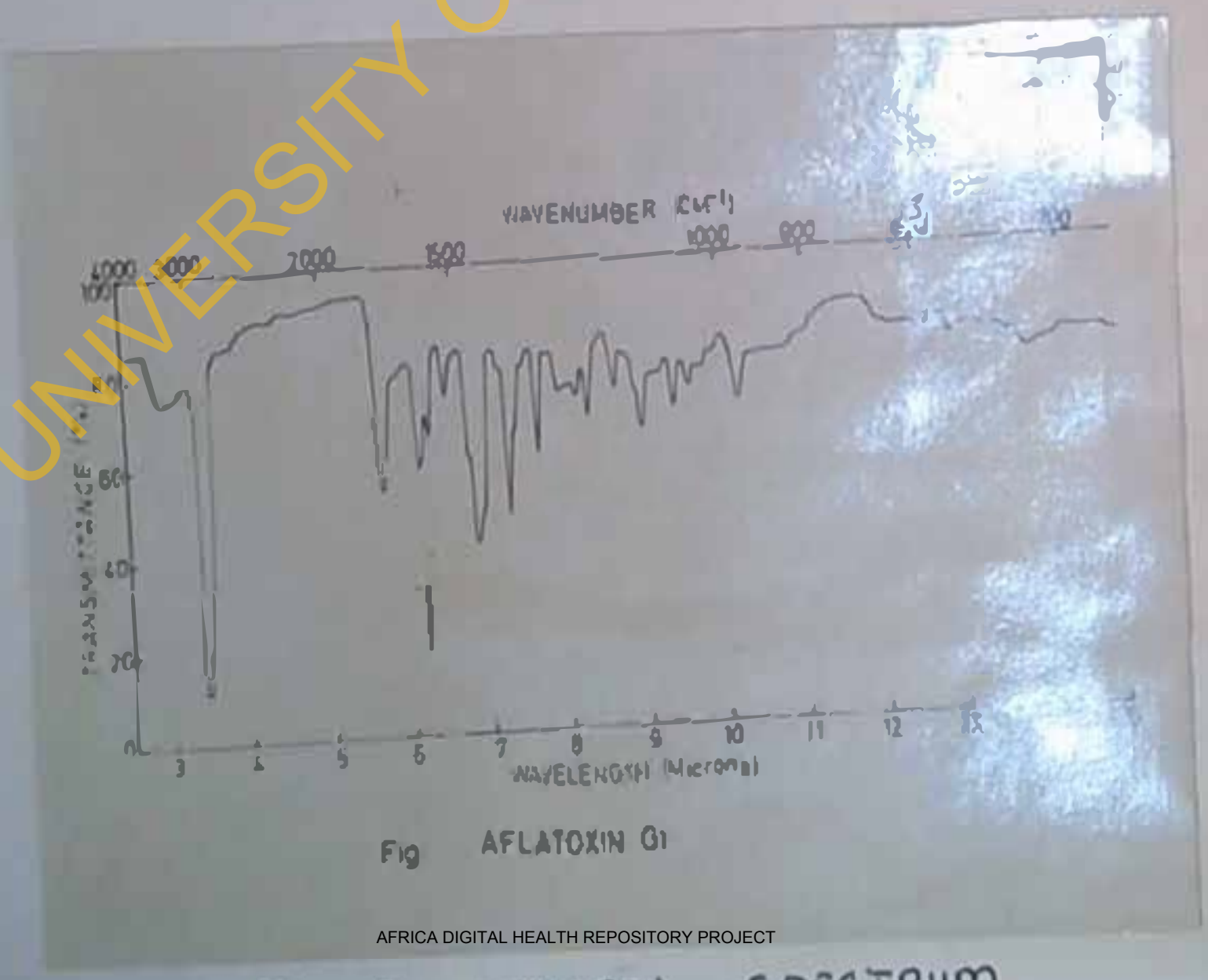

Fig AFLATOXIN G1

Fig. 5 INFRARED SPECTRUM

TABLE 5

Ultraviolet and infra-red characteristics of aflatoxin B_1 and G_1

AFLATOXIN B_1		AFLATOXIN G_1		
Ultraviolet Peaks (Methanol)	Infra-red Peaks (cm^{-1})	Ultraviolet Peaks		Infra-red peaks (cm^{-1})
		Methanol	Water	
223 nm	1750	218 nm	203 nm	1760
265 nm	1680	239 nm	253 nm	1695
362 nm	1630	362 nm		1630
	1590	365 nm	365 nm	1595
	1545			1545

from a Unicam SP 500 Spectrophotometer at 363nm. Emafo (1970) has shown that the optical density bears a linear relationship with the concentration. Concentrations were, however, obtained using the formula of Nabney and Nesbitt (1965) (Page 25).

(b) Serial Dilution Method:

The 'Null fluorescence technique' of Coomes, et al. (1965) was employed in the estimation of residual aflatoxin B_1 after incubation and the aflatoxin M_1 and GM_1 formed during incubation. The minimum amount of aflatoxin B_1 which gave the least observable fluorescence, has been given as 0.0004 µg and for aflatoxin G_1, 0.0003 µg.

In the assay method used, 1 ml. of extract or aflatoxin concentrate was added to tube one of a set of ten test tubes, each containing 1 ml. of the pure solvent. 1 ml. of the mixture in tube one was withdrawn and added to tube two and the process continued until tube ten was reached. 0.04 ml was withdrawn from each tube and applied to chromatoplates of Kiesel gel G (E. Merck, Dermstadt, Germany). Standard of known concentrations

were similarly treated and equal volumes also applied on thin layer plates. The use of standards were found necessary, to correct any variations in fluorescence properties due to either the silica gel or to the solvent.

Spotted plates were developed with chloroform: methanol (96:4 v/v); air-dried in the dark and viewed over a Gallenkamp 3650A Ultraviolet lamp, with the plates placed at a distance of about 20cm. from the light source. The dilution that gave the least fluorescence was noted. Concentration of the test solution was then calculated.

Calculation:

If in a particular experiment, least fluorescence was observed in tube 4 (i.e. dilution 16), and the corresponding concentration under the prevailing conditions was 0.0005 ug; the concentration can be calculated thus:

0.04 ml contained 0.0005 µg

1 ml contained $\frac{0.0005}{0.04} \times 1$

Since the original solution was diluted sixteen times, 1 ml of the original solution, contained

$$\frac{0.0005}{0.04} \times 1 \times 16 = 0.2 \text{ µg.}$$

The same principle and the fact that aflatoxin M_1 and "GM" are three times as fluorescent as aflatoxin B_1 and G_1 respectively, were employed in the calculation of the concentration of the hydroxy aflatoxins.

III. Preparation of Pure samples of Palmotoxin Bo and Go:

Palmotoxin Bo and Go were prepared from mature cultures of Aspergillus flavus (U.I. 81) grown on the palm sap medium. The aflatoxins are also produced along with the palmotoxins in this medium (Adekunle, 1969). Procedures employed here were similar to those for the isolation of aflatoxin B_1 and G_1 except that cultures were incubated for only five days in this medium. The chloroform extracts obtained by liquid - liquid extraction of the broth, were first left to cool, when some fine needle-shaped crystals believed to be mainly kojic acid, separated out (Foster, 1949; and Parrish, et al. 1966). The supernatant was filtered off and concentrated in a rotary film evaporator. All processes took place with minimum exposure to light.

Primary Purification of Extracts:

The concentrated extract was passed through anhydrous sodium sulphate in a double layer of Whatman No. 1 filter

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Primary Purification of Extracts:

The concentrated extract was passed through anhydrous sodium sulphate in a double layer of Whatman No. 1 filter

paper. It was later passed through a very short column of cupric carbonate, previously washed with 2% acetone in chloroform (v/v). This procedure removed some of the adhering pigments (Stubblefield, Shannon and Shotwell, 1968).

Thin layer Chromatography:

Crude extracts were separated by the thin layer chromatographic technique as employed for the isolation of aflatoxin B_1 and G_1. However, in the first thin layer separation, a 3% methanol in chloroform (v/v) solvent system was used. The bands with Rf. value of 0.15 and 0.09 (Plate 2C) and fluorescing blue and green respectively were isolated, (Adekunle, 1969). These corresponded to the palmotoxins Bo and Go respectively.

The substances were eluted from silica gel with 4% methanol in chloroform (v/v). The eluates were taken to dryness using a rotary film evaporator and redissolved in chloroform. The substances were reprecipitated from chloroform with n-hexane or petroleum ether (Shotwell, et al. 1966 and Adekunle, 1969). The residue were separated from the supernatant by centrifugation,

Plate 2: Thin layer chromatogram for the isolation of Palmatosins Board Go

redissolved in chloroform and re-chromatographed in 5% methanol in chloroform (v/v). The substances were once more isolated as above.

Test of Purity:

For the purpose of the metabolic studies, test of purity was largely based on thin layer chromatography. Isolates were chromatographed until they gave single spots on thin layer plates using methanol: chloroform (5:95 v/v) and chloroform: methanol formic: acid (95:5:1 v/v).

The Ultraviolet Spectra were also taken in aqueous solution using a Perkin Elmer Spectrophotometer 137UV (Figures 6 and 7).

IV. Estimation of Palmotoxin Bo and Go:

(a) Reprecipitated samples of palmotoxins Bo and Go were prepared as described above. They were dried in an evacuated dessicator in the dark and later weighed. The weighed samples were dissolved in dry chloroform (analytical grade) and made up to 5 ml. in volumetric flasks. Aliquots of each sample, representing different concentrations, were diluted to 3 ml and their optical densities obtained at 265nm using a Unicam SP 500 Spectrophotometer. Standard curves were obtained for

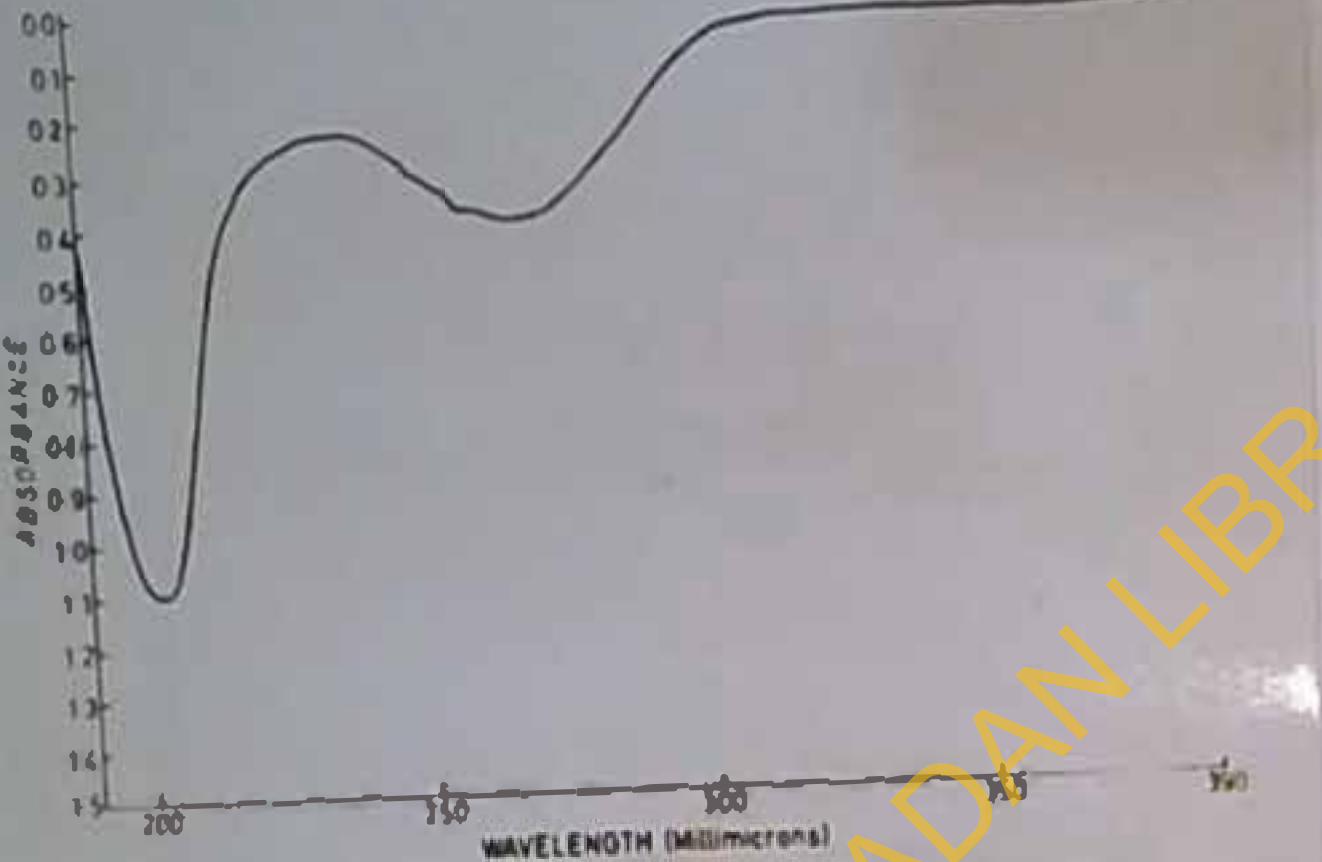


Fig PALMOTOXIN 80 (SOLVENT WATER)

Fig. 6 ULTRAVIOLET SPECTRUM

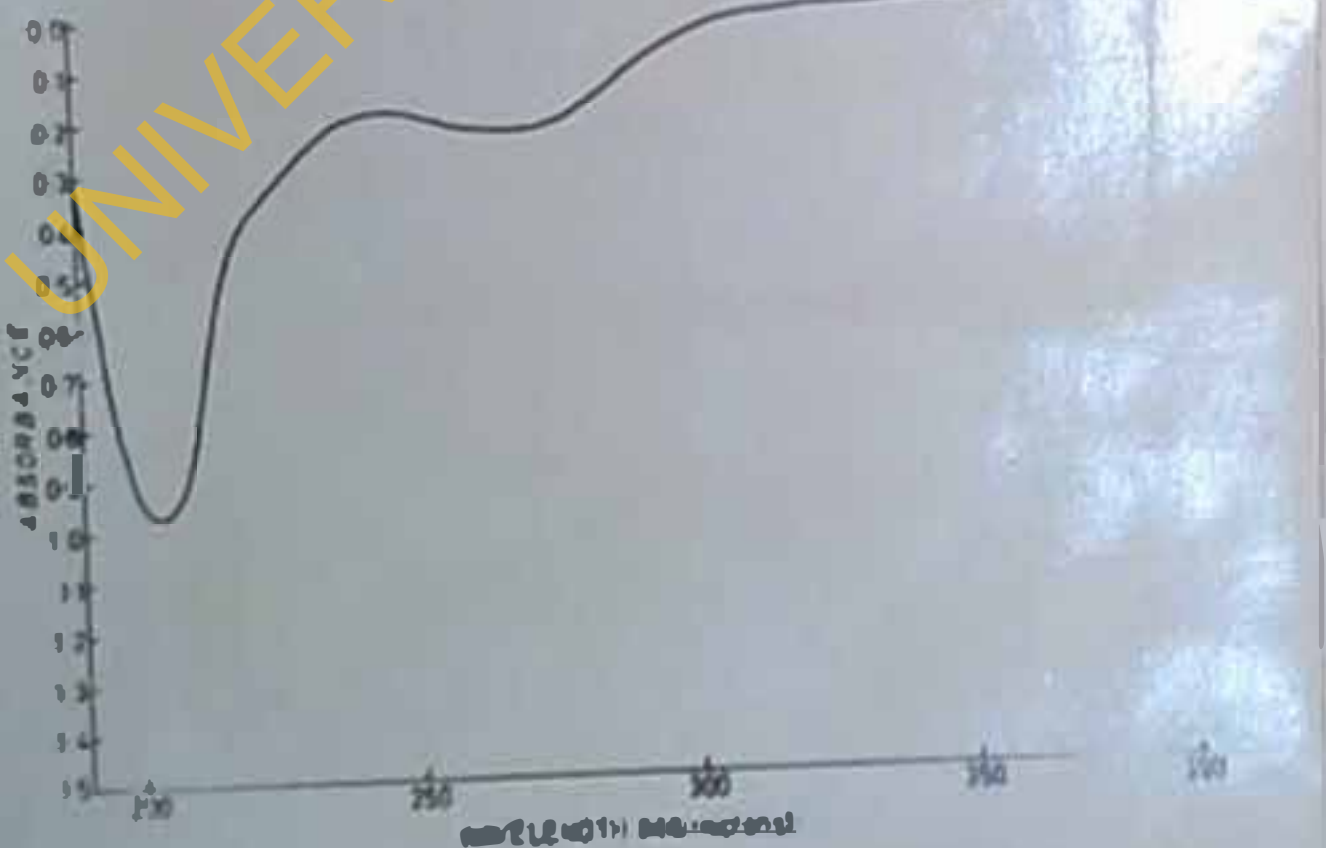


Fig PALMOTOXIN 60 (SOLVENT WATER)

each substance by plotting optical density against concentration (Figures 8 and 9).

(b) Serial dilution technique for the estimation of Palmotoxins Bo and Go:

Standard solutions of the toxins were serially diluted in the same manner as for aflatoxin B_1 and G_1.

0.05 ml. from each test tube was spotted on thin layer plates coated with Kiesel gel G (E. Merck, Dermstadt, Germany) and developed with chloroform: methanol (96:4 v/v) solvent system. The spot with the least observable fluorescence was also obtained by viewing on Ultraviolet light (3650A) placed at about 20 cm from the plate.

Calculation:

Concentration of 1 ml. of solution = 5 ug

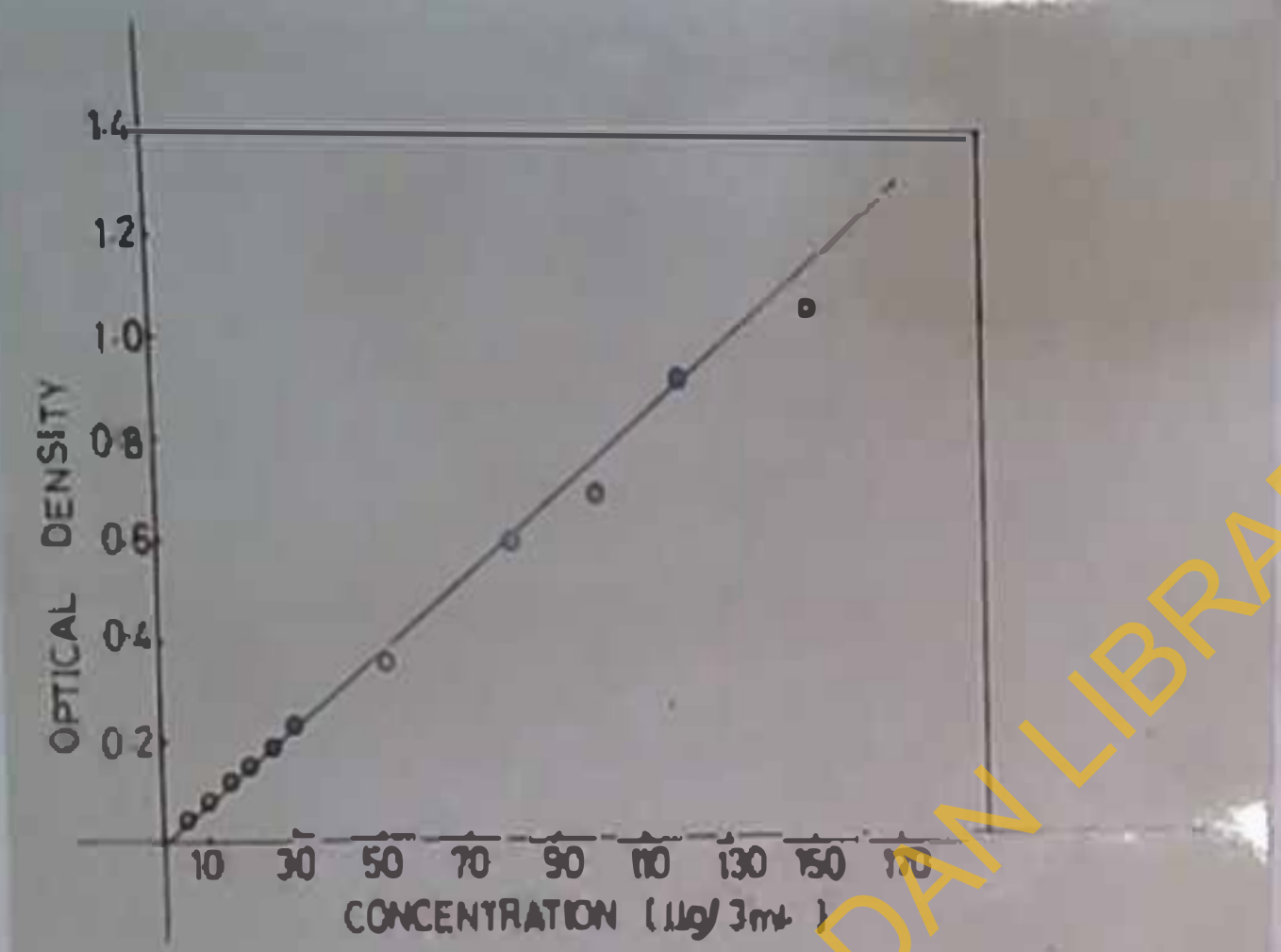
and the dilution with the least fluorescence, was dilution ten.

∴ If X is the concentration equivalent to 0.05 ml in the tenth test tube,

0.05 ml. is equivalent to X µg and 1 ml. of the original solution is equivalent to

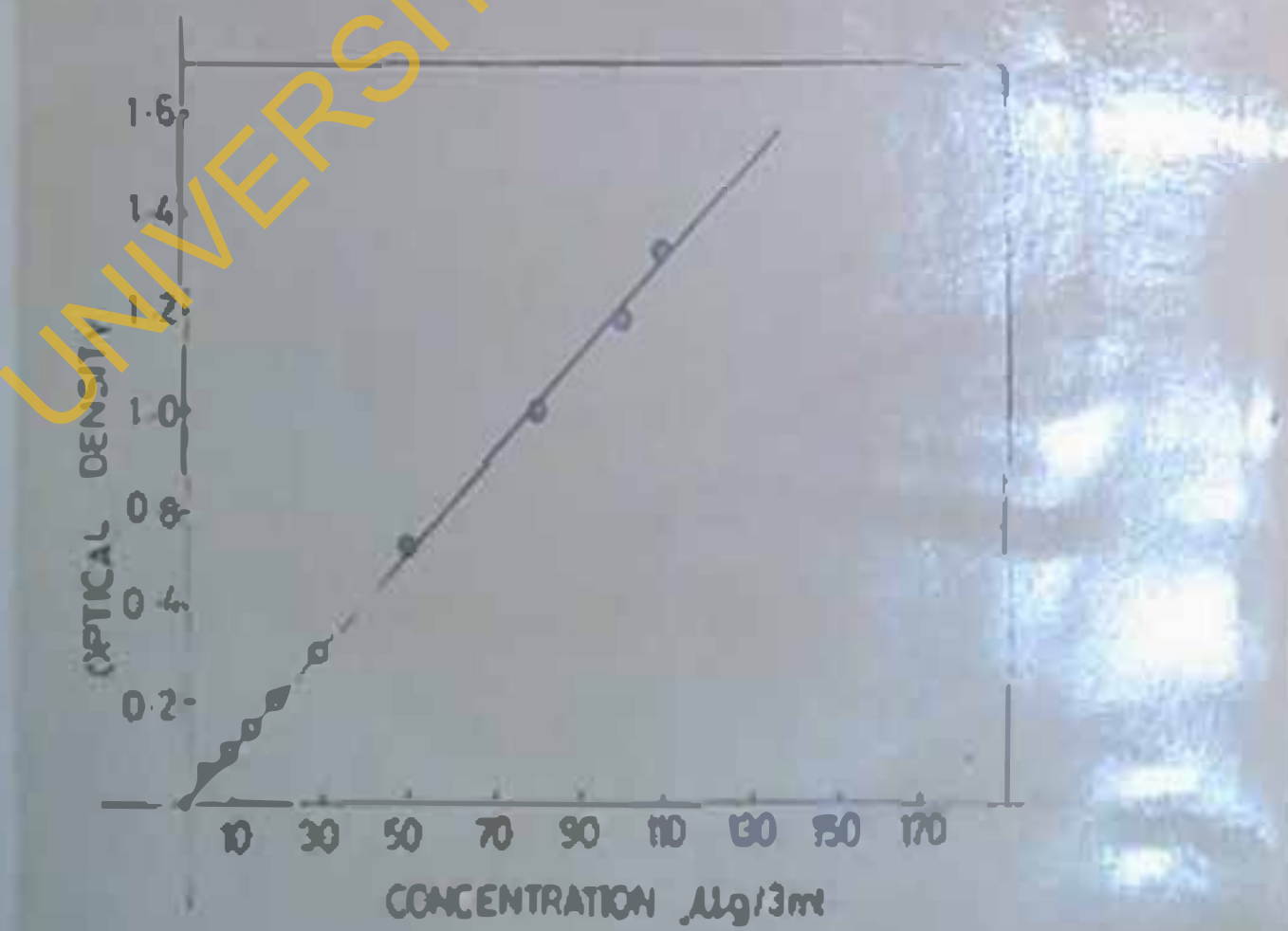
$$\frac{X}{0,05} \times 1 \times 2^{10}$$

$$= 5 \text{ ug}$$



Palmotoxin Bo standard curve.

Fig. 8



Standard curve for palmotoxin Bo

Applying the same principle to palmotoxin Go, it was found that the concentration with the least observable fluorescence was 0.0002 ug.

V. Determination of alkaline phosphatase activity in serum by the Colorimetric method:

The method used in the determination is the method of Bessey, Lowry and Brock (1946). This method depends on the hydrolysis of P-nitro phenylphosphate at pH 10.5.

The substrate used was as in the materials section and supplied in the BDH analytical set (Biochemica test combinations TC-P, Cat. No. 15987 TA&F) for alkaline phosphatase determination.

Four test tubes each containing 1 ml. of the substrate were incubated at 37°C for 30 minutes with 0.1 ml. of the test serum in 3 out of the four tubes. At the end of the 30 minutes, 10 ml of 0.02N sodium hydroxide was added to all the tubes and 0.1 ml. serum added to the fourth tube which served as the blank tube. The optical densities (B) were read immediately at 405nm on a Unicam SP. 600 Spectrophotometer, using 1 cm. glass cuvettes.

Values for alkaline phosphatase were obtained using the following relationship:

E_{405nm} x 200 = milliunits/ml of alkaline phosphatase.

VI. Determination of Serum Glutamic - Oxaloacetic acid transaminase (SGOT) by the Colorimetric method:

This determination is based on the transamination of L-aspartic acid and α-oxoglutaric acid to oxaloacetic acid and glutamic acid respectively.

α-Ketoglutarate + aspartate ---> Oxaloacetate + glutamate.

Glutamic-oxaloacetic acid transaminase mediates this reaction (Reitman and Frankel, 1957). The oxaloacetic acid formed in the reaction is unstable and is decomposed to pyruvic acid by the addition of aniline citrate solution.

Reaction of the pyruvic acid formed with 2.4 dinitrophenyl hydrazine yields the corresponding hydrazone which gives intense brownish colour on the addition of sodium hydroxide solution.

Reagents for this determination are as shown under materials and were supplied in a set (BDH enzyme assay

Set 2; 25002 for the determination of SGOT).

1 ml. of the substrate was pipetted into each of 4 test tubes and each allowed to attain a temperature of 37°C using a Gallenkamp self regulating water bath. After noting the time, 0.2 ml serum was added to three of the tubes which served as sample tubes. The fourth served as blank. The tubes were incbated for 60 minutes and with the tubes still in water, 1 drop of the aniline citrate reagent was added to each tube. After 5 minutes, 1 ml. of 2:4 dinitrophenyl hydrazine solution was added to each tube and 0.2 ml of serum also added to the control tube. Incubation was continued for a further 20 minutes, when the samples were removed from the water bath and 10 ml. of 0.4N sodium hydroxide added to each tube and the tubes allowed to stand for 10 minutes.

Optical densities were measured at 505nm. The concentration of the tests was obtained from a standard curve (Fig 10) obtained by incubating a pyruvate standard for 30 minutes at 37°C and further treating the samples with the other reagents as in the tests.

Set 2; 25002 for the determination of SGOT).

1 ml. of the substrate was pipetted into each of 4 test tubes and each allowed to attain a temperature of 37°C using a Gallenkamp self regulating water bath. After noting the time, 0.2 ml serum was added to three of the tubes which served as sample tubes. The fourth served as blank. The tubes were incbated for 60 minutes and with the tubes still in water, 1 drop of the aniline citrate reagent was added to each tube. After 5 minutes, 1 ml. of 2:4 dinitrophenyl hydrazine solution was added to each tube and 0.2 ml of serum also added to the control tube. Incubation was continued for a further 20 minutes, when the samples were removed from the water bath and 10 ml. of 0.4N sodium hydroxide added to each tube and the tubes allowed to stand for 10 minutes.

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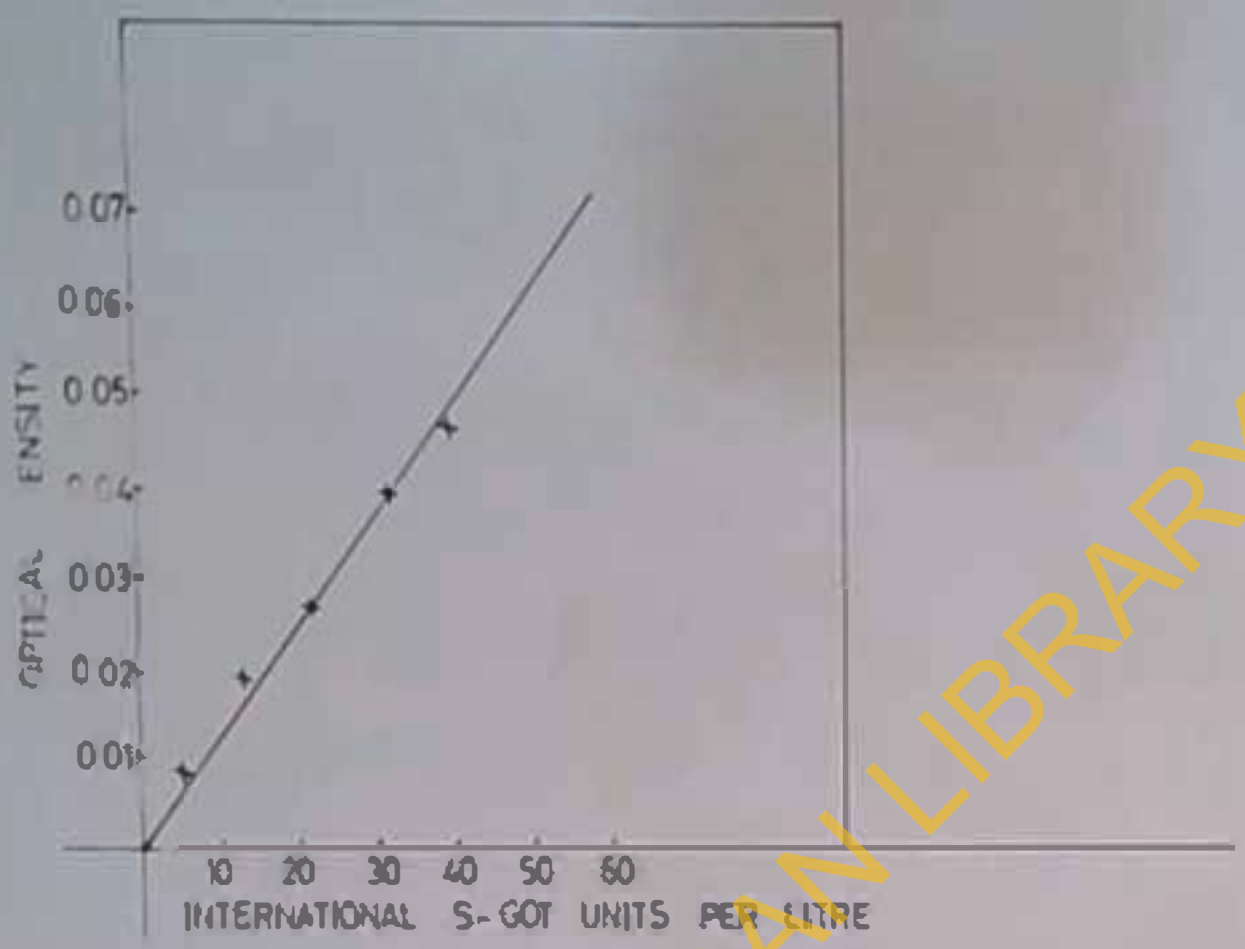
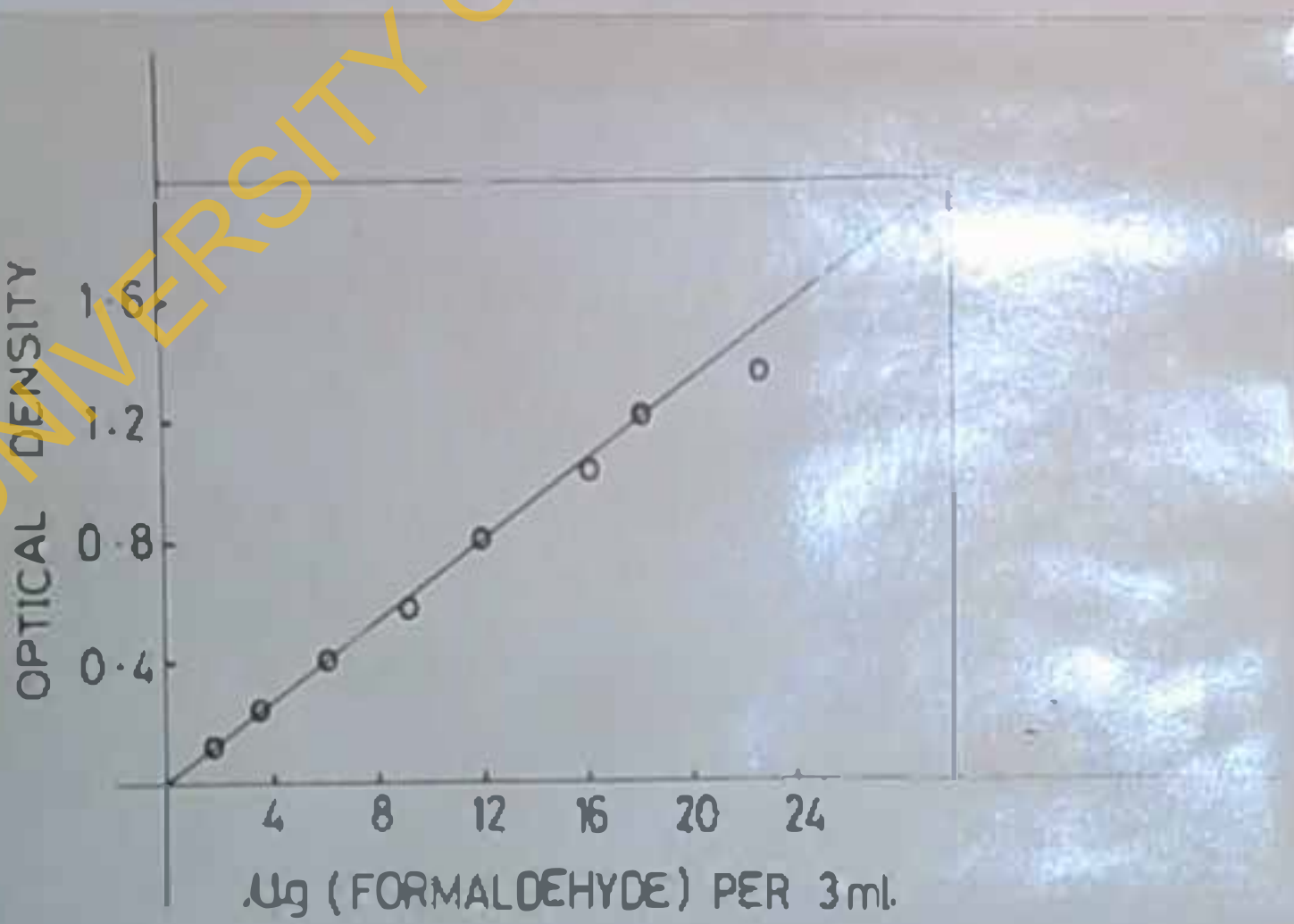


Standard curve for serum glutamic oxaloacetic-transaminase activity

Fig. 10



Standard curve for formaldehyde

VII. Preparation of Liver Slices:

The animals were always killed by decapitation. The livers were removed immediately and rinsed with ice-cold (0 - 2°C) normal saline. Extraneous tissues and gall bladders were removed where necessary. Slices were prepared from weighed samples free-hand, using very sharp razor blade previously cleaned with acetone and rinsed with buffer solution. The temperature was kept at below 4°C all through the process.

VIII. Preparation of the microsomes-plus-soluble fraction of liver homogenate:

The animals used for these experiments were also killed by decapitation and the livers removed immediately and washed in ice-cold (0 - 2°C) 0.1M phosphate buffer (pH 7.6). Weighed liver samples were homogenized in three volumes of ice-cold (0. - 2°C) 0.3M potassium phosphate buffer (Emafo, 1970) using a Waring blender for 20 seconds (Leadbeater and Davies, 1964).

Homogenates were centrifuged at 10,000g for 15 minutes using an MSE Super speed refrigerated centrifuge. Nuclei, mitochandria and unbroken cells were thus

sedimented. The resultant supernatant containing the microsome-plus-soluble fractions was used in the 'in vitro' studies reported in this work.

IX. Formation of acetyl derivatives of aflatoxin M_1 and other hydroxylated metabolites:

Acetyl derivatives were prepared according to the methods of Holzapfel, Steyn and Purchase (1966) and Dutton and Heathcote (1968). The chloroform eluates of the metabolites were evaporated to dryness in a rotary film evaporator at room temperature. To the dry metabolite was added 0.01 ml. pyridine and 0.1 ml. acetic anhydride. The mixture was left overnight in the dark and at room temperature. After evaporation of the pyridine and acetic anhydride, the reaction product was dissolved in chloroform, applied to thin layer plates and developed in 4% methanol in chloroform (v/v). Acetyl-derivatives did not move from the origin.

X. Isolation of metabolites from incubation medium:

Reactions during incubation were usually terminated by the method of Cochin and Axelrod (1959). To each flask containing 5ml. of reaction mixture, 1 ml. of

20% w/v Zinc sulphate solution was added, followed by 1 ml. saturated barium hydroxide solution. These reagents served both to stop the reaction and to precipitate the proteins. The mixture obtained was centrifuged at about 5,000g for 15 minutes in an MSE super speed refrigerated centrifuge. The supernatants were concentrated in a rotary film evaporator at 37°C. The concentrate was applied to silica gel G - coated plates and developed in either 4% methanol in chloroform or in 5% methanol in chloroform (v/v). Samples of aflatoxins B_1 and G_1 and Palmotoxins Bo and Go similarly treated were run alongside the concentrate. An authentic aflatoxin M_1 sample was run alongside. The same procedure was followed in the case of aflatoxin B_1. On illumination with Ultraviolet light, the test substances and their metabolites were located by their Rf. values and their fluorescence.

XI. Estimation of formaldehyde by the Colorimetric method:

This procedure due to Nash (1953) depends on the reaction between formaldehyde with a fairly neutral solution of acetyl acetone and ammonium acetate to

form diacetyldihydrotoluidine.

Formaldehyde was estimated in incubation media by the method of Cochin and Axelrod (1959) as modified by Stitzel, et al. (1966). A standard curve (Fig 11) was obtained by treating known concentrations of formaldehyde (analytical grade) in the same way as the 5,000g supernatant and this was used for the estimation of the formaldehyde produced during the incubation period.

2.5 ml portions of the 5,000g supernatant obtained after centrifuging the incubation media, were placed in test tubes. To each was added 1 ml. of freshly prepared double strength Nash reagent (Nash, 1953). The test tubes were incubated in a Gallenkamp water bath at 60°C for 30 minutes. The tubes were immediately cooled in an ice-water mixture and the optical density obtained using the Unicam SP. 600 Spectrophotometer at 415nm. Seven flasks were usually used for each test and three as blanks. Extra polation of optical densities on standard curves yielded the concentration of test solutions.(Fig)

XII. Identification of Conjugates formed during incubation:

(a) Mercapturic acid conjugate:

The method of Knight and Young (1958) was employed for testing the presence of mercapturic conjugations. 0.1M potassium dichromate mixed with glacial acetic acid (1:1 v/v) was sprayed on the air-dried chromatogram containing the conjugates and finally 0.1N silver nitrate solution was sprayed. The chromatogram was protected from light after spraying to avoid any decomposition of the silver nitrate.

(b) Amino Acid Conjugation:

The reagent used in this test was a modified form of the reagent used by Williams and Kirby (1948) to locate and identify amino acids and applied to the study of aflatoxin metabolites by Emafo (1970).

Thin layer plates containing conjugates of the samples were sprayed with ninhydrin reagent. The plates were kept at 80°C for 15 minutes in a hot air oven.

(c) Sulphate Conjugate Test:

The method of Burma (1953) modified by Schneider and Lambert (1956) and applied in the study of aflatoxin B_1 conjugates (Emafo, 1970) has been retained in these

studies.

Thin layer plates developed in n-butanol: glacial acetic acid: water (10:1:1 v/v), were exposed for 3 hours to the fumes of a mixture of 10 ml concentrated HCl in 90 ml Dioxan, in a closed tank. The chromatogram was air-dried, sprayed with a solution of 20 mg barium chloride in 100 ml of 75% aqueous methanol and further dried. The chromatogram was finally sprayed with potassium rhodizonate reagent.

(d) <u>Glucuronide Conjugation Test:</u>

The naphthoresorcinol spray method of Bridges, Kibby and Williams (1965) has been applied in this test. The quantitative reaction of Fishman and Green (1955), for the micro-analysis of glucuronide has also been adapted for a qualitative assay of glucuronides.

(i) In the spray method (Bridges, Kibby and Williams, 1965), plates developed with n-butanol-glacial acetic acid: water (10:1:1 v/v) were sprayed with the naphthoresorcinol reagent and then heated in a hot air oven at 140°C. Bluish-brown or blue spots on the chromatogram were regarded as positive indication of

the presence of glucuronide.

(11) The Fishman and Green method depends on the elimination of free glucuronic acid which might give colour with naphthoresorcinol, by oxidation with hypoiodite of pH 10.1. Strong acid condition, however, liberates conjugated glucuronide. On reacting with naphthoresorcinol a violet coloured pigment was obtained and thus used as a positive index for the presence of glucuronide.

5 ml of the isolated extract was added to a 50 ml. Erlenmeyer flask containing 2.05 ml carbonate buffer pH 10.1 and shaken with 1.5 ml of iodine solution and stored in the dark for 30 minutes. 0.15 ml of 0.1M sodium bisulphite was then added, shaken and 0.3 ml of 6N sulphuric acid also added. Residual colour was removed with drops of sodium bisulphite solution.

4 ml aliquots of the samples were taken in duplicates, into boiling tubes, 2ml. of 0.4% naphthoresorcinol added and a further 2 ml of 18N sulphuric acid added. A blank obtained from blanks of the incubation, was similarly treated. Tubes were

incubated at 100°C for 90 minutes, with the tubes unstoppered. On cooling, 10 ml of 95% ethanol was added to each tube and colour formed was extracted with 8 ml toluene. Violet colour in the toluene layer was regarded as positive. In cases where the blank was not colourless, comparison of the colours of test at 565nm with that of the blank was used as index of positivity.

(a) Hydrolysis of Conjugates:

1. Acid hydrolysis:

The methods of Garton, Robinson and Williams (1949) and DeNoio and Tocz (1952) were employed. Portions of the concentrated conjugates were added to 0.3N hydrochloric acid or 0.3N sulphuric acid and boiled for 15 minutes. The samples were then examined for hydrolysis by first applying the concentrated hydrolysate to thin layer plates and developing the plates in 4% acetone in chloroform (v/v).

11. Enzymatic hydrolysis of Conjugates:

Enzyme hydrolysis has been employed in the identification of suspected glucuronides. Metabolic

conjugatee isolatcd by thin layer chromatography were concentrated with a rotary film evaporator. The ooncentratee were adjustod to pH 3.3 with 0.1M phosphate buffer. To this wno ndded β-gluouronidase (Ketodase, E. Merck, Dermstadt, Germany) enough to produce 10,000 units per ml. A control to test the activity of the enzyme was run alongside using phenolphthnlcin glucuronide, (supplied from the Biochemistry Department, St. Mary's Hospital, London) ns substrate. Mixtures were incubated at 38.0 ± 0.5°C for 48 hours. The test mixtures were examined on thin layer plates, while the blank tube was testcd for enzyme activity by making the solution alkaline with dilute sodium hydroxide, when a pink colour developed if the enzyme was active.

<u>XIII. Carbon monoxide neration of incubation medium:</u>

The method of carbon monoxide acration had been adapted from Christenson and Wissing (1972). Carbon monoxide was generated from sodium formate by the action of ooncentrated sulphuric acid. Kipps apparatus was used ns a continuous gas gencrating system. The carbon monoxide generatcd was deoxygenated by passing through saturated alkaline solution of sodium dithionite. The

ensuing gas was used to aerate the relevant incubation medium.

XIV. Determination of Iodine number of the toxins:

The method of Bassir (1963) was used. The iodine value of a chloroform blank was first determined and then that of the chloroform solution of the test substances using sodium thiosulphate. The difference between the two titres multiplied by the iodine equivalent of 1 ml of the N/40 sodium thiosulphate used in the titration gave the required iodine number.

To 5 ml chloroform in a conical flask was added 5 ml "Dam's iodine". The flask was corked and left in the dark for 5 minutes. To this then was added 5 ml of 10% potassium iodide and 20 ml water. The mixture was titrated with N/40 sodium thiosulphate using 1% starch as indicator. Using the same procedures, titre readings were taken for the 5 ml of each sample.

CHAPTER IV

INVESTIGATION I

Investigation of the variation in total toxin, pH and mycelial weight when Aspergilluo flavuo wao grown on palm oap medium and yeaot extract medium.

The production of aflatoxin in the yeaot extract-sucroae medium by Aspergillus flavus hao been ahown to vary with the period of incubation (Davis and Diener, 1966, Diener and Davis, 1966 and Emafo, 1970). In this experiment, the period for the maximum production of toxins in the palm oap medium and the aaaociated changes in pH and mycelial weight were dotermined. Thio wao compared with aimilar ohangea in yeaot extract-aucroae medium under identical conditions.

Experimental Procedure:

Freah palm oap obtained aa deacribed previously (page 69), waa diatributed in 50 ml aliquota, into 250 ml. Erlenmyer flaaka. The yeaot extract-aucroae medium waa aimilarly diatributed. The flaaka were plugged with non-abaorbent cotton wool and autoclaved

at 15 lb/sq.in. for 30 minutes and allowed to cool to room temperature. Each flask was inoculated with Aspergillus flavus spores using a wet platinum loop to transfer the spores and shaking each flask gently to obtain a uniform dispersion of the spores in the medium. Flasks were then incubated at $30^{0} \pm 2^{0}C$ as stationary cultures.

Duplicate samples of each set were harvested each day by filtering through a Whatman No. 1 filter paper. The mycelial matt was washed with distilled water and dried overnight to a constant weight at about $100^{0}C$ using a hot-air oven. The samplee were then weighed. The pH of the filtrate was determined with a pH meter (Radiometer, Copenhagen).

To determine the concentration of the total chloroform extractable toxins, 10 ml of filtrate from each flask was extracted twice with 20 ml portions of chloroform in a separating funnel. Extracts were pooled together, dried with anhydrous sodium sulphate and taken to dryness in rotary film evaporator. The residue was taken up in 2 ml of chloroform and the optical densities at 365 nm and 420nm taken. Concentration

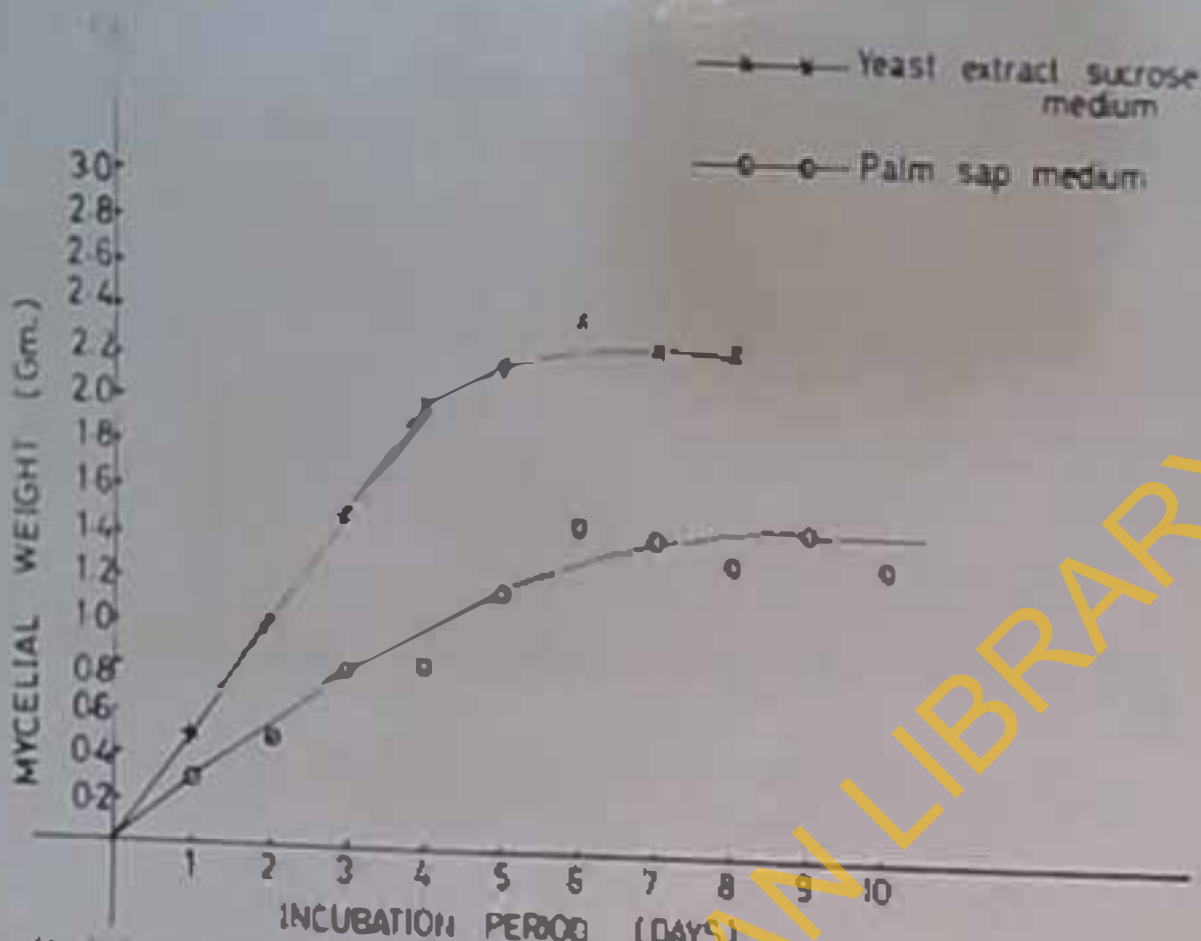
of total toxin was obtained by the method of Nabney and Nesbitt (1965), in terms of aflatoxin B_1.

Results:

A plot of the weight of mycelia against the period of incubation is shown in figure 12. All through the period of incubation, the weight of mycelia from the yeast extract-sucrose medium was found to be greater than that of the palm sap medium. This probably indicates a higher growth rate in the yeast extract-sucrose medium over the palm sap medium. The growth in both cases increased with days of inoubation, reaching a maximum at about the sixth day.

Figures 13 and 14, show the plot of toxin concentration in 10 ml of medium in the yeast extract-sucrose medium and the palm sap medium respectively and the corresponding changes in pH. Total toxin produced in the yeast extract sucrose medium was greater than that of the palm sap medium. A maximum of 0.3 mg/10 ml of solution was obtained in the yeast extract-sucrose medium as compared to an average of 0.26 mg/10 ml in the palm sap medium. This yield was obtained on the sixth day in the yeast extract sucrose medium and on the 5th day in the palm sap

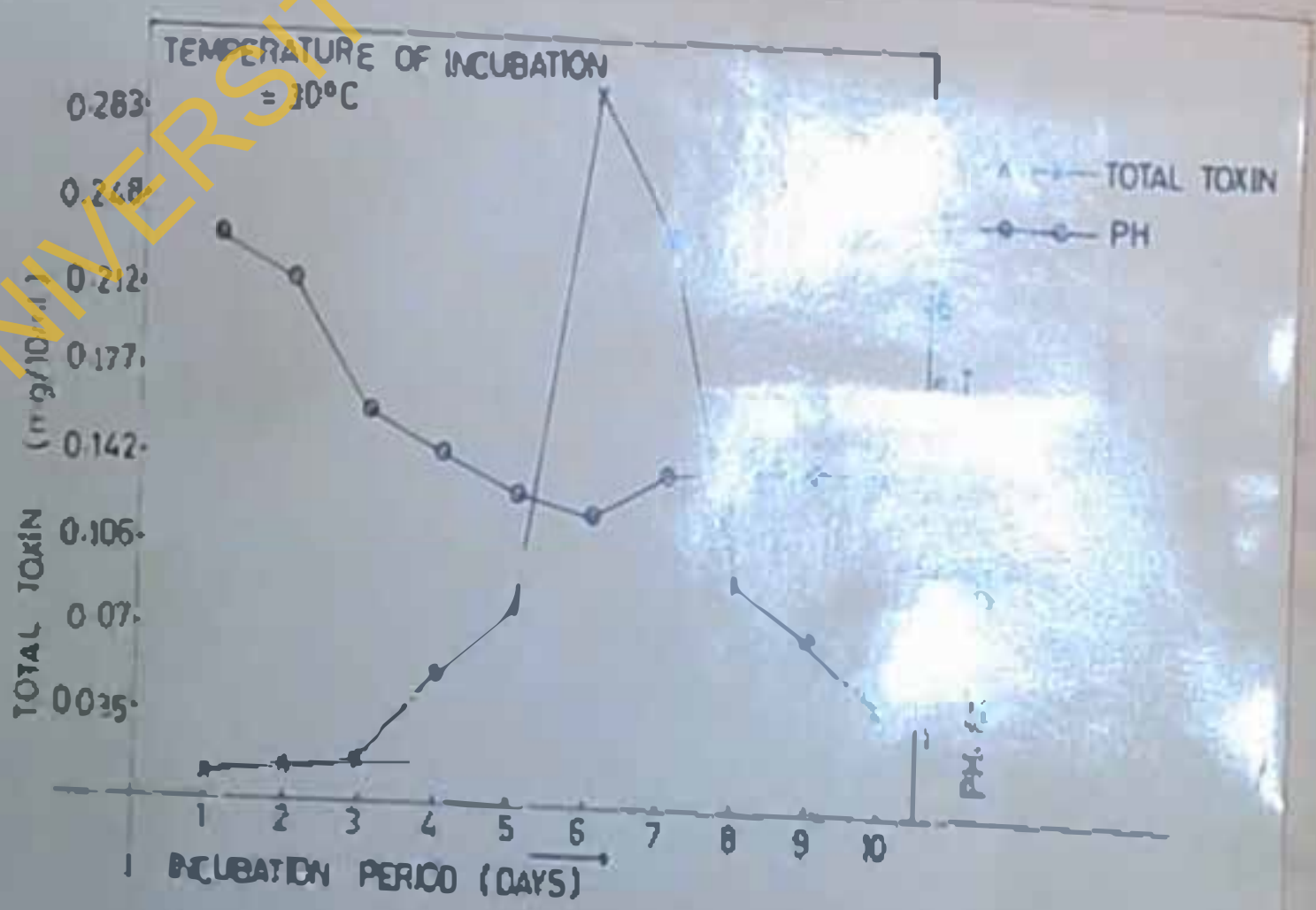
104 B



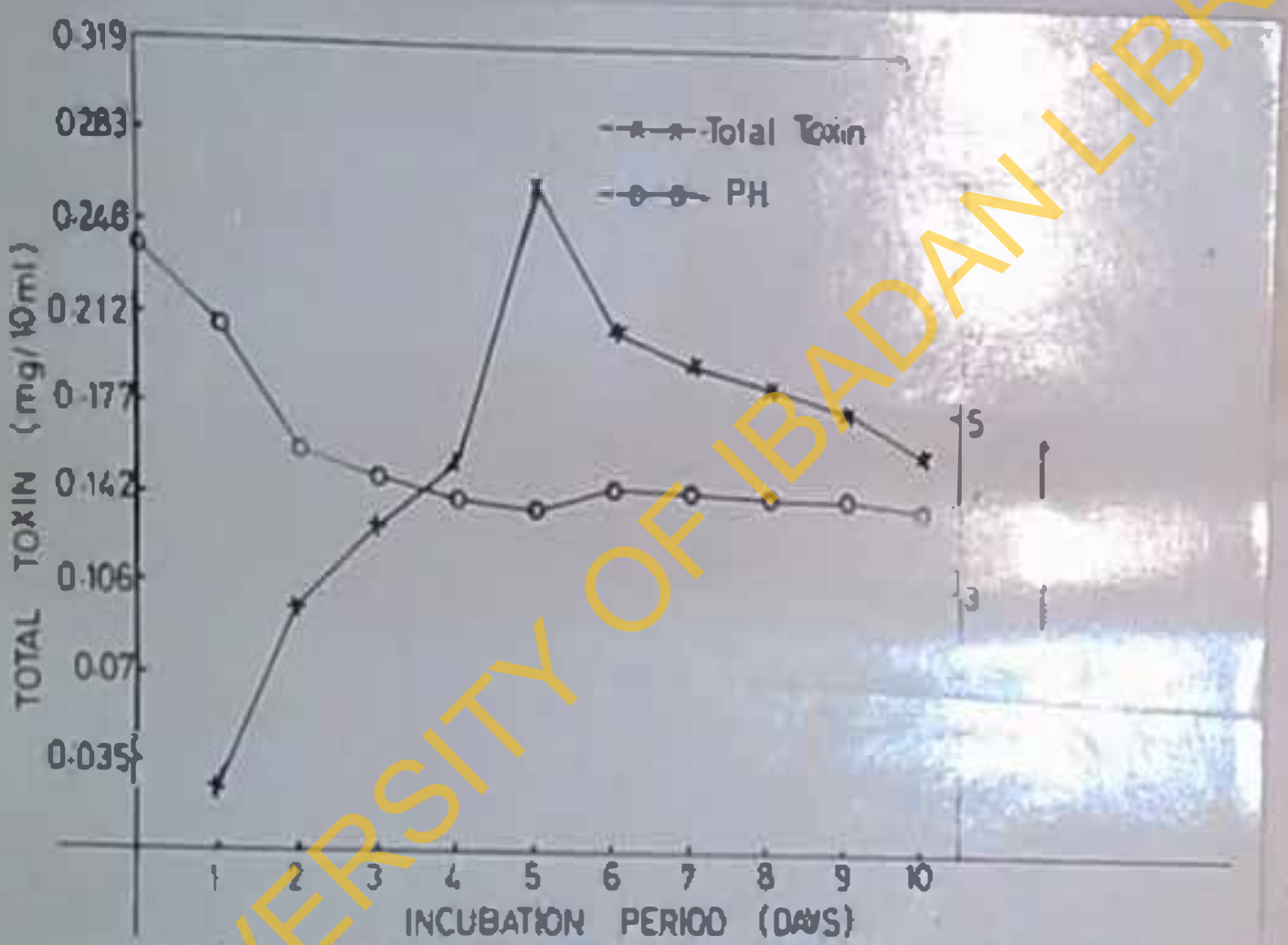
Variation in the dry weight of Mycelial MATT of Aspergillus flavus (Link) with period of incubation (Days) in the yeast-extract-sucrose medium and the palm sap medium

average temperature of incubation = 30°C

Fig 12



Variations in PH and total toxin produced by Aspergillus flavus U I 81 with period of incubation on yeast extract sucrose



Variations in PH and total toxin produced by Aspergillus flavus (U.I.81 with period of incubation on palm sap medium

Average Temperature of incubation = 30°C

Fig. 14

medium, using the same strain of Aspergillus flavus. The rise in the production of toxins was more gradual in the yeast extract-sucrose medium and fell more steeply after peak production than in the palm sap medium, where production rate was faster but fell less rapidly after peak production.

The pH of the medium fell as the toxin concentration increased and reached the lowest value at the point of highest toxin production. In both cases, the final pH was around 4.

Conclusion:

1. Toxin production, pH of the medium and growth of Aspergillus flavus fungus, varied remarkably with the period of incubation in both the palm sap medium and the yeast extract sucrose medium.
2. Yeast extract-sucrose medium was superior to the Palm sap medium in the support of Aspergillus flavus growth and in the production of toxins.
3. In the yeast extract sucrose medium, maximum production of toxin by Aspergillus flavus was on the sixth day while, under the same conditions, maximum production was on the fifth day in the palm sap medium.

INVESTIGATION 2

Further investigation of some physical characteristics of Palmotoxins Bo and Go.

The structuree of the palmotoxins Bo and Go are yet unknown. It has been postulated that they may be hydroxylated long chain fatty acids, of the type described earlier on in this thesis. This postulate has not been found consistent with some of the observed characteristics of the oompounds. It has been found nccessary therefore to re-examine some of the physical characteristics of these compounds. In doing this, the main line of approoch was to compare these characteristics with those of other known metabolites of A. flavus, with a view to discerning any similar features.

Experimental Procedure:

1. Isolation:

The procedure previously used and described under 'methods' was retained except that a test which involved partitioning of the substances between water and diethyl ether has also been introduced.

The scheme given, summarises the proocdurs so far

employed, starting from the crude chloroform concentrate, which has been subjected to a primary purification procedure.

CRUDE EXTRACT

I — thin layer chromatography in chloroform: methanol 97:3 v/v; scraped, eluted and concentrated

First isolate of Palmotoxins Bo and Co

II — Rechromatographed in chloroform: methanol: formic acid (95:5:1v/v).

SECOND ISOLATE

Redissolved in chloroform and aqueous solution prepared from this under Nitrogen.

Aqueous solution of palmotoxins Bo and Co

Partitioned with diethyl ether

Ethereal phase (Non fluorescent)

Fluorescent aqueous phase

Re-extracted with chloroform and run on thin layer plates

Eluted, concentrated and reprecipitated from chloroform with n-hexane or petroleum ether

Ultraviolet Spectral Analysis:

The substances were dissolved in either water or in methanol and their Ultraviolet spectra taken in water, and in methanol respectively, using the Perkin-Elmer, Ultraviolet - visible Spectrophotometer 137UV. The Ultraviolet Spectra of aflatoxins B_1 and G_1 were also run.

Results:

The Ultraviolet Spectra of aflatoxins B_1, G_1 and those of aqueous palmotoxins Bo and Go have been given earlier (Figures 1, 2, 3, 6 and 7). The Ultraviolet Spectra of palmotoxins Bo and Go in methanol are given in figures 15 and 16.

Infra-red spectral analysis:

The infra-red spectrum of each of the substances was obtained neat on sodium chloride discs. A concentrated solution of each of the substances was placed on the disc and the solvent was allowed to evaporate. A Perkin-Elmer Infra-red Spectrophotometer 137 was used to obtain the spectra. The infra-red spectra of aflatoxins B_1 and G_1 were obtained in nujol using the same equipment.

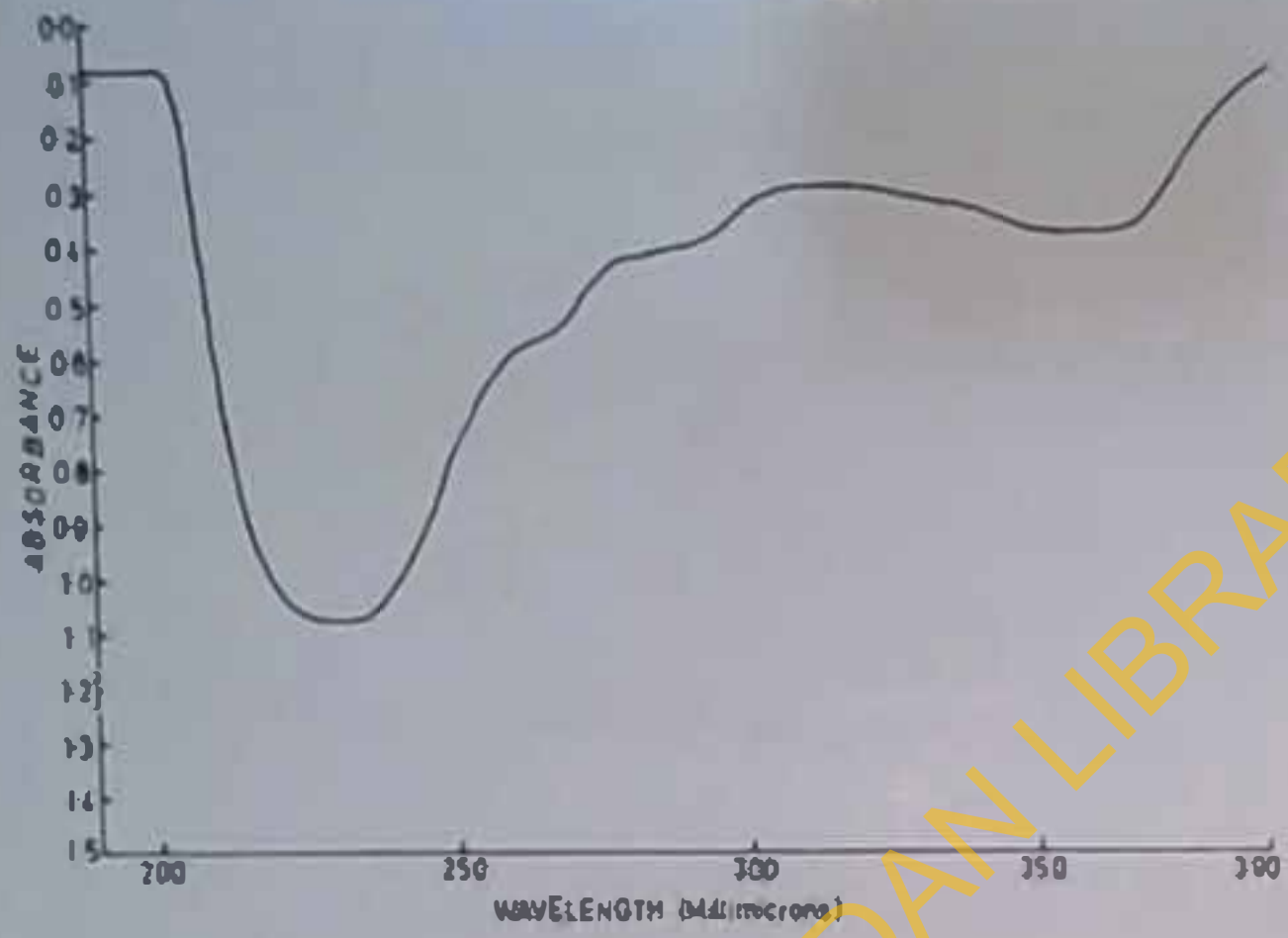


Fig PALMOTOXIN B0 (SOLVENT METHANOL (A R)

Fig 15

Fig PALMOTOXIN B0 (SOLVENT METHANOL)

Results:

The spectra for the aflatoxins B_1 and G_1 have been given already (Figures 4 and 5). The spectra for palmotoxins Bo and Go are shown in figures 17 and 18.

Fluorescence Spectral Analysis:

The fluorescence spectrum of each of the toxins - aflatoxin B_1 palmotoxin Bo and palmotoxin Go, was obtained in dilute chloroform solutions using a Perkin-Elmer fluorimeter, model 203. Readings were taken between 220 nm and 400 nm.

Results:

The fluorescence spectra of aflatoxin B_1 and the palmotoxins Bo and Go are given in figures 19, 20, and 21. All the toxins show an excitation maximum at 365nm; and an emission maximum of 410nm for aflatoxin B_1 and palmotoxin Bo but 425nm for palmotoxin Go.

Nuclear Magnetic Resonance Spectroscopy:

The nuclear magnetic resonance of the substances were obtained in deuterated chloroform with tetramethyl silane as an internal standard. Spectra were obtained using the Varian Associates model of nuclear magnetic

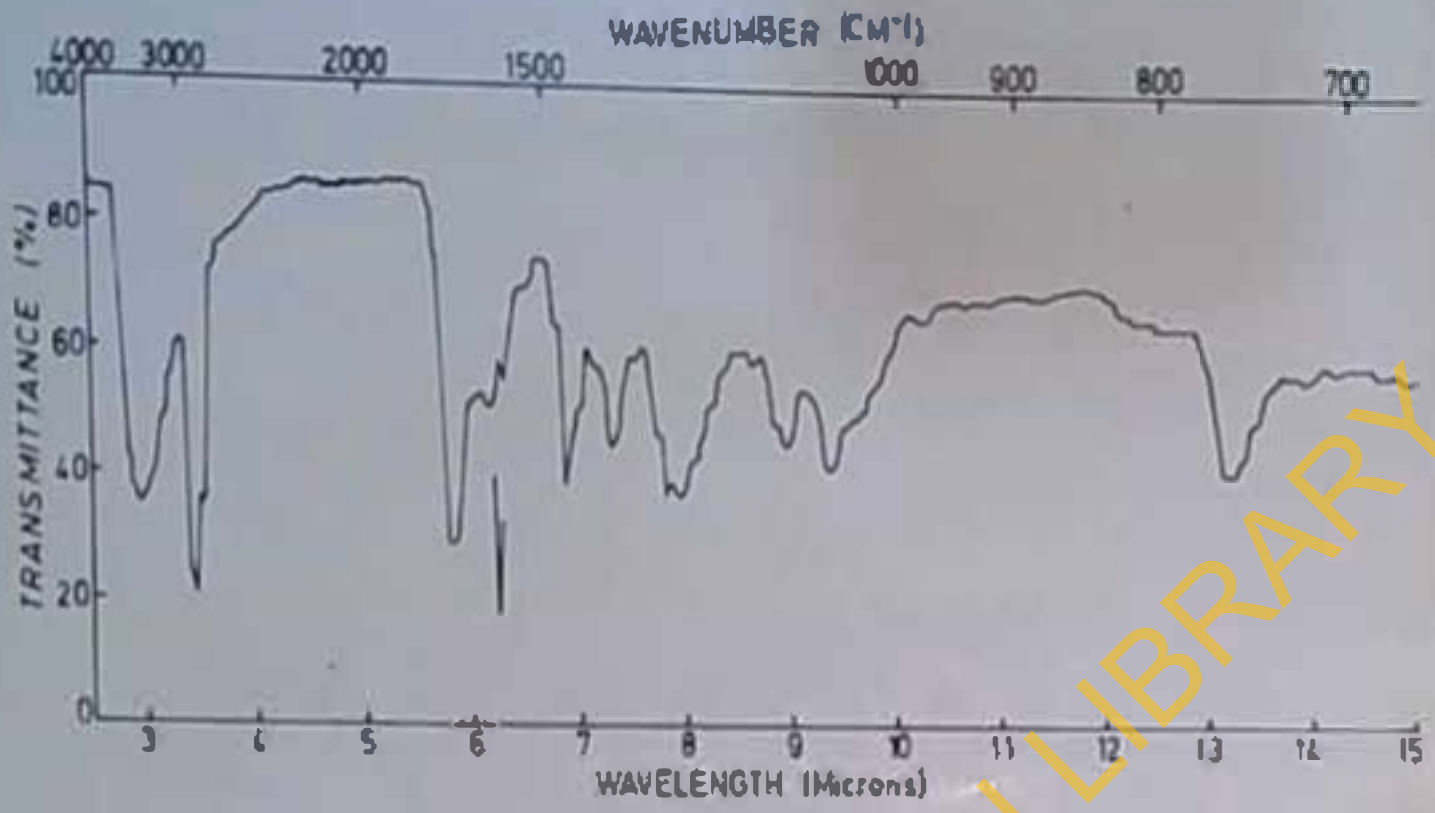


Fig. PALMOTOXIN Bo

Fig. 17. INFRARED SPECTRUM

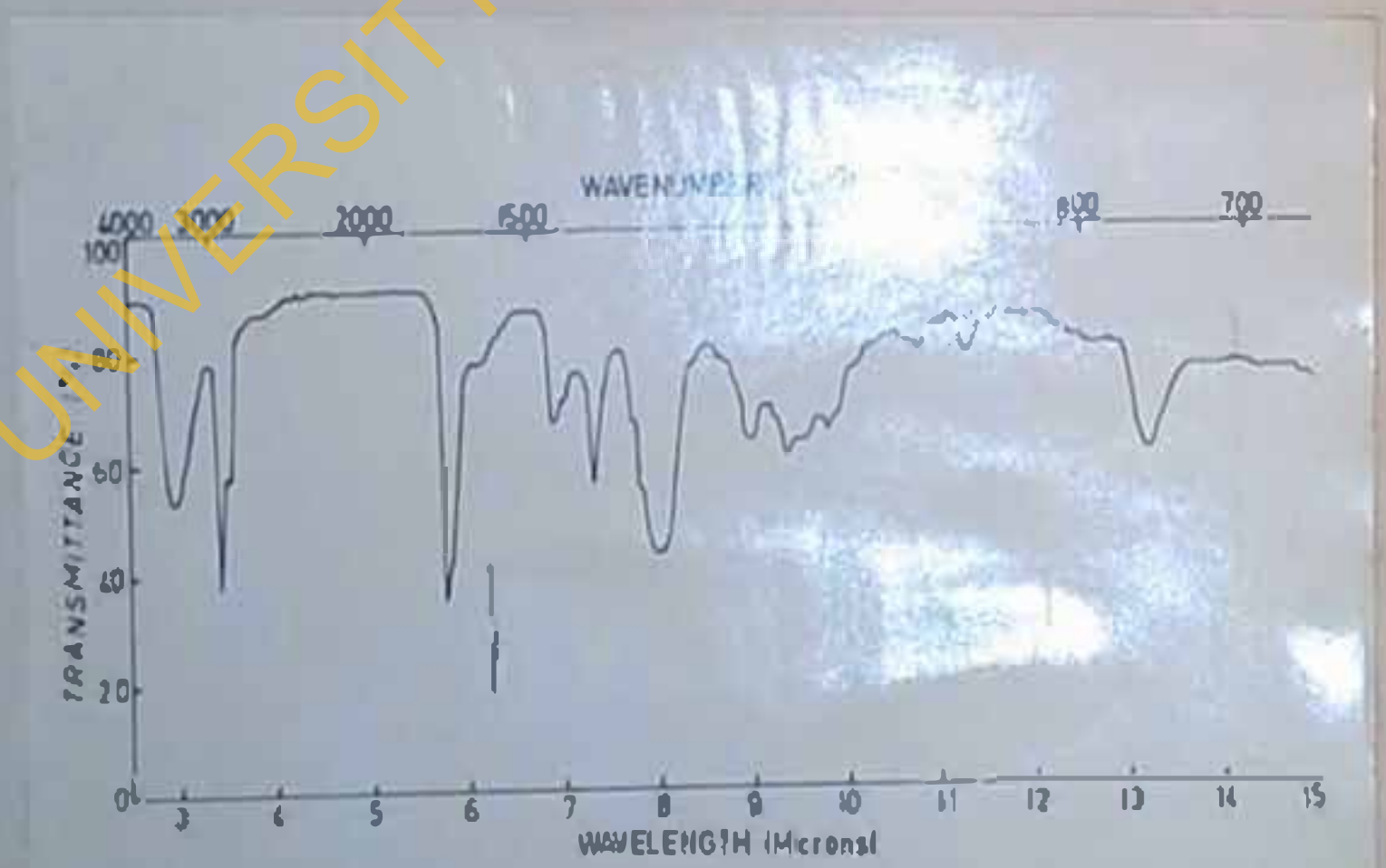
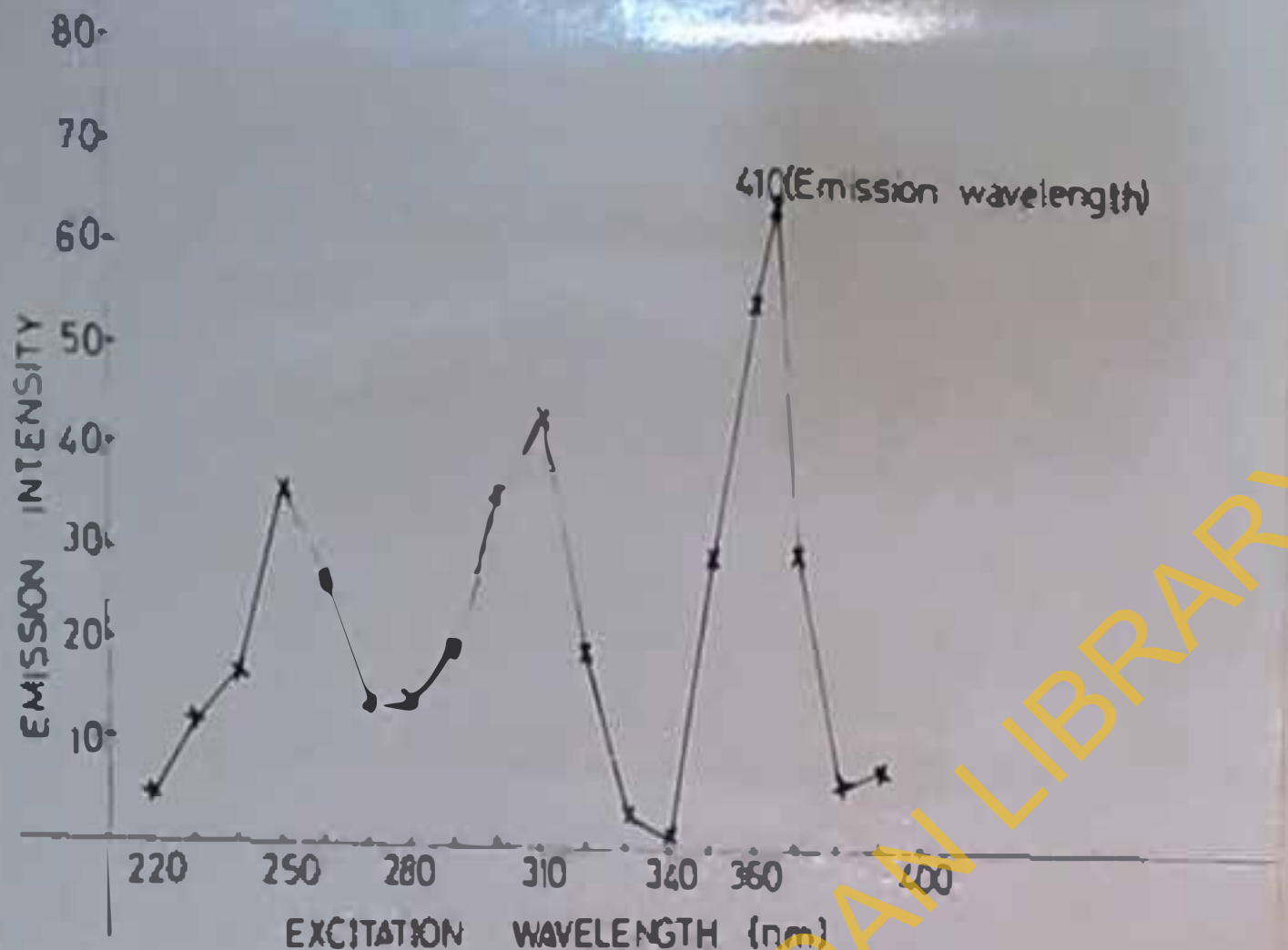


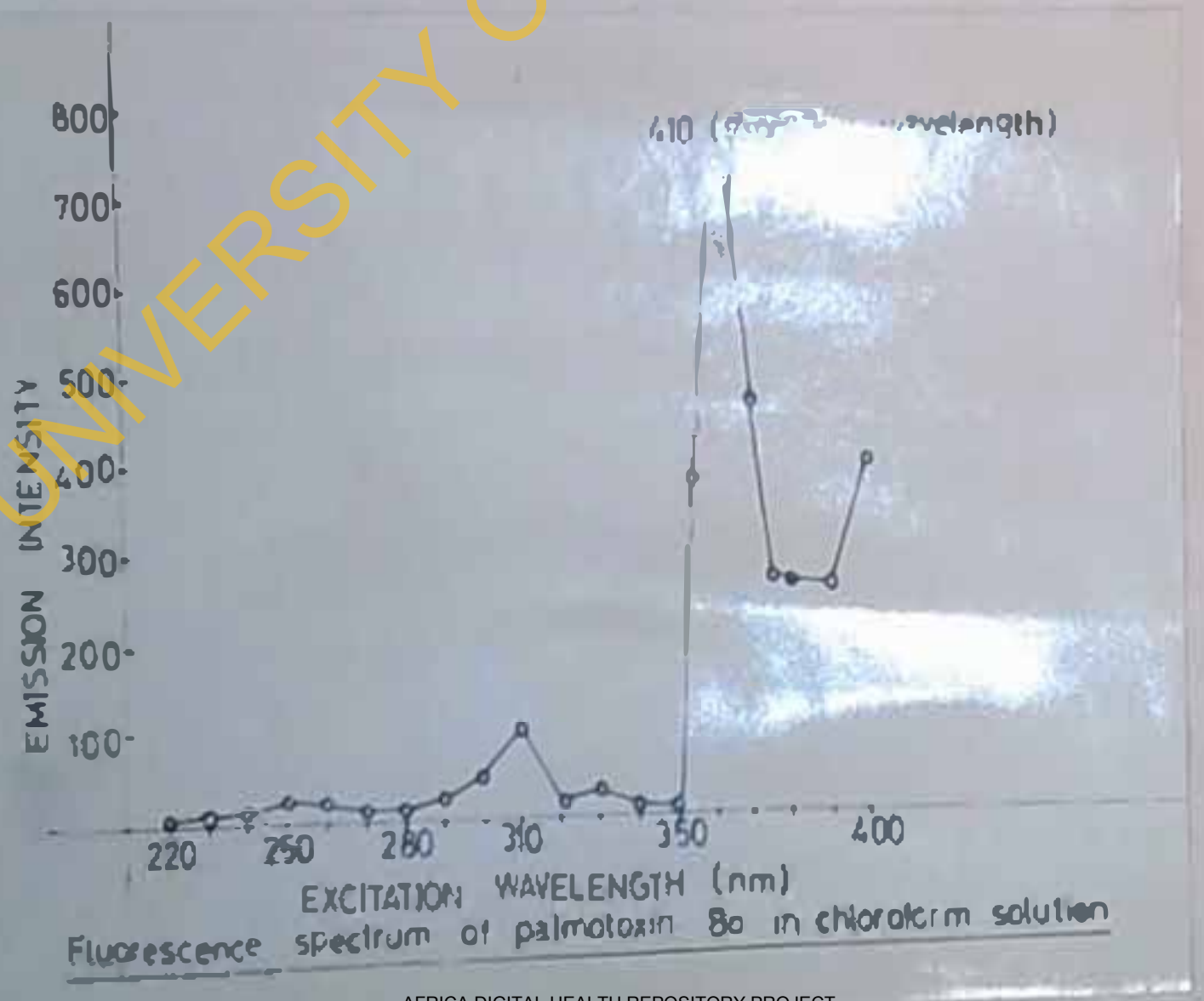
Fig. PALMOTOXIN Go

Fig. 18 INFRARED SPECTRUM



Fluorescence spectrum of Aflatoxin B1 in chloroform solution

Fig. 19



Fluorescence spectrum of palmotoxin Bo in chloroform solution

Fig. 20

Fluorescence spectrum of palmolorin Gb in chloroform solution

Fig. 21

resonance equipment. This was also compared with that of aflatoxin B_1.

Result:

The spectra are given in figures 22, 23, and 24.

A summary of the physical data obtained in this study has been given in Table 6. Data obtained from literature and some which were obtained during this work, on known aflatoxins have been included for comparison.

Determination of Iodine Value of Palmotoxins Bo and Go:

The iodine values of palmotoxins Bo and Go were determined in chloroform by the method of Bassir (1963). Details of the method have been given in Chapter III. The values for aflatoxins B_1, B_2, G_1 and G_2 are also given for comparison (Table 7).

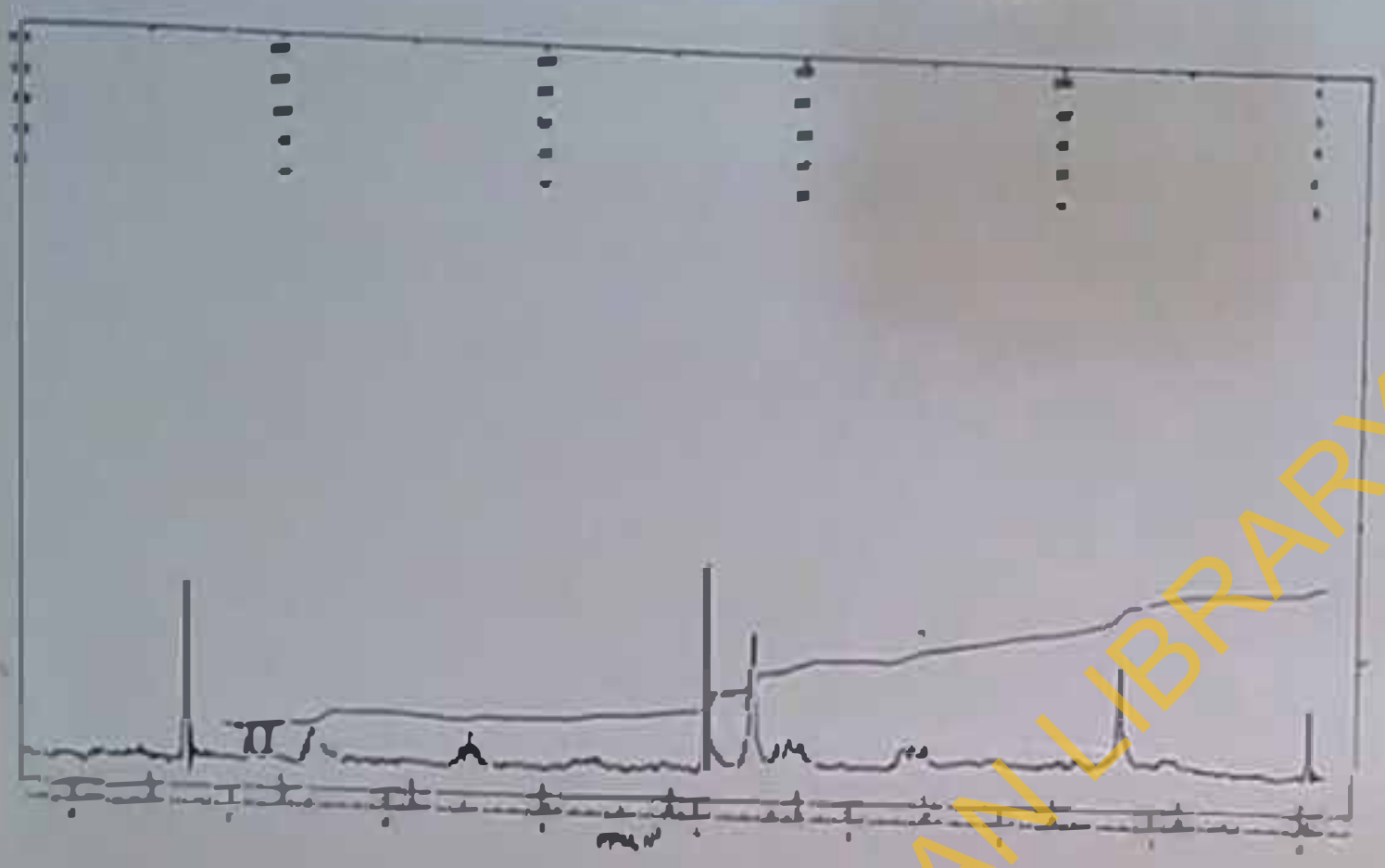


Fig. 22 N.M.R. AFLATOXIN B_1

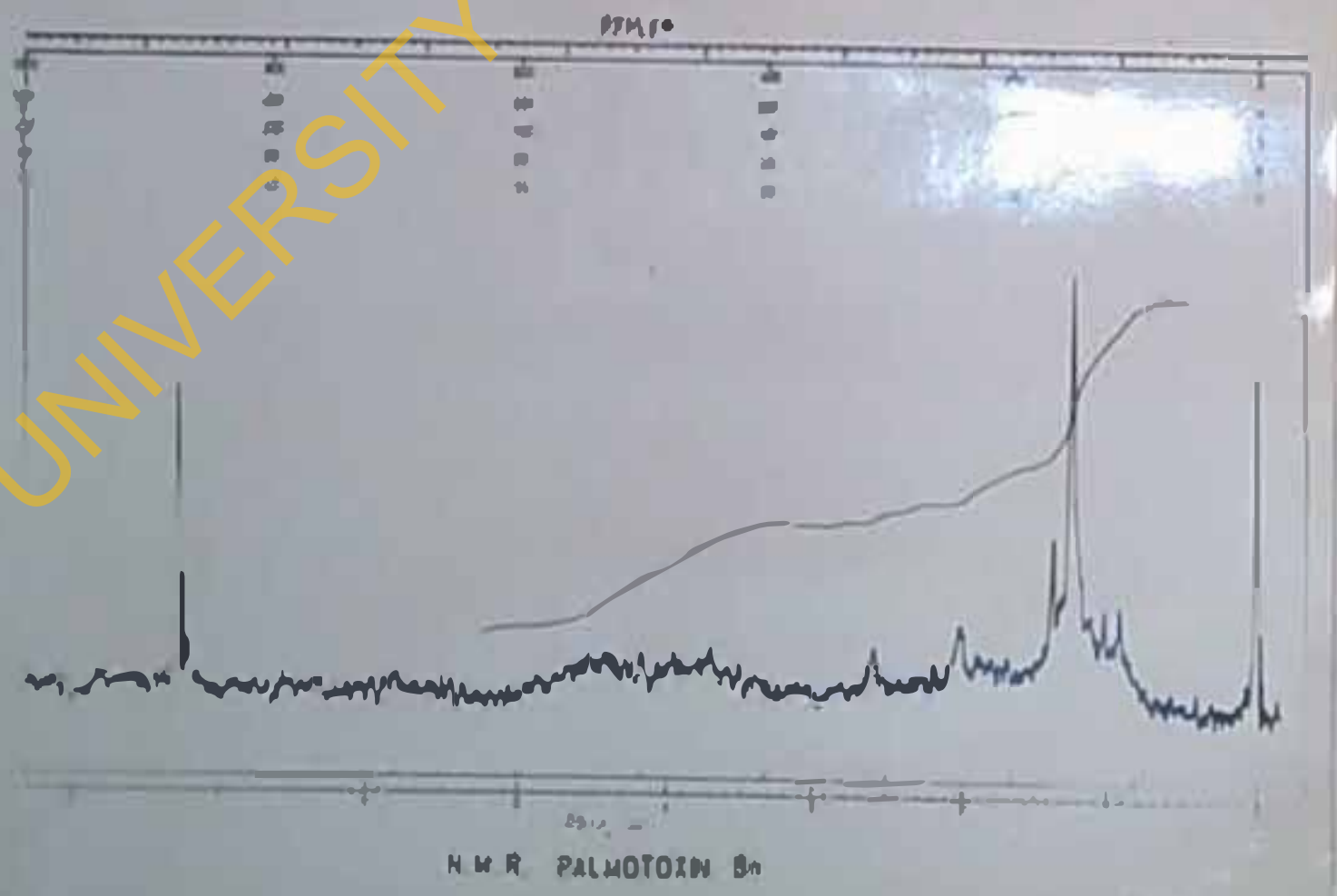


Fig. 23

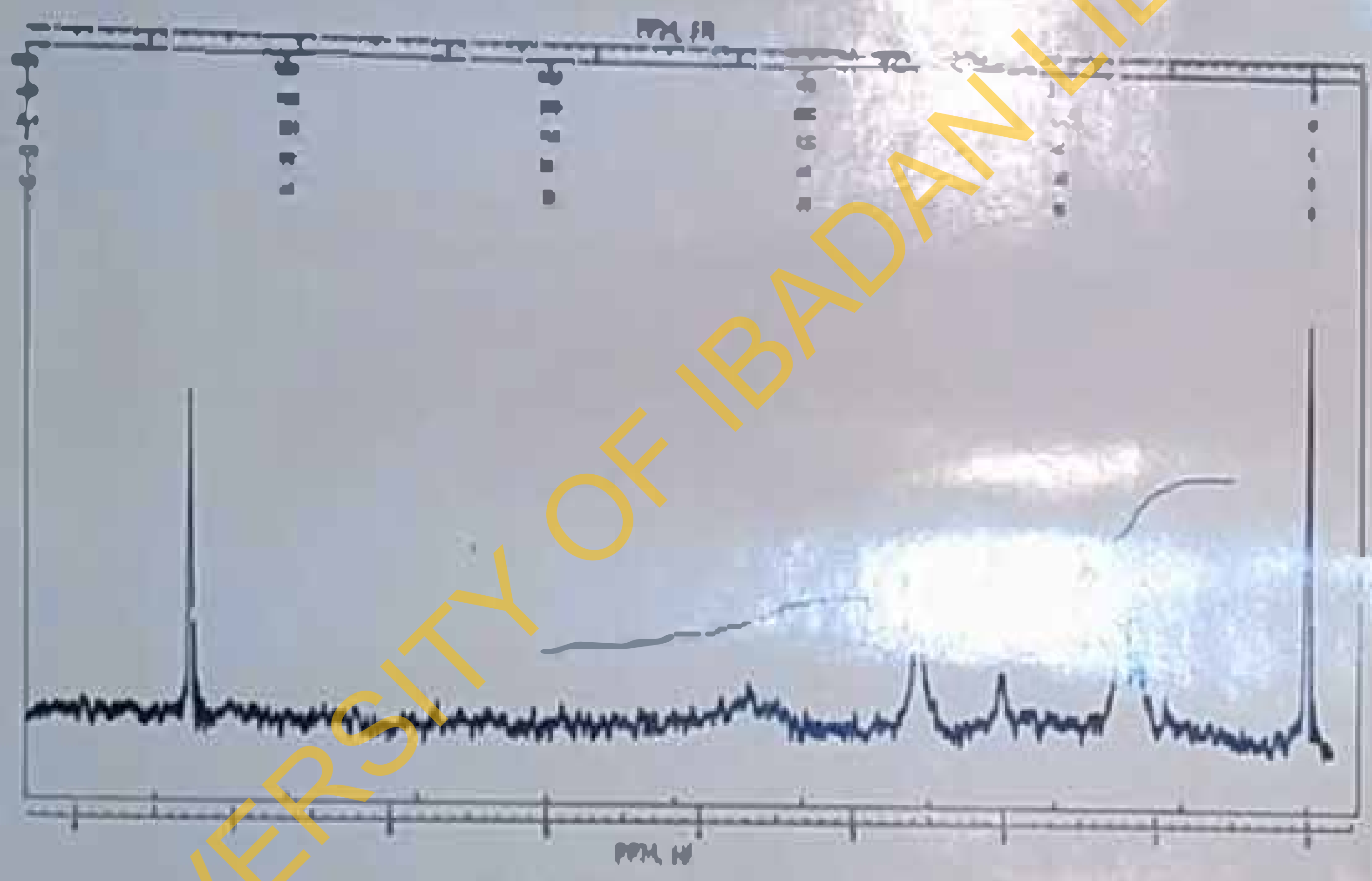


Fig. 24

Table 6

Substance	U.V. Characteristics (nm)	Infrared characteristics cm^{-1}	Fluorescence Excitation	Fluorescence Emission
Palmotoxin Bo	202^{W} (20, 100) 265^{W} (11,400) 232^{m} 265^{m} 292^{m} 365^{m}	3400 1740 1630 1595 1545	365nm	410nm
Palmotoxin Go	204^{W} (12,380) 235^{m} 265^{W} (10,345) 285^{m} 365	3450 1760 1630	365 "	425 "
Aflatoxin B_1	223^{m} 265^{m} 362^{m}	1750 1680 1630 1590 1545	365 "	410 "
G_1	203^{W} 219^{m} 253^{W} 239^{m} 262^{m} 365^{W} 365	1760 1695 1630 1595 1545	365 "	430 "
M_1	226^{E} 265^{E} 357^{E}	3425 1760 1690	-	-
B_5	210 265	3400 1750 1630 1598 1545	365 "	410 "

W = water

m = methanol

E = Ethanol

TABLE 7

Iodine Values

Aflatoxin B_1	Af. B_2	Af. G_1	Af. G_2	Palmotoxin B_0	Palmotoxin G_0
8.5 ± 0.5	7.13 ±0.32	8.9 ±0.4	6.98 ±0.30	12.5 ± 0.6	11.5 ± 0.7

± = standard error

INVESTIGATION 3

Determination of the toxicity of the palmotoxins Bo and Go on 20 day old rats.

The various isolates of Aspergillus flavus have been shown to differ remarkably in their toxicity to different species of animals (Carnaghan, Hartley and O'Kelly, 1963). However, aflatoxin B_1 has been recognized as the most potent of the hitherto, isolated metabolites of Aspergillus flavus.

The toxicity of the palmotoxins Bo and Go has been assessed mainly on chick embryos and they have been shown to induce death, liver lesions and other morphological changes similar to the aflatoxins (Bassir and Adekunle, 1968 and 1969; Bassir and Adekunle, 1970b).

No work has so far been done on the possible toxicity of these substances to mammals. The present experiment has been designed to show the possible effects of sub-acute doses of the palmotoxins Bo and Go to the rat, which has been chosen as representative of this group of animals.

Experimental Procedure:

The methods applied for the assessement of toxicity are similar to those of . Chang, et al. (1963) and Rao and Ghering (1971). These were; loss in body weight, reduction in liver size in relation to the body weight and changes in some serum enzyme activities.

20 day old male rats (wistar strain) were selected from litter mates and arranged in five groups of five animals each. The animals were housed in cages and supplied with food and water ad-libitum.

The animals were starved overnight prior to the administration of the toxins. The rats were weighed and three groups were injected with three different concentrations of palmotoxin Bo or palmotoxin Go; the fourth group was injected with aflatoxin B_1 and the last group served as control, receiving only the carrier solvent.

Each animal (31 - 31.5g-wt.) received concentrations of palmotoxins Bo and Go corresponding to 1.6 mg/kg (50 ug) for the first group; 3.23 mg/kg (100 ug) for

the second group and 6.4 mg/kg (200 ug) for the third group. The fourth was administered with 0.5 mg/kg (15 ug) of aflatoxin B_1 while the fifth group was administered with proylene glycol - the carrier solvent for all the toxins. All injections were given intraperitoneally and at 9.00 a.m. each morning for 15 days. The weights of the animals were taken on alternate days in most cases.

The animals were sacrificed by decapitation, one hour after the last injection. The blood from each group was pooled in centrifuge tubes cooled to about $4^{o}C$ in ice-water mixture. These were centrifuged and the serum immediately separated from the blood cells to avoid any contamination through haemolysis. The serum alkaline phosphatase activity and glutamic oxalo acetic acid (SGOT) transaminase activity were determined as described in page 89/90, for each group of animals. The animals were also dissected and their livers excised immediately, weighed and preserved in formol-saline. Sections of the liver were prepared using the Ultramicrotome (Leitz Wetzelar) and the sections stained with haematoxylin

and Eosin. The sections were examined for histological alterations using a Leitz Wetzlar large research microscope (Ortholux) equipped with a camera.

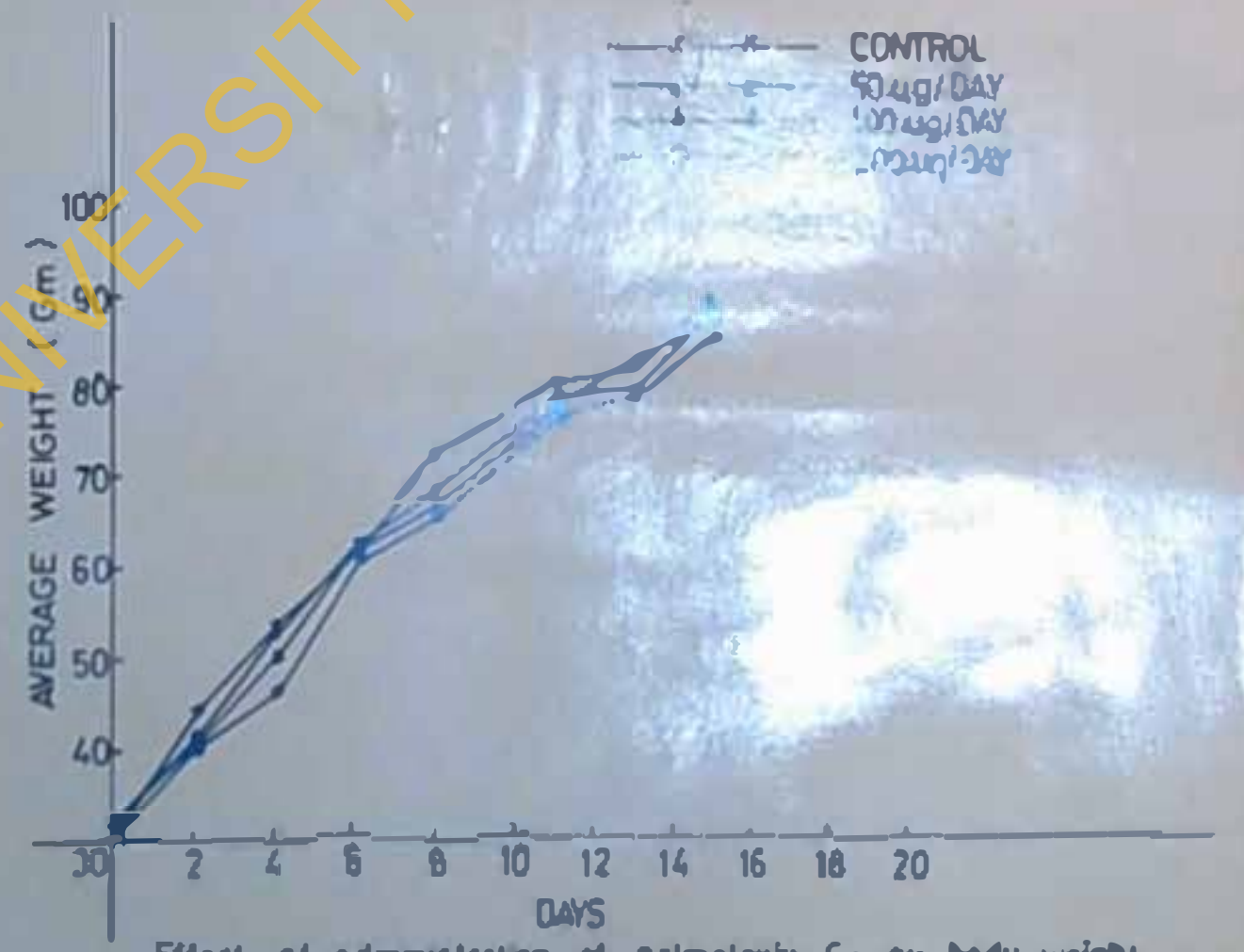
Results:

Rats treated with palmotoxins Bo showed a consistent loss of weight compared with the control. A plot of the body weight against the period of treatment (Fig 25) showed that the loss in weight, was dose dependent for the doses used. Palmotoxin Go treated animals did not exhibit any observable loss in total body weight when compared with the control, (Fig 26). This was also irrespective of the doses used; though lower mean weights were consistently recorded for the group of animals administered with 200 µg of palmotoxin Go.

The palmotoxin Bo and aflatoxin B_1 treated rats, showed a reduction in their respective liver sizes in relation to the total body weight (Table 8). The reduction also varied with the doses of toxin injected. The palmotoxin Go treated animals did not show any remarkable loss in total liver weight with respect to the total body weight except that the lowest liver

Effect of administration of palmotoxin 80 on the body weight of 20 day old rats (wistar strain)

Fig. 25



Effect of administration of palmotoxin Go on body weight of 20 day old rats

TABLE 8

Relationship between liver weights and body weights of the Palmotoxin Bo treated rats

Dose/Rat/day	(Average) Initial weight of rats (g)	(Average) Final Weight (g)	Liver Weight (g)	Mean Liver wt/ body wt.
Control	31.5	98.0	5.0	5.1
50 µg	31.8	85.0	4.1	4.8
100 µg	31.5	81.0	3.16	3.9
200 µg	31.5	75.0	2.6	3.4
15 µg Aflatoxin B_1	31.0	84.0	3.83	4.5

weight/total body weight ratio, was obtained in the group treated with 200 ug. of palmotoxin Go (Table 9).

No deaths were recorded during the period of study. Examination of the livor sections, of the palmotoxins Bo and Go treated rats did not reveal any marked pathological changes when compared with the control. There were changes, however, in the aflatoxin B_1 treated rats as shown in the degeneration of some of the cells. The cell outlines in some cases were no longer distinct (Plate 3a, b, c, and d).

Table 10, shows the serum alkaline phosphatase activity and serum glutamic oxalo acetic acid activity for both palmotoxins Bo and palmotoxin Go treated rats. The values for the aflatoxin B_1 treated animals have been given also.

In the palmotoxin Bo treated rats, there was a remarkable increase in the values of the glutamic-oxalacetic acid transaminase activity when compared with the control. Though there was an increase in the values of the alkaline phosphatase activity, this was not as pronounced as in the case of the transaminase.

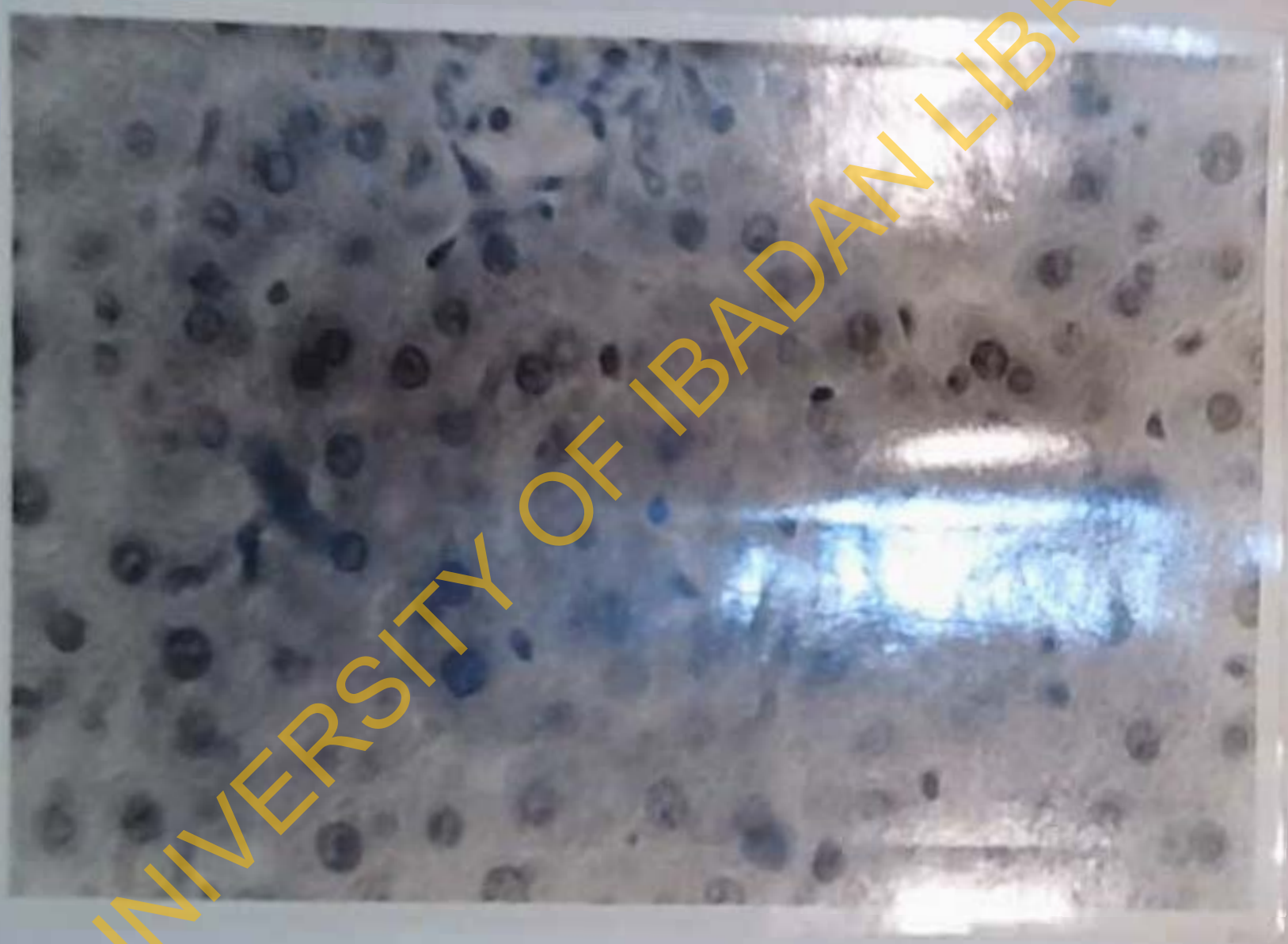


Plate 3. PHOTOMICROGRAPH OF LIVER SECTIONS OF CONTROL RATS. [HAEMATOXYLIN & STAINED × 40]

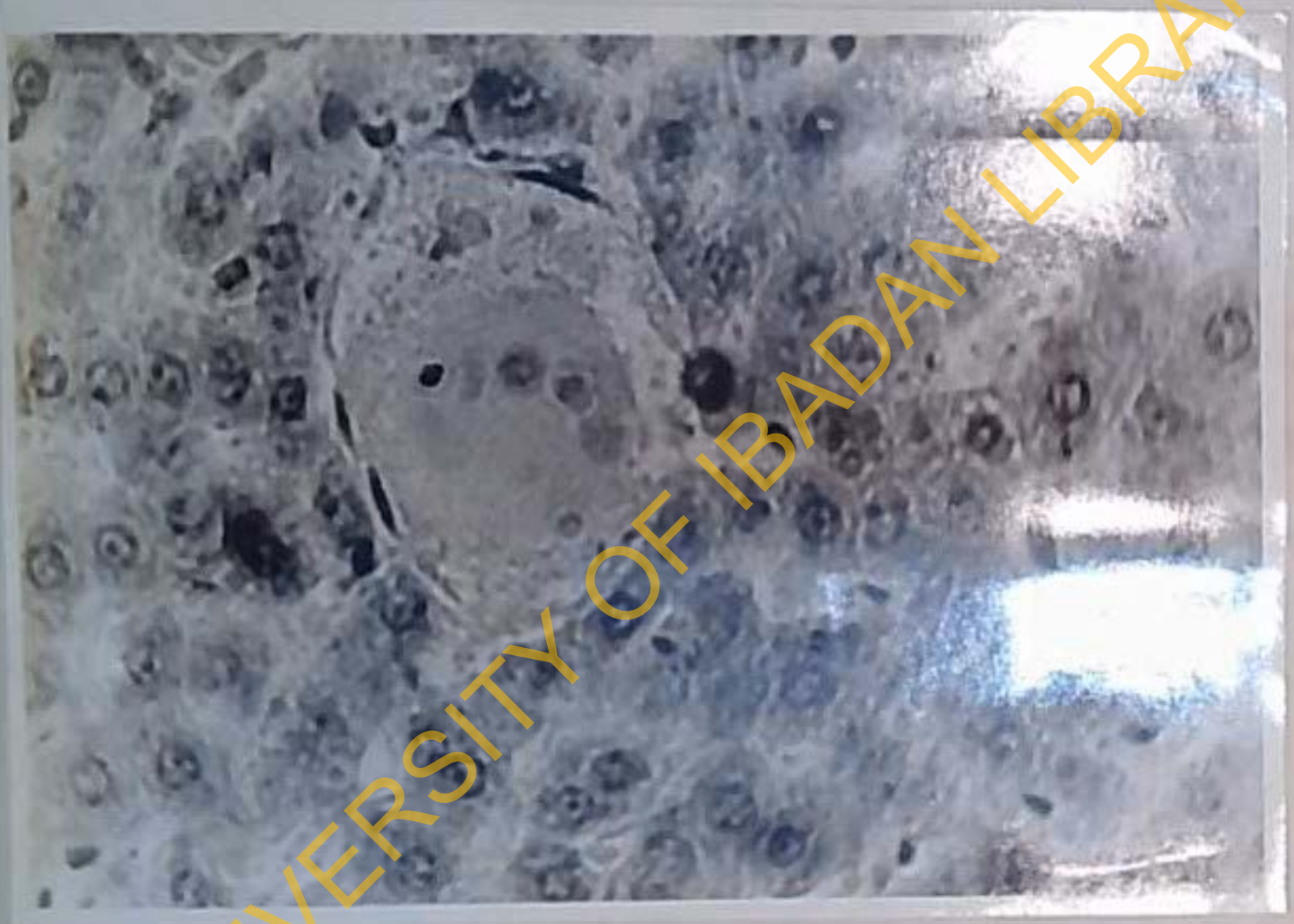


Plate 36. PHOTOMICROGRAPH OF LIVER SECTIONS OF PALMOTOXIN B_0 TREATED RATS. [HAEMATOXYLIN & EOSIN STAINED) x 40]

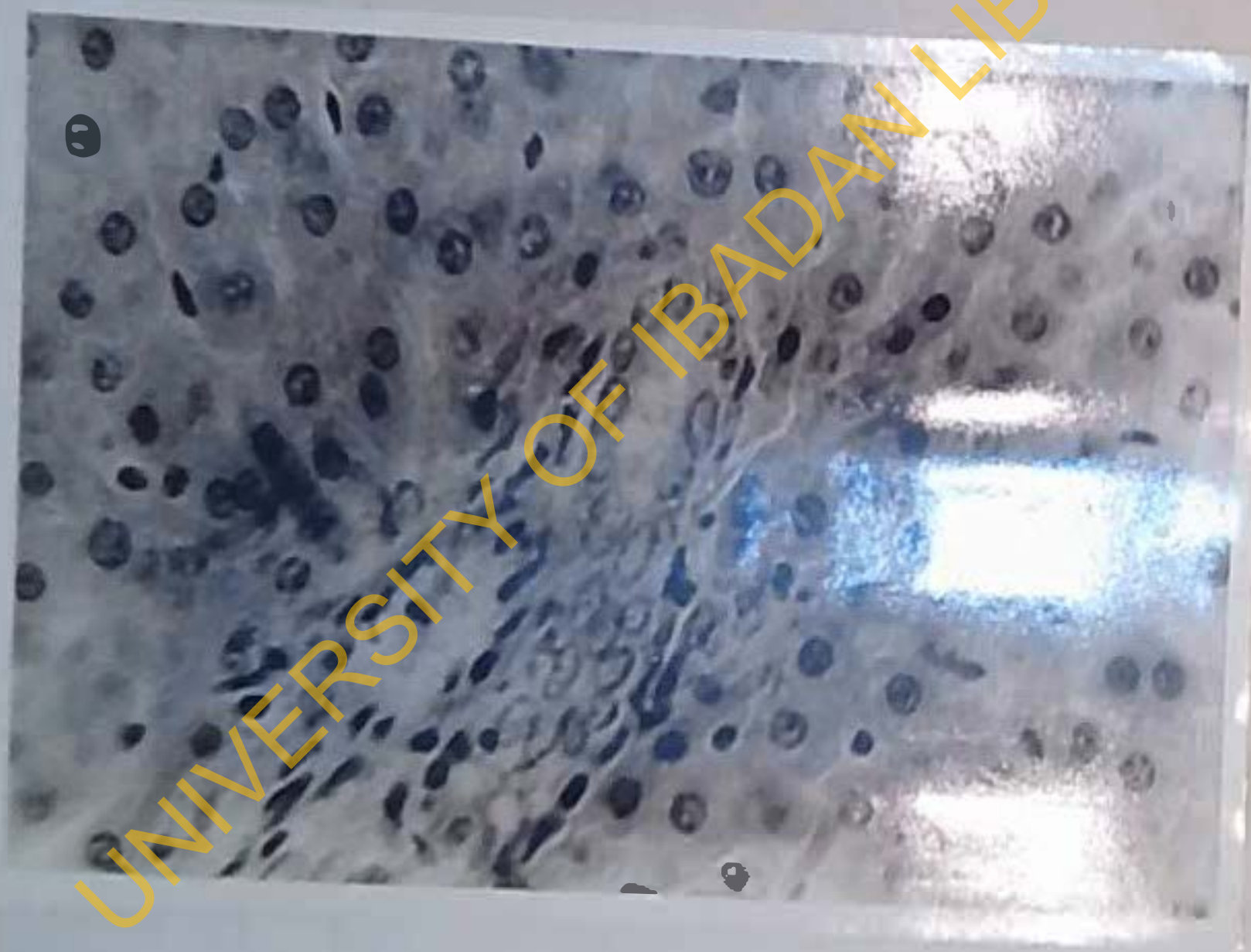


Plate 3C. PHOTOMICROGRAPH OF LIVER SECTIONS OF PALMOTOXIN Go TREATED RATS [HAEMATOXYLIN & EOSIN STAINED x 40]

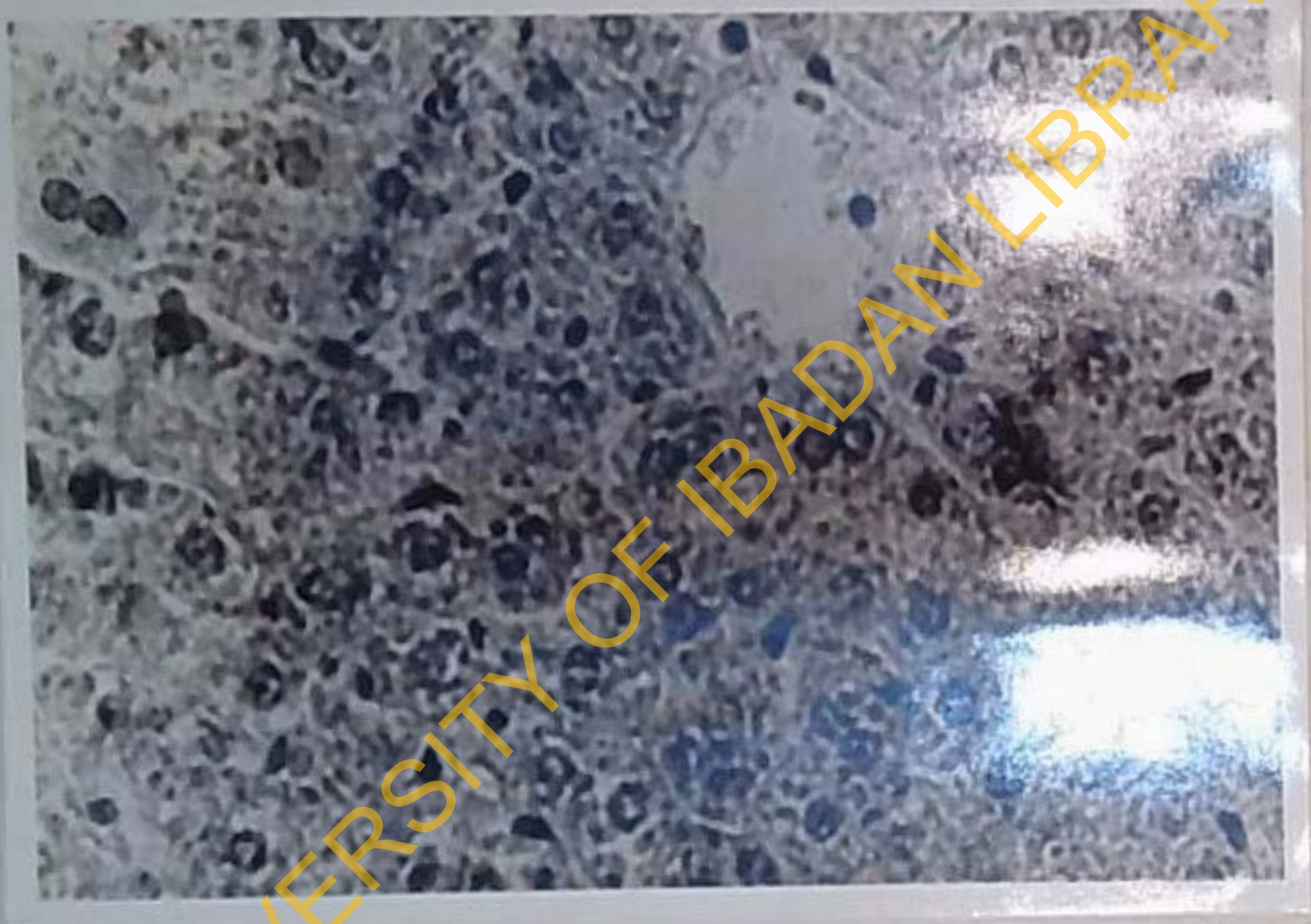


Plate 3b. PHOTOMICROGRAPH OF LIVER SECTIONS OF AFLATOXIN B_1 TREATED RATS (HAEMATOXYLIN & EOSIN STAINED) ×40)

Table 9

Relationship between liver weights and body weights of the Palmotoxin Go treated rats

Dose/Rat/day	Initial Average weight of Rats (g)	Final Average weight of Rats (g)	Liver weight (g)	Ave. Liver weight/ Body weight
Control	31.5	91.0	4.8	5.3
50 µg	31.5	89.0	4.5	5.00
100 µg	31.7	89.0	4.6	5.20
200 µg	31.6	86.0	4.2	4.9
15 µg Aflatoxin B1	31.0	84.0	3.8	4.5

TABLE 10

Glutamic oxaloacetic acid transaminase and Alkaline phosphatase activity in the serum of rats treated with palmotoxin Bo and Co and Aflatoxin B_1

	PALMOTOXIN Bo TREATED RATS		PALMOTOXIN Co TREATED RATS	
Dose/Rat	GOT level (International Units/Litre	Alkaline phosphatase Level (Mu)	GOT Level (International Units/litre)	Alkaline phosphatase Level (Mu)
Control	12.5 ± 1.0	204.0 ± 3.0	12.4 ± 0.5	203.0 ± 3.0
50 µg	19.0 ± 1.5	214.0 ± 2.0	11.9 ± 1.0	205.0 ± 2.5
100 µg	24.5 ± 0.5	226.0 ± 1.5	12.5 ± 1.0	204 ± 2.6
200 µg	33.0 ± 3.5	230.0 ± 1.0	13.0 ± 1.5	206 ± 3.0
15 µg Aflatoxin B_1	26.0 ± 2.5	215 ± 316	26.0 ± 2.0	215 ± 1.5

Mu = milliunits = 0.06 mmole units (Bessey, Lowry and Brock, 1946).

Activity of these enzymes in the palmotoxin Go treated animals did not alter significantly, for any deductions to be made from it. The values were compared both with those of the control and aflatoxin B_1 treated animals.

Conclusion:

1. At the dose levels studied 20-day old rats appeared susceptible to aflatoxin B_1 and palmotoxin Bo toxicity. Palmotoxin Go did not appear toxic to the rats at the same doses.

SECTION 2

INVESTIGATION 4

Comparison of the metabolism of aflatoxin B_1 and aflatoxin G_1 by rat liver microsomal-plus-soluble fractions.

Aflatoxin B_1 is hydroxylated by the liver of rats and most other animals either *in vivo* or *in vitro*, to the 4-hydroxy-aflatoxin B_1 referred to as aflatoxin M_1 (Holzapfel, Steyn and Purchase, 1966). The rat also converts it to the 2-hydroxy aflatoxin B_1 (aflatoxin B_{2a}) (Schabort and Steyn, 1969; Steyn, Pitout and Purchase, 1971). It is also metabolized by the cleavage of the methoxy group yielding formaldehyde which is further converted to carbon dioxide in the body (Schunk and Wogan, 1965; Wogan, Edwards and Schunk, 1967; Osiyemi, 1968 and Bassir and Emafo, 1970).

Not much is known about the metabolism of aflatoxin G_1. It is believed, however, to undergo similar degradative processes as aflatoxin B_1. In the present

SECTION 2

INVESTIGATION 4

Comparison of the metobolism of aflatoxin B_1 and aflatoxin G_1 by rat liver microsomal-plus-soluble fractions.

Aflatoxin B_1 is hydroxylated by the liver of rats and most other animals either '*in vivo*' or '*in vitro*', to the 4-hydroxy-aflatoxin B_1 referred to as aflatoxin M_1 (Holzapfel, Steyn and Purchase, 1966). The rat also converts it to the 2-hydroxy aflatoxin B_1 (aflatoxin B_{2a}) (Schabort and Steyn, 1969; Steyn, Pitout and Purchase, 1971). It is also metobolized by the cleavage of the methoxy group yielding formaldehyde which is further converted to carbon dioxide in the body (Schank and Wogan, 1965; Wogan, Edwards and Schank, 1967; Osiyemi, 1968 and Bneoir and Emrfo, 1970).

Not much is known about the metabolism of aflatoxin G_1. It is believed, however, to undergo similar degradative processes as aflatoxin B_1. In the present

experiment, the hydroxylation and demethylation of aflatoxin G_1 are established and compared with the hydroxylation and demethylation of aflatoxin B_1 using the rat-liver microsomal-plus-soluble fractions.

Experimental Procedure:

Pure aflatoxins B_1 and G_1 obtained as described on page 76 , were dissolved in chloroform and established to have single spots by thin layer chromatography. The chloroform solution was gradually added to a small quantity of distilled water and the chloroform blown off with Nitrogen. This procedure was adopted because of the poor solubility of aflatoxin in water especially when available as a solid. The concentration of the aqueous solution was then estimated by the Spectrophotometric method of Nabney and Nesbitt (1965).

Incubations for enzymic activity were in 50 ml Erlenmeyer flasks each containing 50 µ mole nicotinamide; 50 µ mole glucose 6-phosphate, 0.52 µ mole NADP, 50 µmole MgCl, 25 µ mole semi carbazide hydrochloride (pH 7.6) and 50 m µ mole of either aflatoxin B_1 or G_1 and 2 ml. of microsome-plus-soluble fraction corresponding to 0.5 g

fresh liver, in a total volume of 5ml. Flasks were shaken in a Gallenkamp reaction incubator with shaker, in air, for one hour and at 37.0° ± 0.5°C. Controls were applied also.

After precipitating the proteins with 20% zinc sulphate and saturated barium hydroxide and centrifuging, the unmetabolized aflatoxins B_1 and G_1 in the supernatant were estimated. and the amount used up obtained by difference. The formaldehyde content of the supernatant was estimated by the Nash reaction. The fluorescent metabolites were also estimated by the 'Null fluorescence technique'. Fluorescent metabolites were obtained as described on page 93 and the Ultraviolet spectra obtained in methanol (analytical grade), using the Perkin-Elmer Spectrophotometer 137UV.

Results:

The Rf values of the two metabolites of aflatoxins B_1 and G_1 on silica gel G plates ('chromalay' brand) are given in Table 11a and their Ultraviolet absorption peaks are shown in Table 11b. Figure 27 is the Ultraviolet tracing of the metabolite of aflatoxin B_1

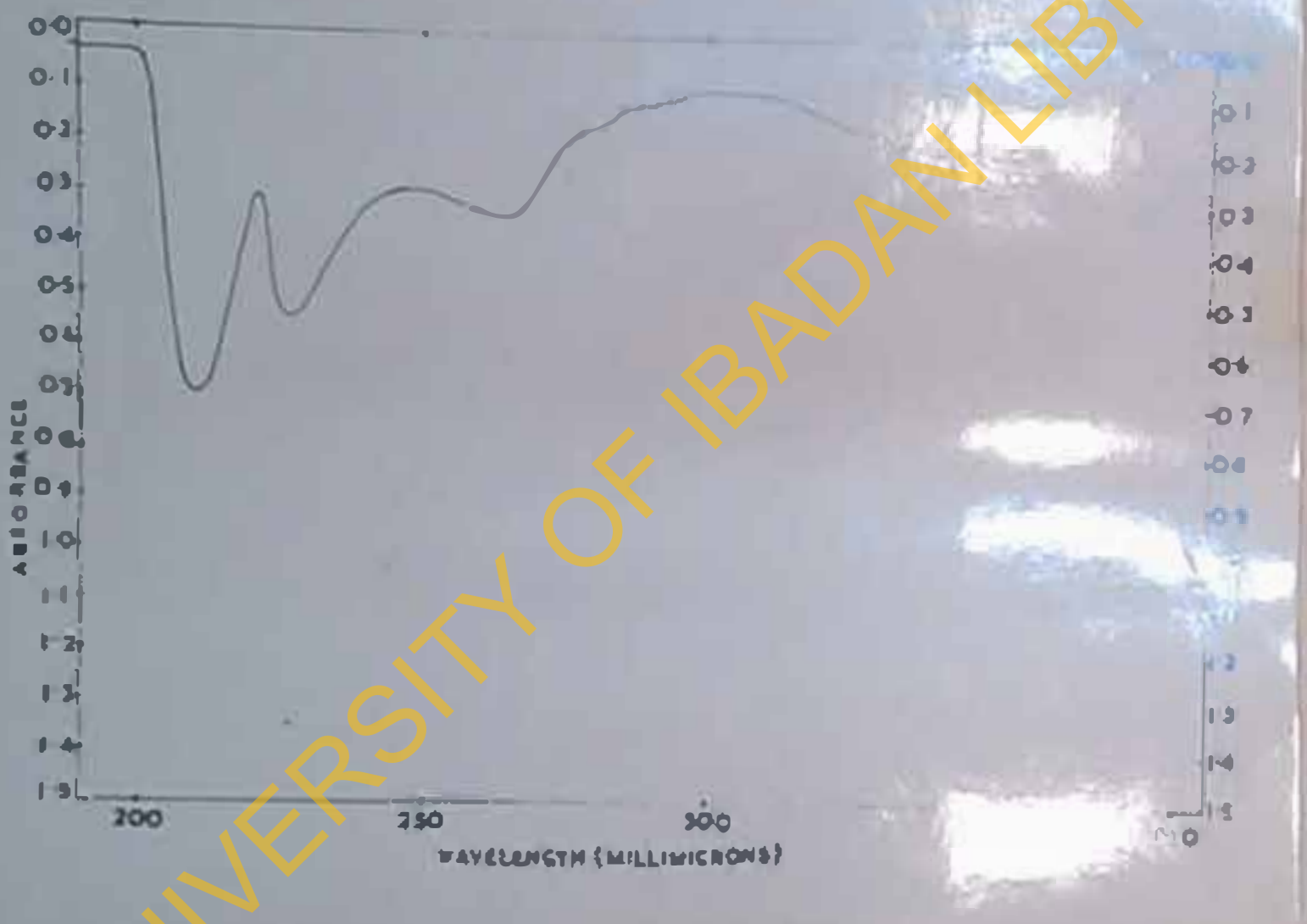


Fig 27. ULTRAVIOLET SPECTRUM OF AFLATOXIN B_1 METABOLITE BELIEVED TO CORRESPOND TO AFLATOXIN M_1.

TABLE 11(a)

Rf values of derivatives of Aflatoxins B_1 and G_1

	AFLATOXIN B_1		AFLATOXIN G_1	
	Mn	Mx	'GM'	"GMx
a	0.25	0.15	0.047	0.04
b			0.170	0.120

Mx and "GMx" represent the second metabolites respectively of each of the toxins B_1 and G_1

(a) Solvent: 10% acetone in chloroform v/v
(b) Solvent: 3% methanol in chloroform v/v

TABLE 11(b)

Ultraviolet Absorption Peaks (n m) of aflatoxins B_1, G_1 and the corresponding derivatives M_1, Mx and 'GM'

B_1	M_1	M_x	G_1	GM
223 nm	226 nm	226 nm	218 nm	232 nm
265 "	265 "	257 "	262 "	261 "
365 "	357 "	360 "	365 "	-

identified as aflatoxin M_1 on the bases of its Rf value and Ultraviolet absorption peaks. Figure 28 is the Ultraviolet spectrum of the aflatoxin G_1 metabolite probably corresponding to 'GM'. The two minor metabolites were not investigated further. Reaction of the isolated aflatoxin M_1 and 'GM' with acetic anhydride, yielded a product which did not migrate from the base line when applied to thin layer plates and developed with 10% acetone in chloroform (v/v). This has been taken to suggest the presence of hydroxy groups (Emafo, 1970). Plate 4 shows the aflatoxin G_1 metabolites on thin layer plates.

Production of aflatoxins M_1 and 'GM' and demethylation of aflatoxins B_1 and G_1 are given in Table 12. The results are given as means of duplicate experiments with ten incubation flasks in each set and their standard errors and represent yields of a one hour incubation and utilizing microsome-plus-soluble fractions from the same liver pool. The value for the total amount of aflatoxin G_1 metabolized was slightly higher than that of aflatoxin B_1 but the difference was not significant. However, more hydroxy product and formaldehyde were formed in one hour from aflatoxin B_1 than aflatoxin G_1, the differences

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PLATE 4: Thin layer chromatogram showing metabolites from aflatoxin B_1 (IN VITRO)

Fig AFLATOXIN 'BM' (ISOLATED)

Fig. 28: ULTRAVIOLET SPECTRUM OF AFLATOXIN G_1 METABOLITE BELIEVED TO CORRESPOND TO AFLATOXIN 'GM' (INVITRO)

TABLE 12

Comparative Metabolism of Aflatoxins B_1 and G_1

Toxin	Concentration	% age total toxin metabolized	%age converted to M_1/"GM"	Formaldehyde formed (M µ mole)	No. of Animals
B_1	50_{m} umole	86.1 ± 1.9	1.87 ± 0.40	25.0 ± 2.6	20
G_1	50m umole.	90.7 ± 2.1	0.78 ± 0.07	9.8 ± 0.65	20
		P 0.05 (NS)	P 0.001(S)	P 0.001 (S)	

± = Standard error

NS = Not significant

S = Significant difference

being very significant. Despite the seemingly high value of the total toxin metabolized, the amount of the hydroxy products were very small in comparison.

Conclusion:

1. Aflatoxin G_1 is probably hydroxylated and demethylated by the rat liver, in a manner similar to aflatoxin B_1.
2. Aflatoxin B_1 is, however, more easily demethylated and hydroxylated than aflatoxin G_1.

INVESTIGATION 5

Comparison of the 'in vitro' metabolism of palmotoxin Bo and Go using rat liver microsomal-plus-soluble fraction:

While the fate of aflatoxin B_1 in biological systems, such as described in the last experiment, has been well documented (de Iongh, Vles and Van Pelt, 1964; Holzapfel, Steyn and Purchase, 1966; Masri, et al. 1967 and Wogan, Edwards and Schank, 1967), the fate of the palmotoxins Bo and Go is yet unknown. 'In vitro' methods as applied in the study of aflatoxin B_1, have been utilized to investigate the biotransformation of palmotoxins Bo and Go.

Experimental Procedure:

Pure palmotoxins Bo or Go, was dissolved in chloroform and the chloroform solution added to distilled water with a stream of Nitrogen to blow off the chloroform from the water. The concentration of the aqueous solution was determined from the standard curve.

50 m μ mole of each toxin was incubated with 50 μ mole glucose-6-phosphate; 50 μ mole nicotinamide, 50 μ mole magnesium chloride, 0.52 μ mole NADP and 25 μ mole

semi-carbazide hydrochloride and 2·ml· of microsome-plus-soluble fraction equivalent to 0.5g liver weight, in a total volume of 5ml· and using 50 ml· Erlenmeyer flasks.

Three control tubes were also included. The first tube contained all the reagents but no toxin. Tube two contained the toxin and the other reagents but no liver fractions. And tube threo contained the reagents and the toxins but incubated with liver fractions deactivated by heat. The inclusion of tube one, was to compensate for any endogenous chromogenic substances (Christensen and Wissing, 1972). Seven flasks containing the test were used. The ten flasks were incubated in air at 37.0 ± 0.5°C for one hour in a Gallenkamp reaction incubator with shaker.

Proteins were precipitated by the method of Stitzel, et al. 1966. Supernatants obtained after centrifuging at 5,000 g for 15 minutes were extracted with chloroform. The chloroform extracts were concentrated and examined on thin layer for any fluorescent products. Part of the supernatant was used for formaldehyde determination by the Nash reaction.

Results:

Two derivatives of palmotoxin Bo were identified on thin layer. These had lower Rf. values than the parent compound. (0.22 and 0.21 respectively) in 5% methanol in chloroform (v/v), thus indicating their more polar nature. Only one derivative of palmotoxin Co, with Rf. values of 0.2 was isolated. Application of the Nash reaction to the supernatant gave a golden yellow colour indicating the presence of formaldehyde in the mixture.

Fluorescence was also observed at the point of application of the samples. This was suspected to be a conjugate and was further examined.

Further examination of the isolates from incubation of palmotoxins Bo and Co with the rat liver-microsomal-plus-soluble fraction:

The major derivatives of palmotoxins Bo and Co with Rf 0.22 and 0.2 respectively were examined further for their characteristics. Study on the second palmotoxin Bo metabolite was not carried out in view of the minute amount produced, thus hindering any

meaningful study. However, the major metabolites were isolated as described on page 93 . The eluates from the silica gel scrapings were concentrated and redissolved in chloroform. An aqueous solution was prepared from this under nitrogen. Ultraviolet characteristics were determined on a Perkin-Elmer Spectrophotometer 137UV. The fluorescence spectra were determined in chloroform, using a Perkin-Elmer fluorimeter model 203.

The isolates were further reacted with acetic anhydride and the reaction mixture run on thin layer plates, using a 4% methanol in chloroform (v/v) solvent system.

The fluorescent group at the base line of the thin layer chromatogram was extracted with a methanol-chloroform-water mixture, (5:2:2 v/v) and the extract concentrated in a rotary film evaporator. The concentrate was again run on thin layer plates using n-butanol-glacial acetic acid-water 10:1:1 (v/v) as developing solvent. A single fluorescent moiety was discerned in both polarographs Ba and Ga and with Rf. values of 0.40 and 0.34 respectively. The substances were tested

for mercapturic acid, glucuronic acid, sulphate and amino acid conjugates.

Results:

Figures 29 and 30 show the Ultraviolet spectra of the palmotoxins Bo and Go metabolites respectively. Absorption peaks for the palmotoxin Bo metabolite in water were at 215nm and 265nm while that of palmotoxin Go were at 212nm and 272nm. Figures 31 and 32 show the fluorescence spectra of the palmotoxins Bo and Go metabolites respectively. Both showed excitation maxima at 365nm. The emission maximum was at 410nm for palmotoxin Bo metabolite while that of palmotoxin Go metabolite was at 425nm.

Products of the reaction of the metabolites with acetic anhydride did not move from the base when run on thin layer plates using 4% methanol in chloroform or 10% acetone in chloroform (v/v).

Positive test for conjugates was obtained only for glucuronide conjugation. Hydrolysis with β-glucuronidase yielded fluorescent substances with the same Rf. value as the parent compounds (palmotoxins Bo and Go).

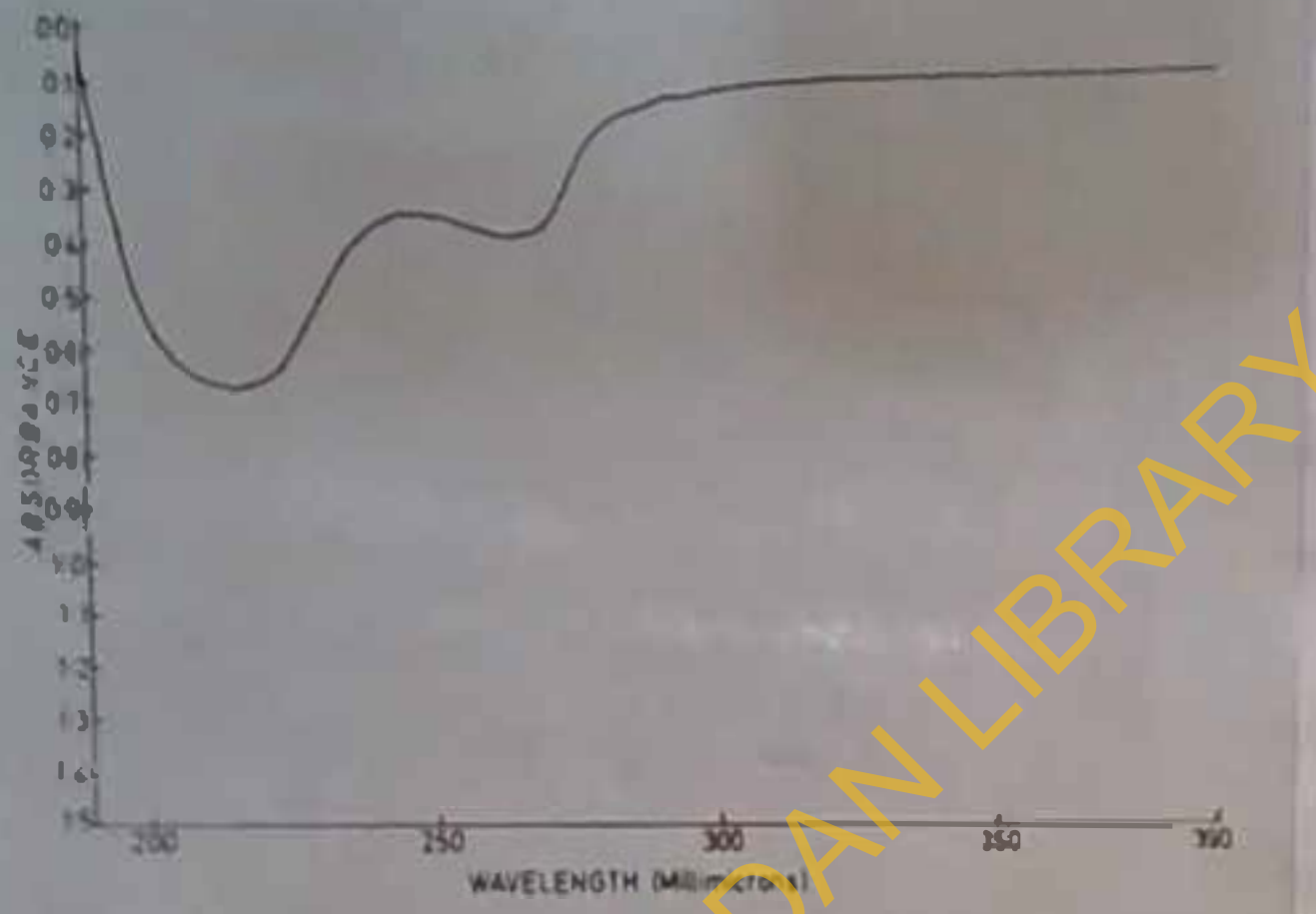


Fig PALMOTOXIN Bo METABOLITE (SOLVENT WATER)

Fig. 29.

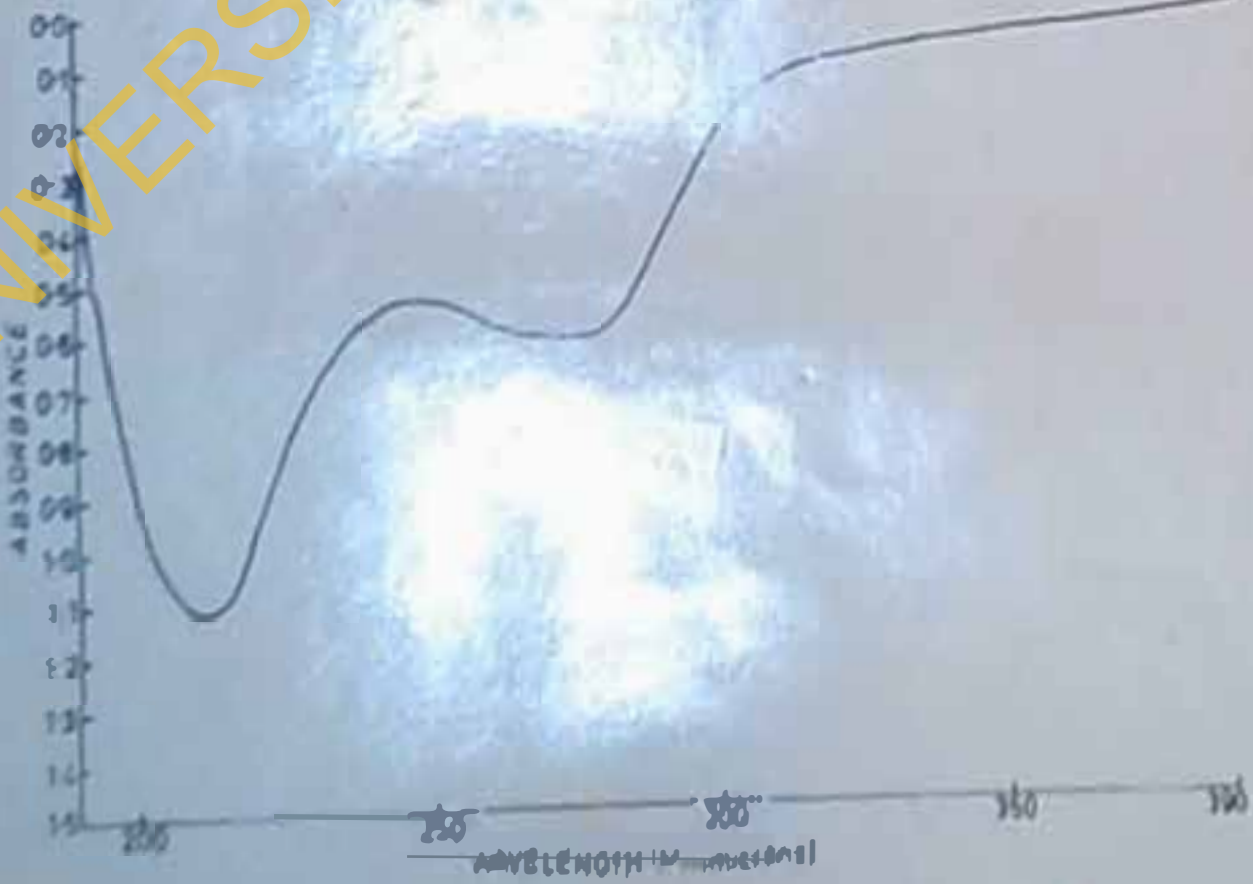
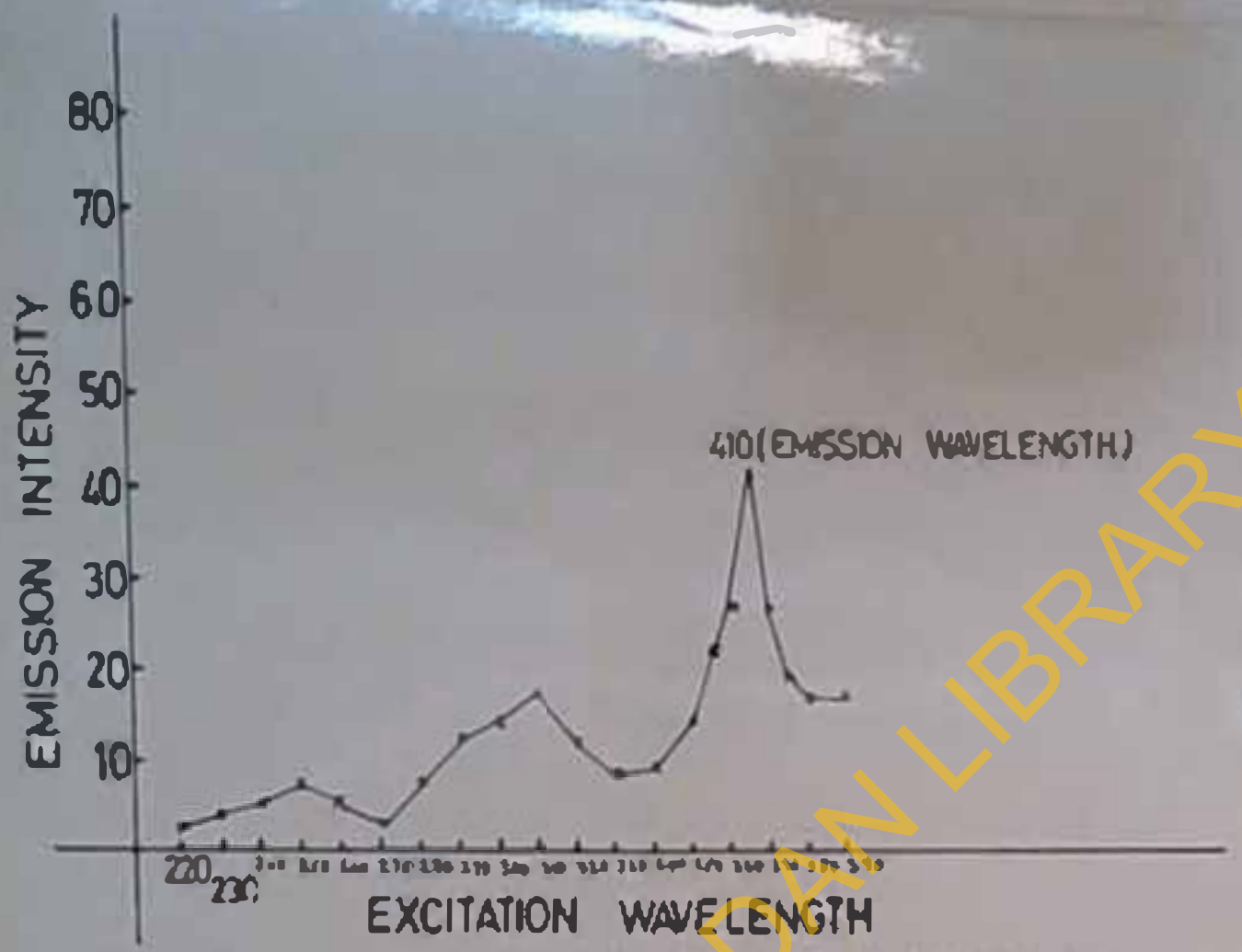


Fig PALMOTOXIN Bo (metabolite) (SOLVENT WATER)



Fluorescence spectrum of the isolated metabolite of palmotoxin Bo in chloroform

Fig. 31

Fluorescence spectrum of isolated palmoloxin Go metabolite in chloroform

III. Comparison of the fluorescence intensity of palmotoxins Bo and Go with those of their respective metabolite:

Having established the fluoreocence characteristics of palmotoxins Bo and Go metabolites their relative intensities were compared with those of the parent compounds in chloroform solution, using the emme Perkin-Elmer fluorimeter model 203.

Experimental Procedure:

A solution of either palmotoxin Bo or palmotoxin Go in chloroform was diluted with more chloroform until their optical densities taken at 265nm, using a Unicam Sp. 500 Spectrophotometer were the same as those of the respective metabolites. The fluorescence intensities at an excitation of 365nm and emission of 410nm for palmotoxin Bo and its metabolite and 425nm for palmotoxin Go and its metabolite were read off the scale.

Results:

In both cases, it was found that the metabolites were twice as fluorescent as their parent compounds.

Conclusions:

1. The rat liver microsomal-plus-soluble fraction,

biotransformed palmotoxin Bo into two other fluorescent compounds detectable by thin-layer chromatography. It however, transformed palmotoxin Co into only one new compound. These are probably hydroxy products.

2. Demethylation occurred when rat-liver microsomal-plus-soluble fraction was incubated with either palmotoxin Bo or palmotoxin Co.

3. The fluorescence intensities of the metabolites of palmotoxins Bo and Co, were twice as much as their parent compounds.

4. Glucuronide conjugates are formed when either palmotoxin Bo or palmotoxin Co was incubated with rat-liver microsomal-plus-soluble fractions.

EXPERIMENT B

Determination of the co-factor requirements in the 'in vitro' metabolism of palmotoxins Bo and Go using rat-liver microsomal-plus-soluble fractions:

The incubation medium used in the 'in vitro' studies so far, has been reported to be ideal for the study of the metabolism of aflatoxin B_1 and to result in maximum activity of the drug-metabolizing enzymes of the rat liver (Emafo, 1970; Bassir and Emafo, 1970). It has been shown that withdrawal of any component or its substitutions with other co-factors might affect the enzyme activity. Using the same method, a study of the dependence of the metabolism of palmotoxins Bo and Go on these factors have been investigated and compared with similar effects on aflatoxin B_1.

Experimental Procedure:

Four batches of five 50ml. Erlenmeyer flasks were used in the experiment. Into the first batch was added 50 µ mole of magnesium chloride only. The second batch contained 50 µ mole of magnesium chloride and 50 µ mole of glucose-6-phosphate. In the third was added 0.52 µ mole

NADP, 50 µ mole glucose-6-phosphate and 50 µ mole nicotinamide but no magnesium chloride. The final batch contained the complete system of Bassir and Emafo (1970), comprising all the above factors in equivalent concentrations. To four of the flasks in each batch was added; 50m µ mole of either aflatoxin B_1 or palmotoxin Bo or palmotoxin Go; 2ml of the liver extract, equivalent to 500mg. liver and 25 µ mole semi-carbazide hydrochloride (pH 7.6). The fifth flask in each batch was used as control and contained liver extract but no toxin. One of the four flasks containing toxins was also used as control and was incubated with liver extract deactivated by heat. Each flask was made up to 5ml. with double distilled water. The flasks were incubated for one hour at 37.0 ± 0.5°C in the Gallenkamp reaction incubator equipped with a shaker. The amount of toxin left after incubation was estimated as before by the 'Hull fluorescence technique and the formaldehyde produced estimated by the Nash reaction.

Results:

The influence of co-factors on the *in vitro*

metabolism of aflatoxin B_1, palmotoxins Bo and Go have been tabulated in Table 13. Table 14 also shows the effect of the co-factors on their demethylation. There was no metabolism of the toxins in the medium containing magnesium chloride only. No demethylation took place in the medium in which glucose-6-phosphate was added to magnesium chloride. Values obtained for total metabolism in this medium were rather small, almost insignificant.

The introduction of NADP and nicotinamide in the presence of glucose-6-phosphate, without magnesium chloride appeared to have triggered off the reaction to a reasonable extent.

From the values obtained in the last batch, magnesium chloride appeared to have had a tremendous activating influence on the reactions.

Conclusion:

1. Biotransformation of palmotoxin Bo and palmotoxin Go, appeared to involve NADP dependent enzymes similar to those for the metabolism of aflatoxin B_1.
2. Magnesium chloride activated the metabolism of aflatoxin B_1, palmotoxin Bo and palmotoxin Go.

TABLE 13

Percentage toxin metabolized by the equivalent of 0.5 g of Liver

Cofactors present in medium	Aflatoxin B_1	Palmotoxin Bo	Palmotoxin Go
Magnesium chloride	0	0	0
Magnesium chloride + Glucose-6-phosphate	2.45 ± 0.9	3.6 ± 0.4	4.5 ± 1.0
NADP + Nicotinamide + Glucose-6-phosphate	50.5 ± 0.5	26.2 ± 1.3	25.5 ± 4.2
Magnesium chloride, Nicotinamide, NADP and Glucose-6-phosphate	86.1 ± 1.9	47.5 ± 2.20	49.5 ± 3.0

± = Standard error.

TABLE 14

Influence of co-factors on the demethylation of aflatoxin B_1 and palmotoxins Bo and Co.

Cofactors present in Medium	Formaldohyde formed by the equivalent of 0.5g liver after 1 hour incubation (in millimicromoles).		
	Aflatoxin B_1	Palmotoxin Bo	Palmotoxin Co
Magnesium chloride	0	0	0
Magnesium + Glucose-6-chloride phosphate	0	0	0
NADP + nicotinamide + Glucose-6-phosphate	14.0 ± 1.3	8.25 ± 1.0	4.45 ± 0.8
Magnesium chloride + nicotinamide + NADP Glucose-6-phosphate	25.0 ± 1.0	20.0 ± 2.0	10.0 ± 1.5

± = Standard error.

EXPERIMENT C

Investigation of the effect of changes in NADP (Nicotinamide Adenine dinucleotide Phosphate) concentration on the demethylation of palmotoxin Bo and palmotoxin Go:

In view of the role of NADP in the activity of NADP dependent drug-metabolizing enzymes (Posner, Mitoma, and Udenfriend, 1961; Nilsson and Johnson, 1963), a study of the influence of the variation in concentration of NADP on the demethylation of palmotoxins Bo and Go was carried out. The reliability of the formaldehyde estimation has made it readily acceptable as an index for the assessment of metabolic activity.

Experimental Procedure:

The other co-factors were kept constant, while the NADP concentration was varied. Hence the medium was made up of 50 μ mole magnesium chloride, 50 μ mole Tris-5-phosphate 50 μ mole nicotinamide 25 μ mole semi-carbazide hydrochloride (pH 7.6) and 50 μ mole of each toxin. Various concentrations of NADP representing, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 μ mole

were used. Triplicate samples were used for each concentration. 2ml of rat-liver microsomal-plus-soluble fraction was added to each in a total volume of 5ml. A control in which the liver fraction was incubated without the toxin was applied for each sample. Flasks were incubated in air for one hour at 37.0 ± 0.5°C using the Gallenkamp reaction incubator with a shaker.

At the end of the one hour, reactions were stopped by the method of Stitzel, et al. (1966) and the formaldehyde content of each flask estimated by the Nash method.

Results:

Variations in the concentration of NADP affected the formaldehyde yields from palmotoxins Bo and Co. This effect has been represented graphically in figure B3. The formaldehyde yield rose with the concentration of NADP until at a concentration of about 0.4 μ mole when addition of more NADP did not appear to result in increased formaldehyde yield.

Conclusion:

A concentration of 0.4 μ mole of NADP appeared adequate for maximum demethylation of the palmotoxins at the toxin concentration studied and under the prevailing conditions of the experiment.

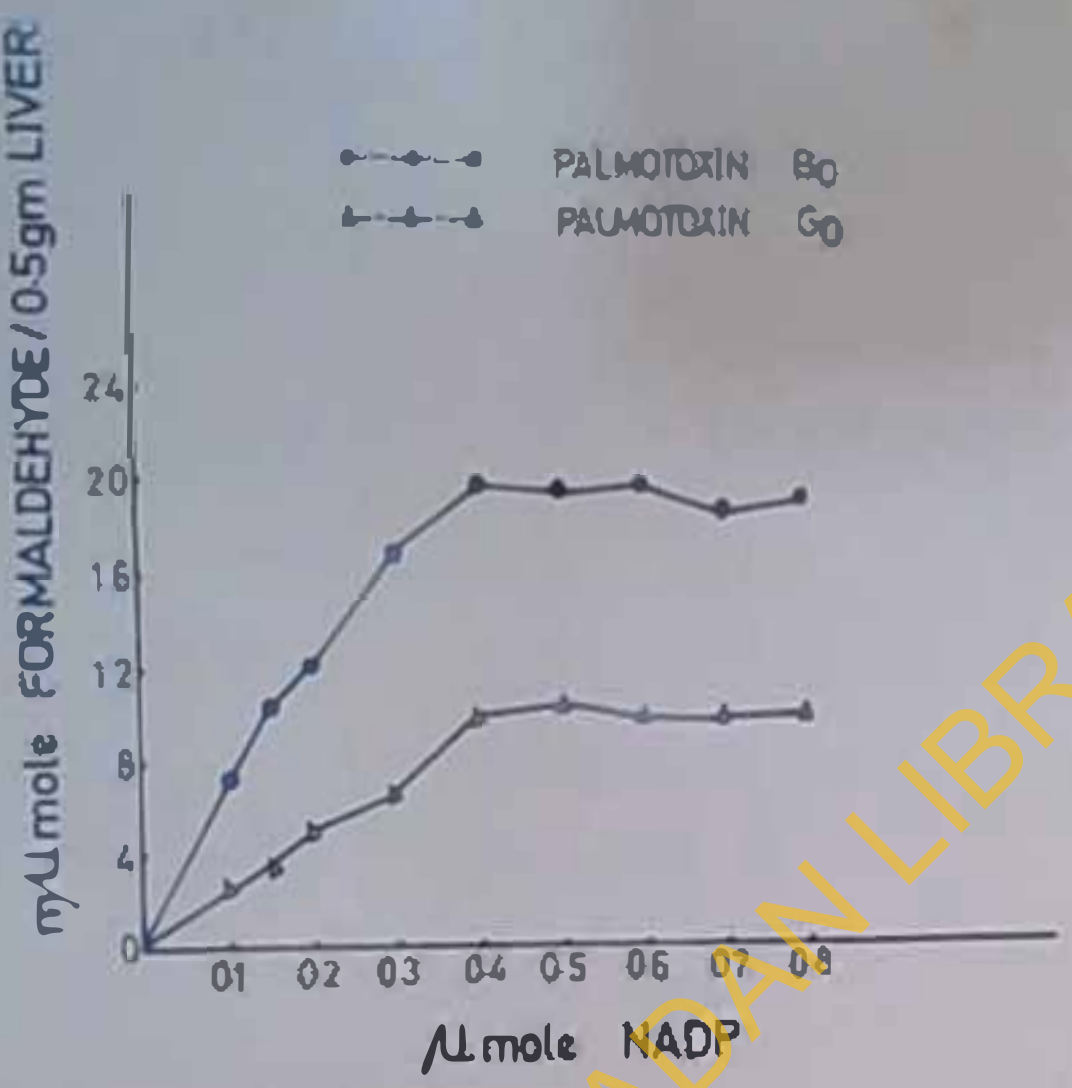
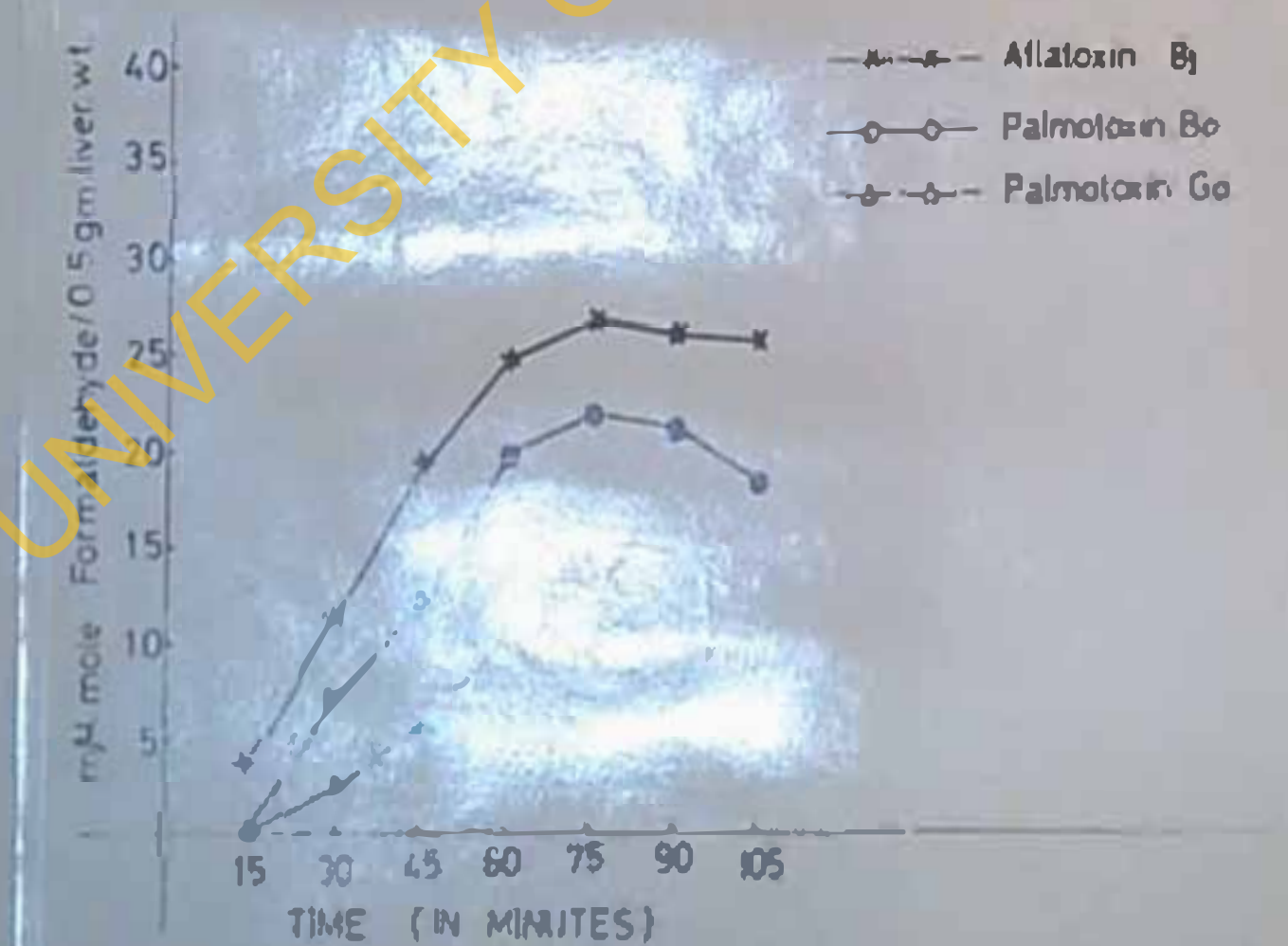


Fig. 33. INFLUENCE OF VARIATION OF NADP CONCENTRATION ON THE DEMETHYLATION OF PALMOTOXINS Bo AND Go.



Amount of formaldehyde formed from Aflatoxin B1, Palmotoxins Bo and Go, as a function of time.

Fig. 34

EXPERIMENT D

Variation in formaldehyde formation from aflatoxin B_1, palmotoxins Bo and Go as a function of time of incubation:

Emafo (1970) has shown that demethylation of aflatoxin B_1 varies with the time period of incubation with rat liver homogenates. The effect of period of incubation of palmotoxin Bo and Go on their demethylation has thus been studied using rat liver microsomal-plus-soluble fractions.

Procedure:

Incubation mixture containing 50 µ mole nicotinamide, 50 µ mole magnesium chloride, 50 µ mole glucose-6-phosphate; 0.52 µ mole NADP, 25 µ mole semi-carbazide hydrochloride (pH 7.6) and 50m µ mole of each toxin - aflatoxin B_1, palmotoxin Bo and palmotoxin Go, and 2ml of the microsomal-plus-soluble fractions, were used in a total volume of 5ml in 50ml Erlenmeyer flasks. Controls of inactivated (boiled) microsomal-plus-soluble fraction and another in which fresh liver fraction was added without the toxins, were employed in each case.

The flasks were incubated in air at 37.0 ± 0.5°C, using the Gallenkamp reaction incubator with shaker. Duplicate samples were stopped at 15 minute intervals by precipitating the proteins which were later separated by centrifugation using an MSE refrigerated centrifuge. The supernatant from each sample was analysed for the formaldehyde content as described in page 94 .

Results:

A plot of the formaldehyde concentration against time is given in figure 34. After 15 minutes, demethylation had only been observed for aflatoxin B_1. There was, however, a continuous rise in the concentrations of formaldehyde in the three samples as the incubation progressed. The greatest amount of formaldehyde was produced in 75 minutes.

Conclusion:

Demethylation of aflatoxin B_1 palmotoxins Bo and Go 'in vitro' varied with time, with maximum production at about the 75th minute.

EXPERIMENT E

Influence of pH on the demethylation of palmotoxins Bo and Go:

Demethylation of aflatoxin B_1 '*in vitro*' is reported to depend both on pH and the buffer solution (Emafo, 1970). 0.3M phosphate buffer; pH 7.6 has been found to be ideal for the demethylation of aflatoxin B_1 by rat liver microsomal-plus-soluble fraction. Since the reactions involved enzyme catalysis, it is known from basic studies that the hydrogen ion concentration (pH) of the medium in which an enzyme acts, influences the activity of the enzyme remarkably. Thus, it is known that an enzyme has a particular pH at which it acts best (optimal pH) and this varies from enzyme to enzyme. The present experiment was designed to find the optimal pH of palmotoxins Bo and Go demethylating enzymes, when rat-liver-microsomal-plus-soluble fraction was used.

Experimental Procedure:

40 male rats selected from litter mates, were distributed into 8 groups and their livers homogenized

in 0.3M potassium phosphate buffers of pH 6, 6.5, 7, 7.4, 7.5, 7.6, 7.7, and 8. The microsomal-plus-soluble fractions was obtained as usual.

Each group was incubated with 50 μ mole of either palmotoxin Bo or palmotoxin Co, using the same co-factors as previously described and including 25 μ mole of semi-carbazide hydrochloride adjusted to the respective pH and in a total volume of 5ml. The flasks were inoubated in air for one hour at 37.0 ± 0.5°C, using the Gallenkamp reaction incubator with shaker. Controls were applied for each group as in earlier experiments. Proteins were precipitated and the formaldehyde content of each supernatant corresponding to the different pH values, was estimated by the Nash reaction.

Results:

No detectable formaldehyde was formed from palmotoxin Co *in vitro* at pH of 6. There was, however, a gradual rise in both cases till a maximum at pH 7.5 and 7.6 respectively was attained (Figure 35).

Conclusion:

Optimal pH for the '*in vitro*' demethylation of the palmotoxins Bo and Co was between 7.5 and 7.6.

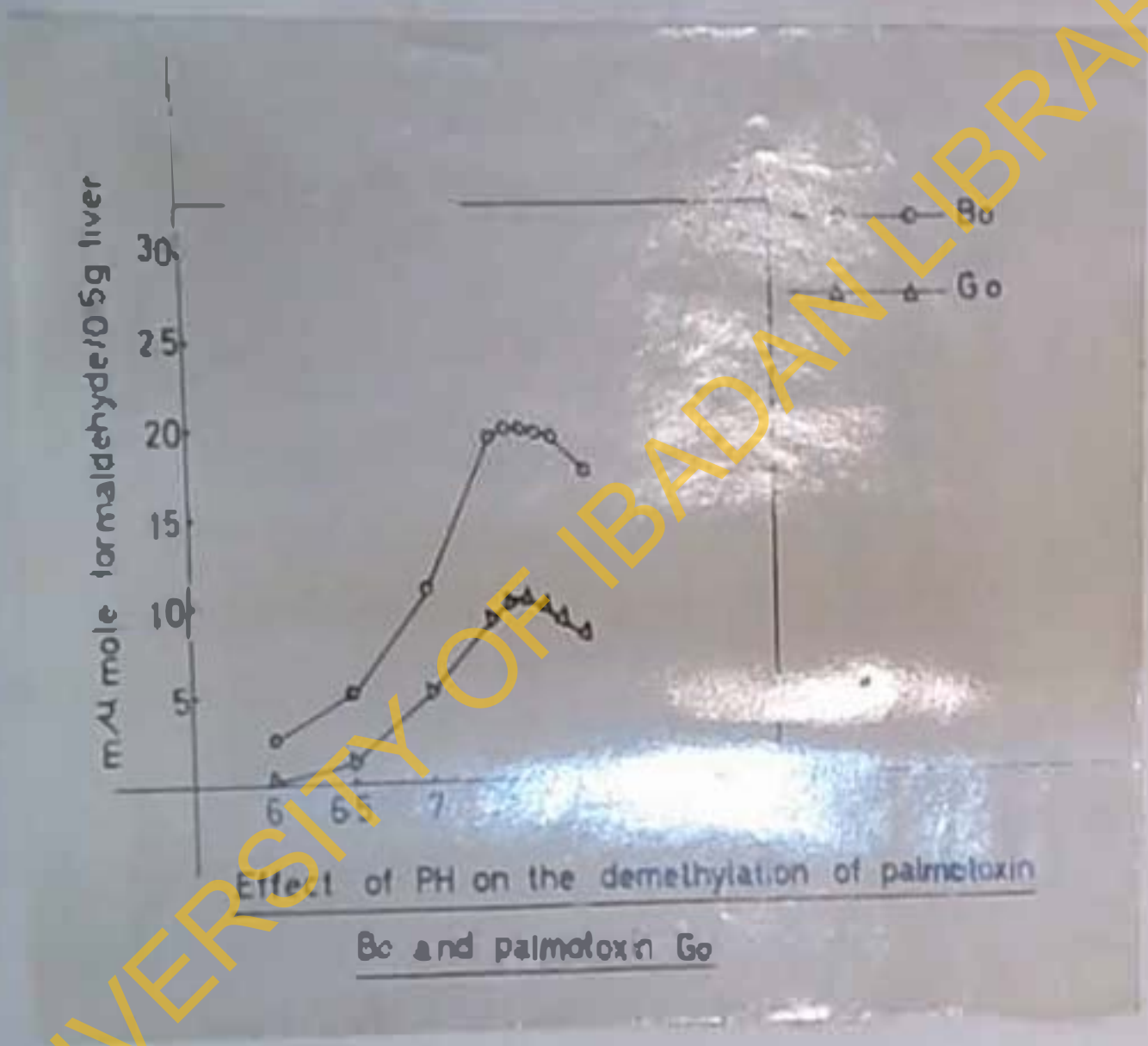


Fig. 35.

SECTION 3

INVESTIGATION 6

Investigations of the effect of species differences in the metabolism of aflatoxin B_1, palmotoxin Bo and palmotoxin Go:

Response of animals to drugs and biologically active compounds have long been known to vary according to species (Dubois, Thurah, and Murphy, 1957; Quin, Axelrod, and Brodie, 1958). These varied responses are also associated with the metabolic pattern of the substances in the different species. Quin, Axelrod and Brodie (1958) have demonstrated a variable capacity of enzymes in the liver microsomes from different species to demethylate amino pyrine. Thus, it is recognized that enzyme preparations from different species vary in their capacity to handle a particular substance and this might be an index for estimating the relative activities of enzymes involved in such transformations.

In the work reported here, the species differences in the metabolism of aflatoxin B_1, palmotoxins Bo and Go

have been investigated using both liver slices and microsomal-plus-soluble fractions of the livers of the respective animals.

Experimental Procedure:

The procedure adopted here has been described by Emafo (1970) and is similar to the method of Quin, Axelrod and Brodie (1958). The amount of each toxin metabolized, the corresponding amount of major fluorescent metabolites formed, and the formaldehyde formed by each species were estimated using both liver slices and microsomal-plus-soluble fractions isolated from the livers of the respective species. This has been regarded as indicating the ability of the respective species to biotransform the toxins.

Incubation with Liver Slices:

Liver slices equivalent to 0.5g fresh liver weight, were incubated with 50m μ mole of each toxin in a McEwan solution. Semi-carbazide hydrochloride (pH 7.6) was added (25 μ mole) during incubations for formaldehyde estimation, in a final volume of 5ml. After incubation for one hour at 37° ± 0.5°C, except for the case of the toad and lizard, where incubations

were at 25° - 26°C, proteins were precipitated and the remaining unmetabolized toxin estimated. The fluorescent metabolites formed during the incubation were identified using the rat metabolites as reference (Plates 5a, b, and c) and then estimated by the 'Null fluorescence technique' on thin layer plates.

Control flasks were used in the following order

1. flask containing the medium, with liver slices but no toxin.
2. flask containing the toxin in the medium but no liver slices, and
3. flask containing the toxin in the medium but incubated with liver slices deactivated by boiling.

This procedure was retained for all the species studied.

Incubation with microsome-plus-soluble fraction:

2ml of microsome-plus-soluble fractions from each of the animals was incubated with 50 µ mole magnesium chloride, 50 µ mole glucose-6-phosphate, 50 u mole nicotinamide, 0.52 µ mole NADP and 50m µ mole of each toxin; in a total volume of 5ml and using 50ml Erlenmeyer flasks for the incubation. Except for the lizard and

149B

Plate 5a Thin layer Chromatogram

PLATE 6D. Thin layer chromatogram showing metabolites of Palmatain Bo from sheep, rat, goat and mice (in vitro)

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Plate 5C.

Thin layer chromatogram showing metabolites of Palmotoxin Go from duck, rat, mice, Sheep and Goat (in vitro)

tond where incubations were carried out in air, at 25° - 26°C, all othors were done, nlso in nir nt 37.0 ± 0.5°C for one hour. Controls were applied ns in the liver slices. 25 µ mole oemi-carbnzido hydrochloride (pH 7.6) was added in casos whore formoldehydo hnd to be determinod.

Protcins were prccipitnted by the method of Stitzel, et nl. (1966). Supernntants were obtained by centrifuging nt 5,000g using an MSE referigernted centrifuge. The supernatants were exnminod, for fluorescent metnbolites, on thin layer and where present thnse metabolites were nccordingly estimnted. Similnrly, the residual toxins were estimnted ond the differnnce between this nnd the total recoverable toxin in the control expressed nn percentnges.

Formaldehyde wns estimnted in the supernntnnt. The fluorescent metnbolite of the bnse line of the the thin layer chromatogram wns examined qualitntively for each nnimnl.

Results:

The aflatoxin B_1 metnbolized by 0.5g liver slices in

shown in Table 15. The comparative distribution of these values is shown on the histogram (Fig 36). The lowest value of 55.6% was recorded in sheep and the highest of 95.5% in toad, Values for the metabolism by microsomal fractions are given in Table 16 and their comparative distribution in figure 37. Higher values were recorded here and it ranged from 75.5% to 99.8%.

Palmotoxin Bo metabolized by 0.5g liver slices is given in Table 17 and the comparative distribution in figure 38. The amount metabolized by microsomal-plus-soluble fraction is given in Table 18 and the comparative distribution in figure 39. In all cases, lower values were recorded here than for aflatoxin B_1. While aflatoxin B_1 was almost totally metabolized in most of the animals studied, most of the animals did not metabolize more than 60% of the palmotoxin Bo used.

Values for the palmotoxin Go metnbolized are given in Tables 19 and 20 for the liver slices and microsomal-plus-soluble fraction, respectively. Their comparative distribution is given in figures 40 and 41. Here again values were lower, generally, than for aflatoxin B_1. Except in the dog the values were in many cases lower

TABLE 15

Influence of species differences on the metabolism of aflatoxin B_1 by liver slices.

	Rat	Mice	Guinea Pig	Rabbit	White Rock Cock	Duck	Goat	Sheep	Dog	Lizard	Toad
Formaldehyde produced during incubation m mole	12.4 ± 1.6	6.5 ± 0.7	4.1 ± 0.5	12.2 ± 1.3	8.3 ± 0.8	0	2.0 ± 0.3	2.4 ± 0.3	5.4 ± 0.3	4.8 ± 0.2	5.2 ± 0.5
Percentage of aflatoxin B_1 metabolized	81.0 ± 2.5	90.5 ± 3.2	87.1 ± 3.6	92.8 ± 2.5	96.0 ± 5.5	97.0 ± 3.0	92.5 ± 2.5	55.6 ± 2.0	82.1 ± 3.5	80.8 ± 3.0	95.5 ± 2.5
Percentate Aflatoxin M_1 formed	1.5 ± 0.1	0.9 ± 0.05	0.74 ± 0.02	0.88 ± 0.1	0	0	1.75 ± 0.25	2.1 ± 0.15	2.0 ± 0.25	1.0 ± 0.12	1.1 ± 0.14
Number of Animals	25	40	8	8	5	5	5	5	5	30	30

± = Standard error for Seven determinations.

TABLE 16

Influence of species differences on the metabolism of Aflatoxin B1 by microsomal-plus - soluble fraction

	Rat	Mice	Guinea Pig	Babbit	White Rock Cock	Duck	Goat	Sheep	Dog (adult)	Lizard	Toad
Formaldehyde produced during incubation (m mole)	25.0 ± 2.6	22.7 ± 3.0	0	15.25 ± 2.2	16.5 ± 3.2	0	2.5 ± 0.5	5.0 ± 1.0	9.96 ± 1.6	0	0
Percentage Aflatoxin B_1 during incubation	87.1 ± 1.0	95.5 ± 0.65	95.5 ± 1.0	97.5 ± 0.75	98.3 ± 1.0	99.0 ± 0.5	97.5 ± 3.0	75.5 ± 3.0	86.0 ± 2.6	99.5 ± 4.5	97.8 ± 1.5
Percentage concentration of Aflatoxin M_1	1.87 ± 0.4	1.00 ± 0.1	0.86 ± 0.05	0.95 ± 0.07	0	0	1.9 ± 0.25	4.1 ± 0.5	2.1 ± 0.2	1.35 ± 0.2	1.49 ± 0.3
Number of Animals	25	40	8	8	5	5	5	5	5	30	30

± = Standard error for seven determinations.

TABLE 17

Influence of species differences on the metabolism of Palmotoxin Bo by Liver slices.

	Rat	Mice	Guinea Pig	Rabbit	White Rock Cock	Duck	Goat	Sheep	Dog (adult	Lizard	Toad
Formaldehyde produced during incubation u mole	11.5 ± 2.0	8.5 ±0.5	3.5 ±0.05	4.8 ±0.1	4.5 ±0.12	0	0	2.6 ±0.04	5.0 ±1.2	3.4 ±0.3	3.2 ±0.1
Percentage palmotoxin Bo metabolized during incubation	45.2 ±1.5	50.3 ±2.5	35.5 ±3.5	52.0 ±2.0	45.0 ±3.0	45.2 ±1.0	56.0 ±2.5	50.5 ±0.5	43.4 ±2.3	45.5 ±3.5	49.5 ±1.0
Percentage concentration of metabolite Bo(m) formed	0.55 ±0.1	0.63 ±0.02	1.0 ±0.12	0.21 ±0.01	0	0	1.1 ±0.12	1.0 ±0.03	0.75 ±0.15	0.2 ±0.05	0.3 ±0.01
No. of Animals	25	40	8	8	5	5	5	5	5	30	30

± = Standard error. for seven determinations.

Bo(m) = metabolite of palmotoxin Bo

TABLE 18

Influence of species differences on the metabolism of Palmotoxin Bo by microsomal-plus-soluble fraction

	Rat	Mice	Guinea Pig	Rabbit	Duck	Goat	Sheep	Dog (adult)	Lizard	White Rock cock	Toad
Formaldehyde produced during incubation m mole	20.00 ±2.1	15.6 ±1.2	0.00	5.10 ±0.15	0	0	3.4 ±0.08	10.4 0.1	0	5.2 ±0.33	0
Percentage Palmotoxin Bo metabolized	47.25 ±2.25	58.6 ±1.6	37.1 ±3.0	60.0 ±5.0	50.4 ±3.5	59.5 ±1.5	67.0 ±5.0	42.9 ±2.1	51.2 ±1.5	50.1 ±2.5	52.0 ±1.1
Percentage concentration of the metabolite Bo(m) formed	0.61 ±0.1	0.7 ±0.15	1.2 ±0.2	0.31 ±0.02	0	1.15 ±0.2	1.10 ±0.2	0.82 ±0.01	0.4 ±0.01	0	0.35 ±0.01
No. of Animals	25	40	8	8	5	5	5	5	30	5	30

± = standard error for Seven determinations

Bo(m) = metabolite of Palmotoxin Bo

Table 19

Influence of species differences on the metabolism of Palmotoxin Go by liver Slices

	Rat	Mice	Guinea Pig	Rabbit	White Rock Cock	Duck	Goat	Sheep	Dog	Lizard	Toad
Formaldehyde produced during incubation (m mole)	7.5 ±0.8	6.5 ±1.2	2.1 ±0.1	3.8 ±0.9	1.9 ±0.3	0	0	2.5 ±0.35	5.2 ±0.5	3.2 ±0.15	3.00 ±0.05
Percentage Palmotoxin Go metabolized	46.5 ±0.6	54.5 ±1.0	28.5 ±0.5	58.5 ±0.5	35.5 ±0.5	38.5 ±1.0	35.3 ±1.3	57.1 ±1.6	49.5 ±0.9	33.4 ±1.5	38.6 ±2.0
Percentage concentration of metabolite Go(m) formed	0.3 ±0.02	0.9 ±0.15	0.23 ±0.01	0.08 ±0.01	0.00	0.00	0.4 ±0.02	1.0 ±0.13	0.38 ±0.05	0.35 0.06	0.30 ±0.04
Number of Animals	25	40	8	8	5	5	5	5	5	30	30

± = standard error for seven determinations.

Go(m) = metabolite of Palmotoxin Go

TABLE 20

Influence of species differences on the metabolism of Palmotoxin Go by microsomal plus-s soluble fractions.

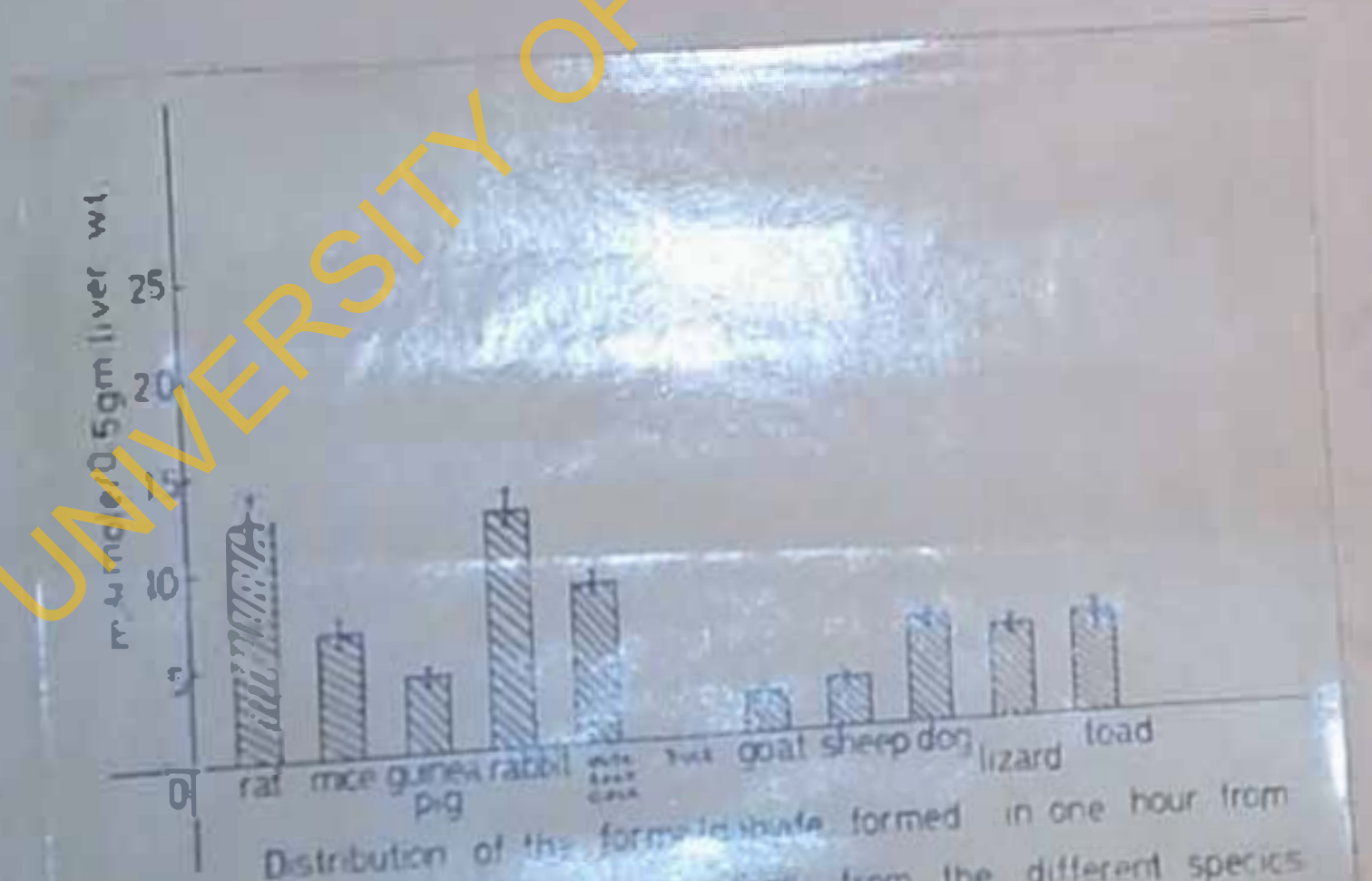
	Rat	Mice	Guinea Pig	Rabbit	White rock cock	Duck	Goat	Sheep	Dog	Lizard	Toad
Formaldehyde formed during incubation m mole	10.5 ±0.9	7.3 ±0.64	0	7.8 ±0.3	4.3 ±0.2	0	0	3.05 ±0.26	9.1 ±0.26	0	0
Percentage Palmotoxin Go metabolized	49.5 ±3.3	57.5 ±1.3	30.0 ±0.4	62.0 ±2.5	39.1 ±2.0	40.0 ±1.5	39.5 ±2.0	65.9 ±2.5	55.0 ±1.5	39.5 ±0.5	62.0 ±2.0
Percentage concentration of the metabolite Go(m) formed	0.38 ±0.05	1.0 ±0.35	0.3 ±0.04	0.1 ±0.01	0.00	0.00	0.5 ±0.05	1.2 ±0.2	0.48 ±0.05	0.42 ±0.01	0.40 ±0.02
Number of Animals	25	40	8	8	5	5	5	5	5	30	30

± = [illegible] of Seven determinations

Go(m) = metabolite of Palmotoxin Go

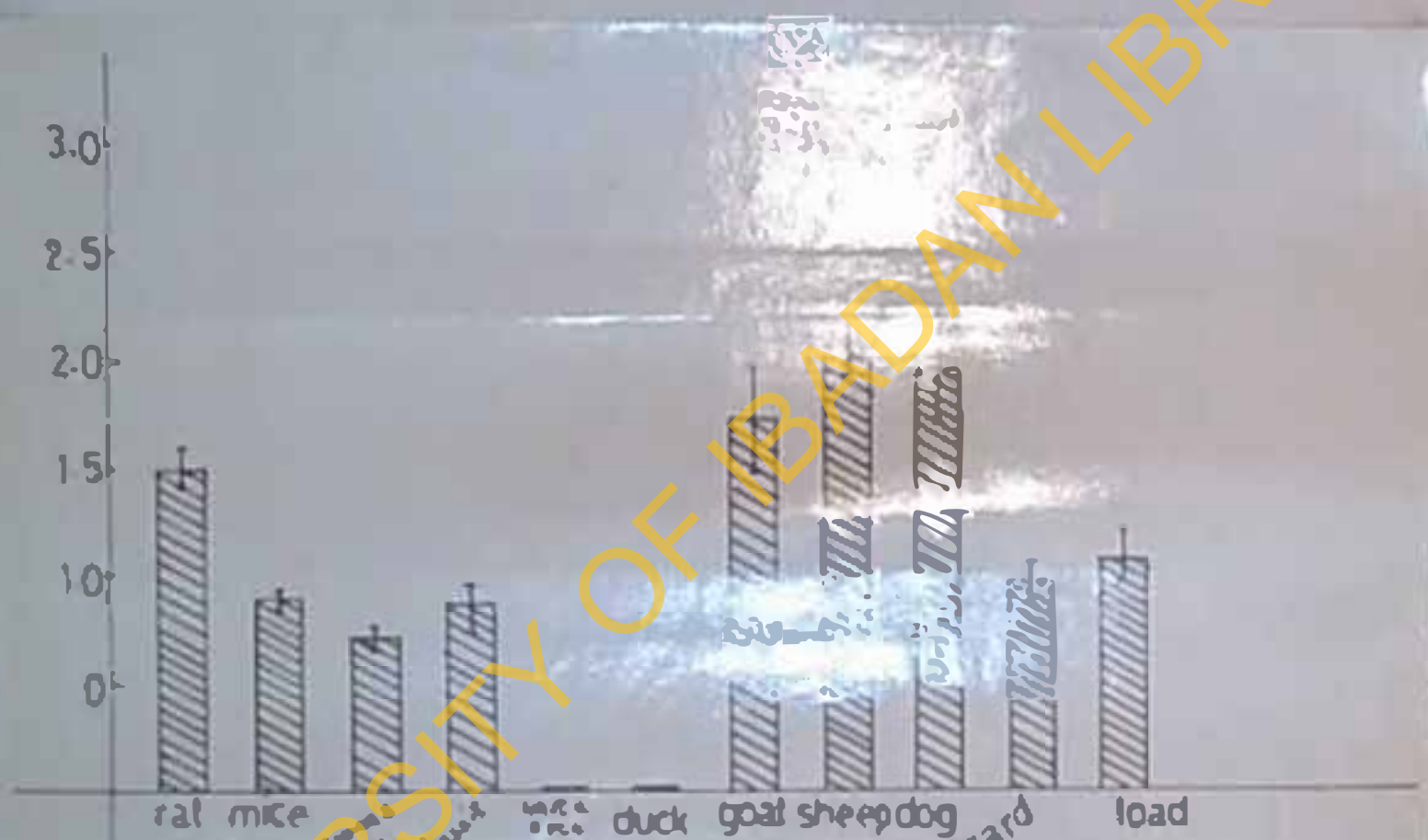
Distribution of total aflatoxin B1 metabolized in one hour by the liver slices from the different species of animals

Fig. 36a



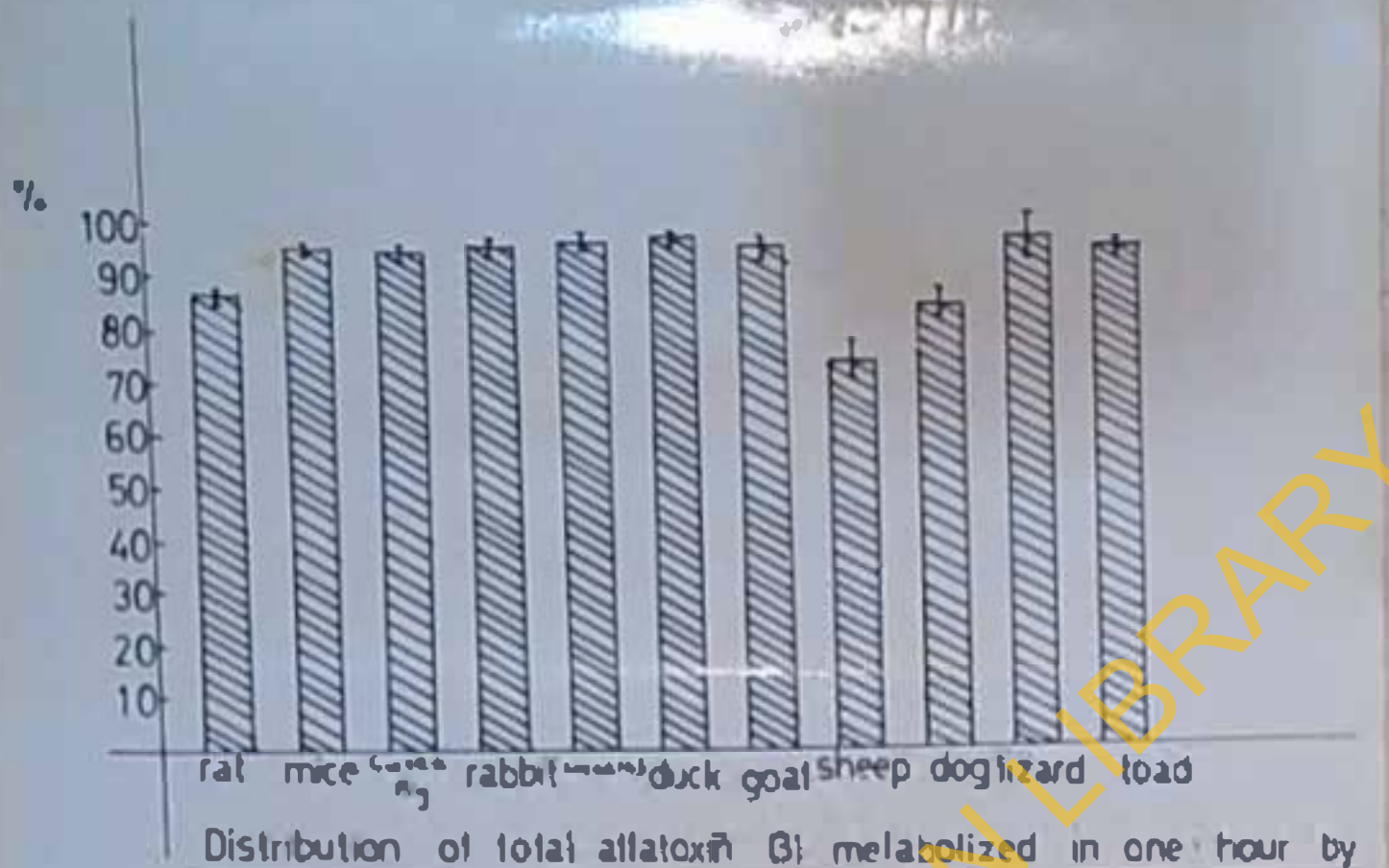
Distribution of the formed in one hour from aflatoxin B1 by slices from the different species of animals.

Fig 36b



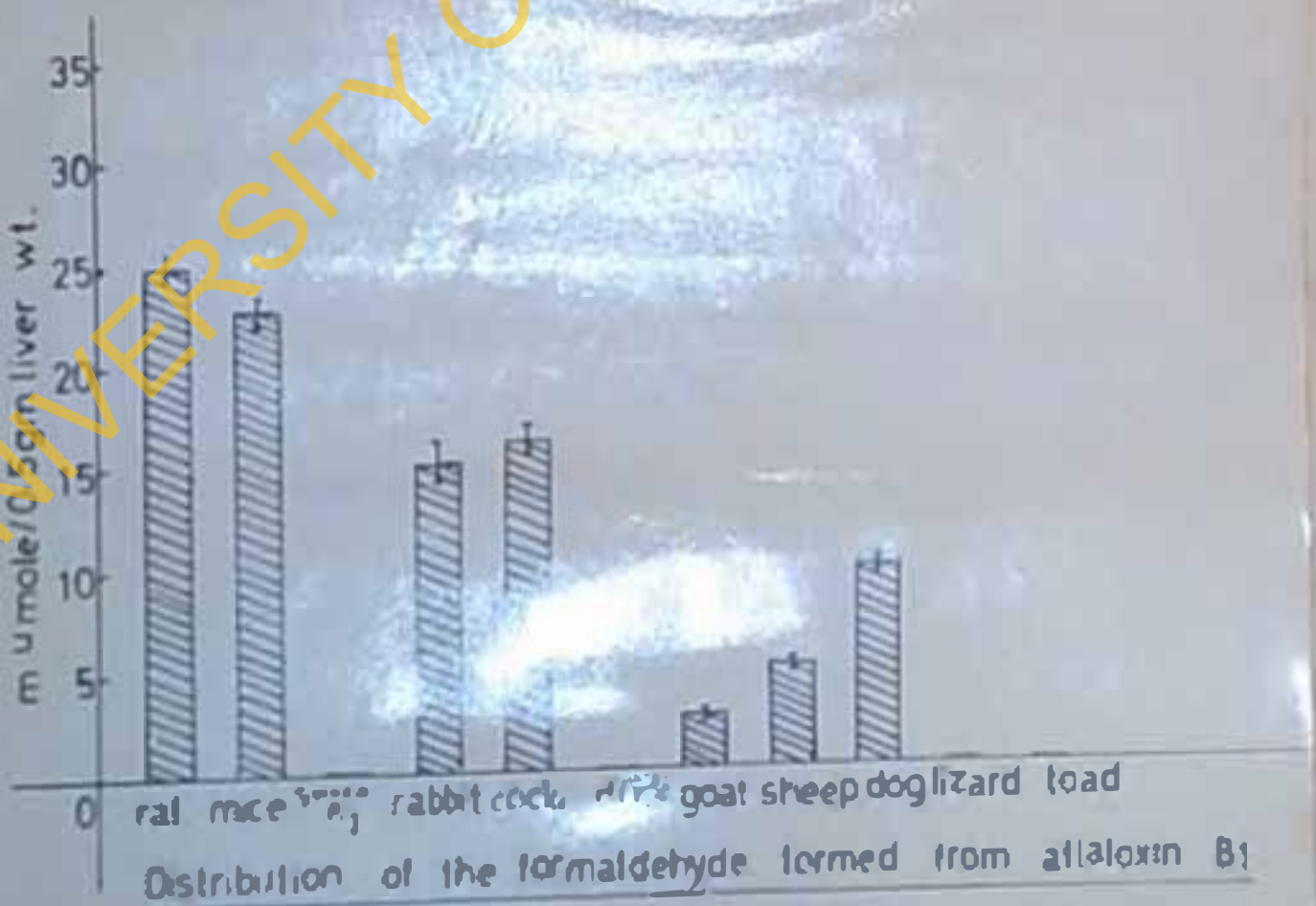
Distribution of the aflatoxin M1 formed in one hour by the liver slices of the different species of animals

Fig 36c



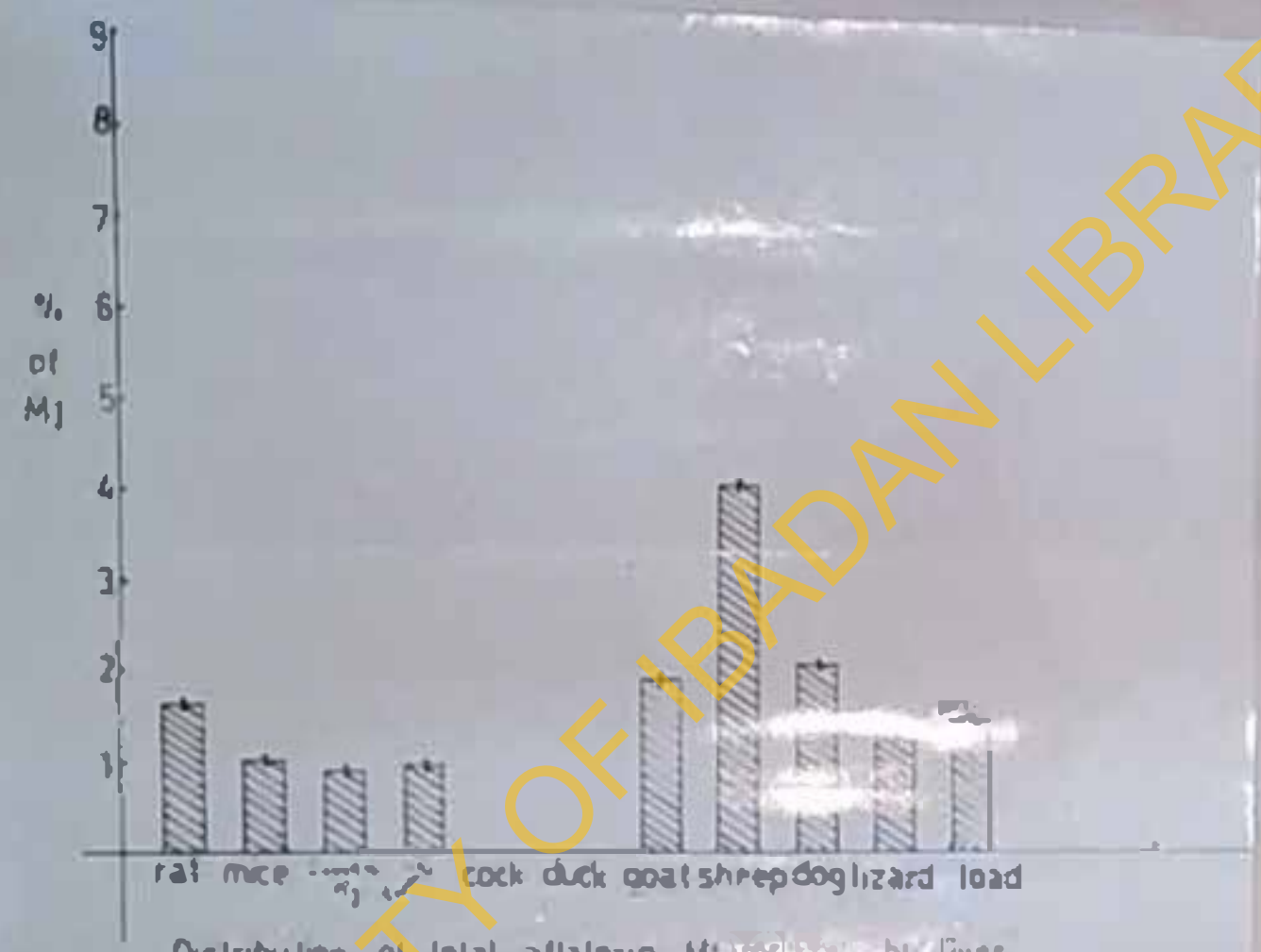
Distribution of total aflatoxin B1 metabolized in one hour by microsomal fractions of the different species of animals

Fig. 37a



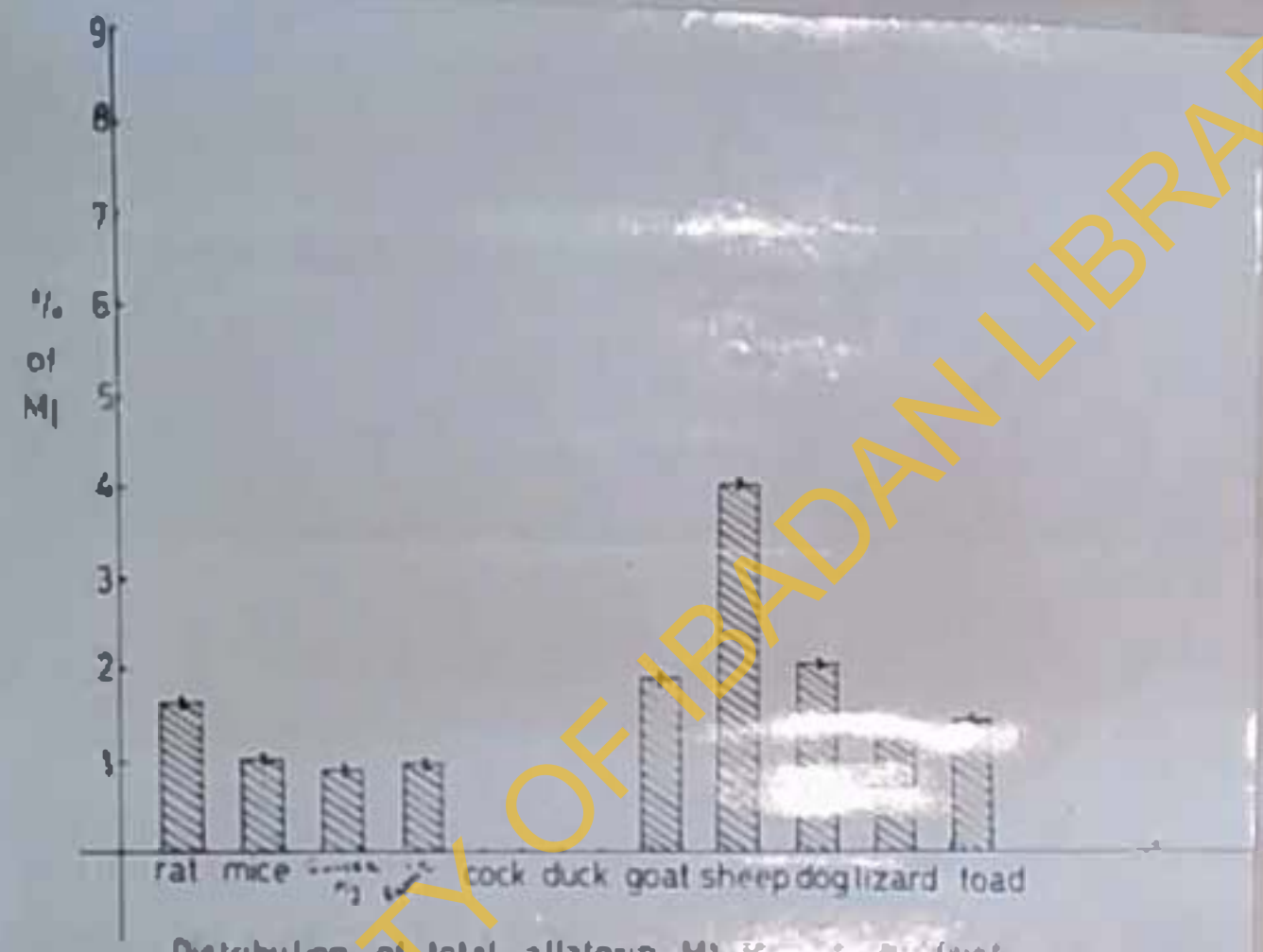
Distribution of the formaldehyde formed from aflatoxin B1 in one hour by the microsomal fractions of the different species of animals.

Fig. 37b



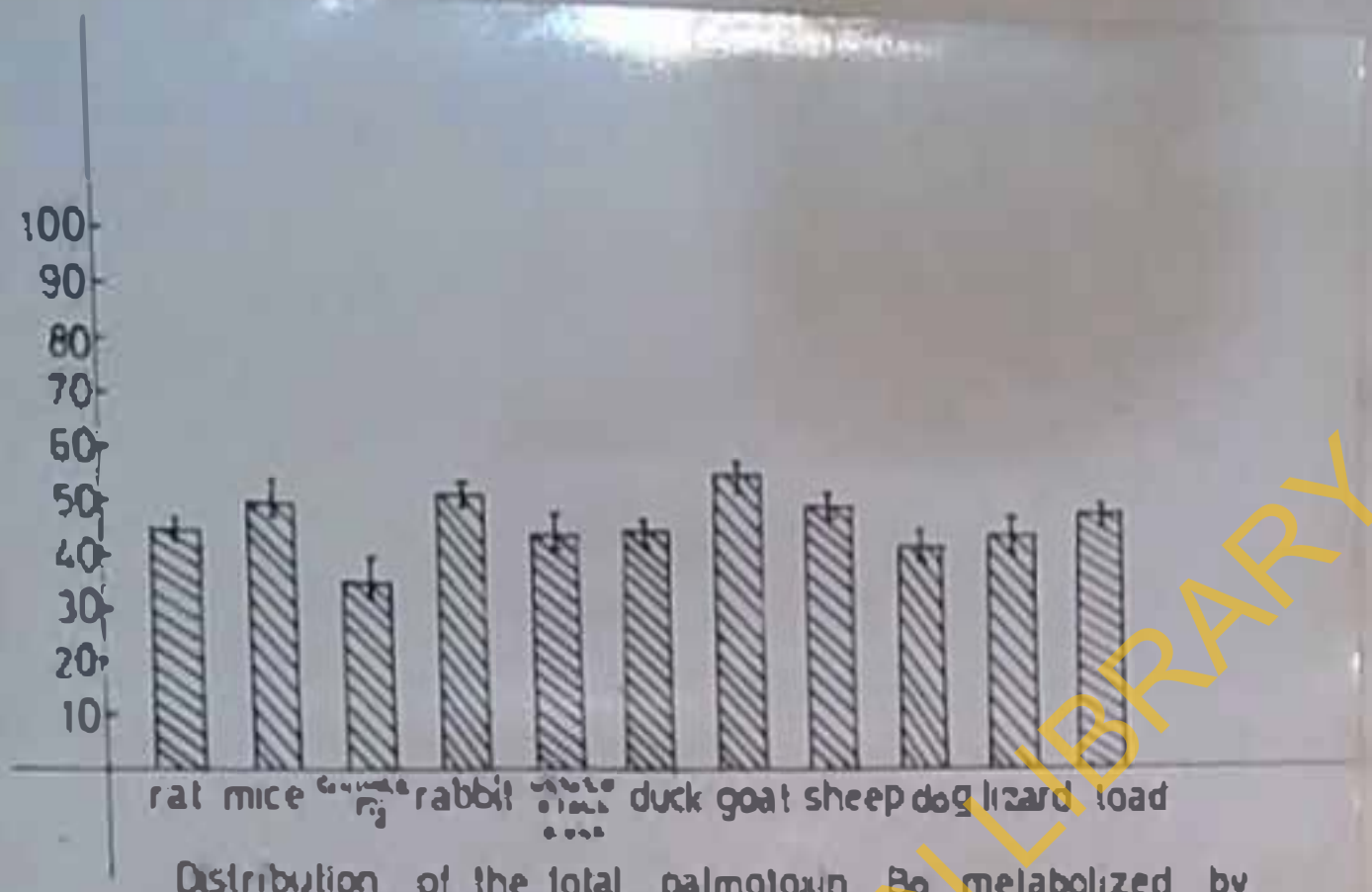
Distribution of total aflatoxin [illegible] by liver microsomal fractions of the different species of animals.

Fig 37c



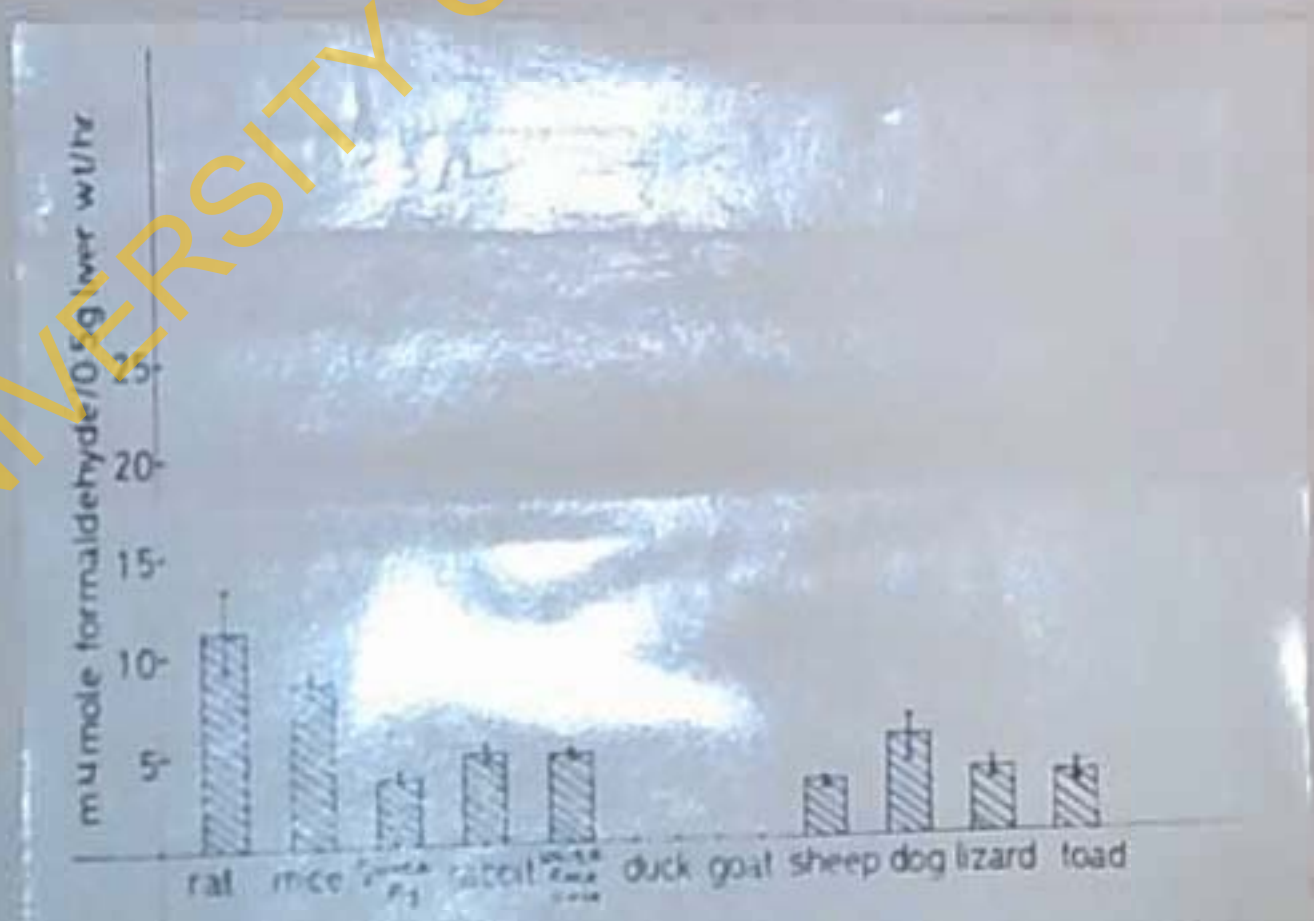
Distribution of total aflatoxin M1 [illegible] level microsomal fractions of the different species of animals

Fig. 37c



Distribution of the total palmotoxin Bo metabolized by the liver slices from the different species of animals.

Fig. 38a



Distribution of the formaldehyde formed from palmotoxin Bo in one hour by the liver slices of the different species of animals.

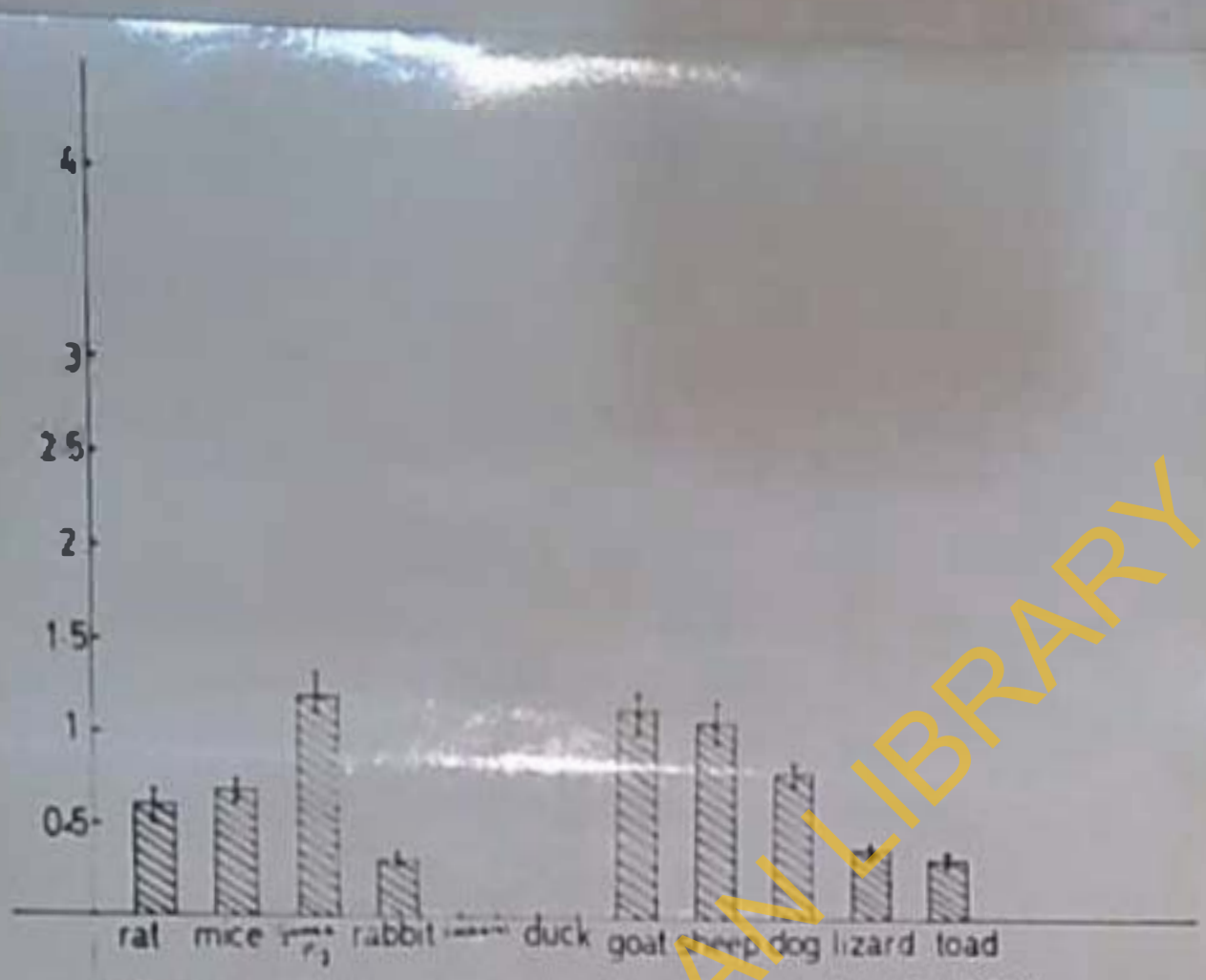
Fig. 38b

157 (W)

Distribution of the total palmotoxin [illegible] fabolized by the liver microsomal fractions of the [illegible] species of animals

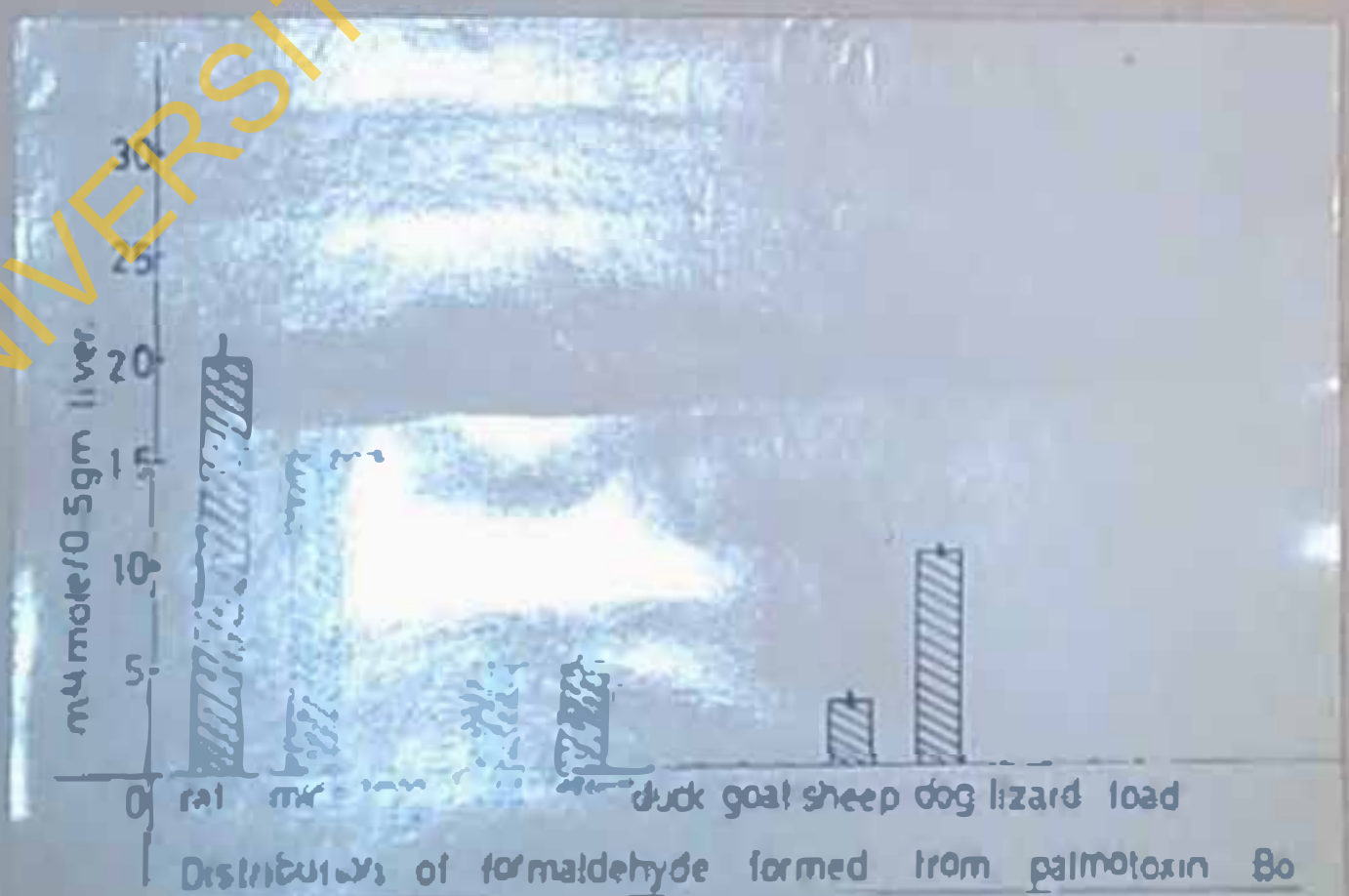
Fig. 39

157 (iii)



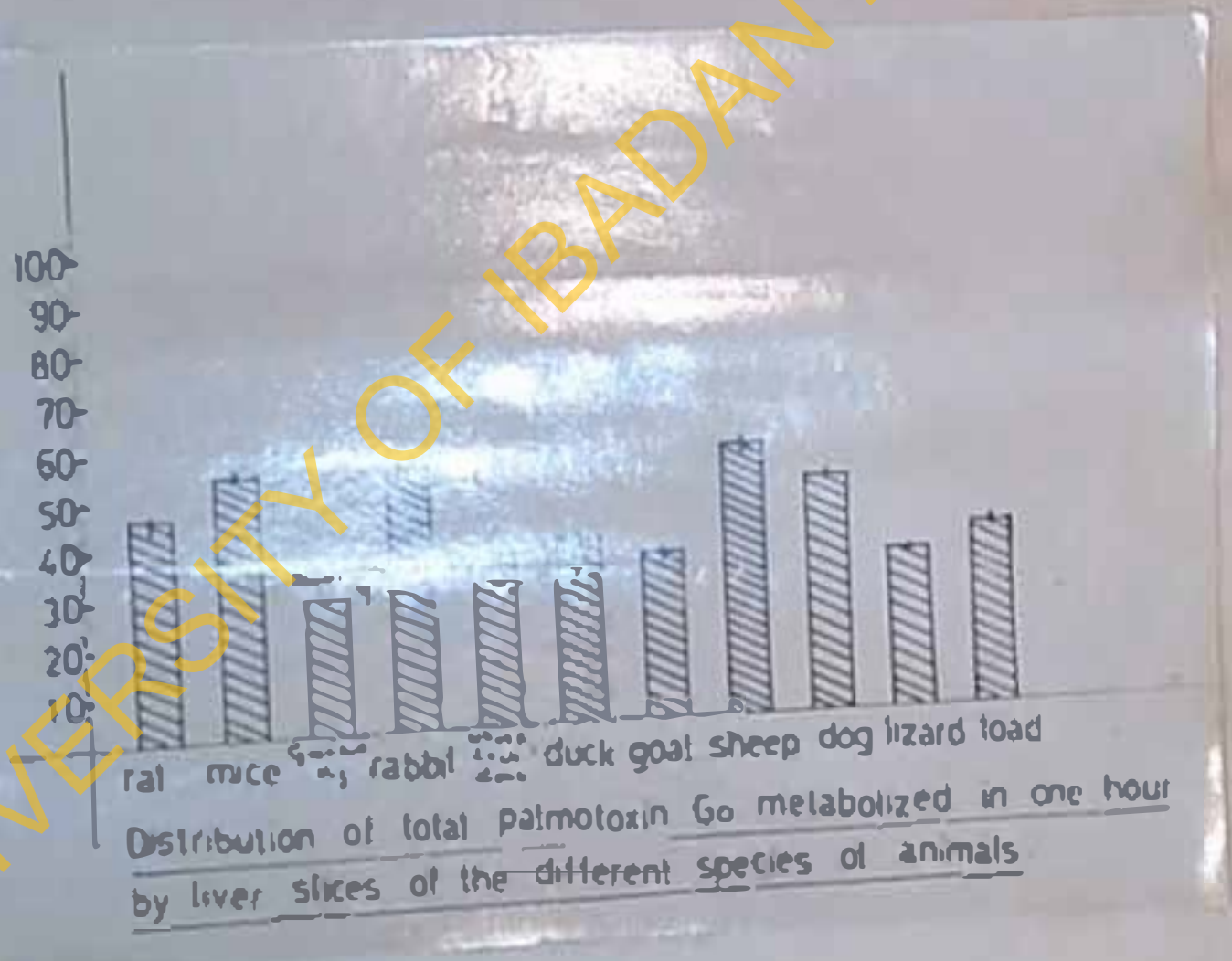
Distribution of metabolite of palmotoxin Bo (Rf 0.22) formed in one hour by liver microsomal fractions of the different species of animals

Fig. 39b



Distribution of formaldehyde formed from palmotoxin Bo in one hour by microsomal fractions of the different species of animals

Fig. 39c



Distribution of total palmotoxin Go metabolized in one hour by liver slices of the different species of animals

Fig. 40 a

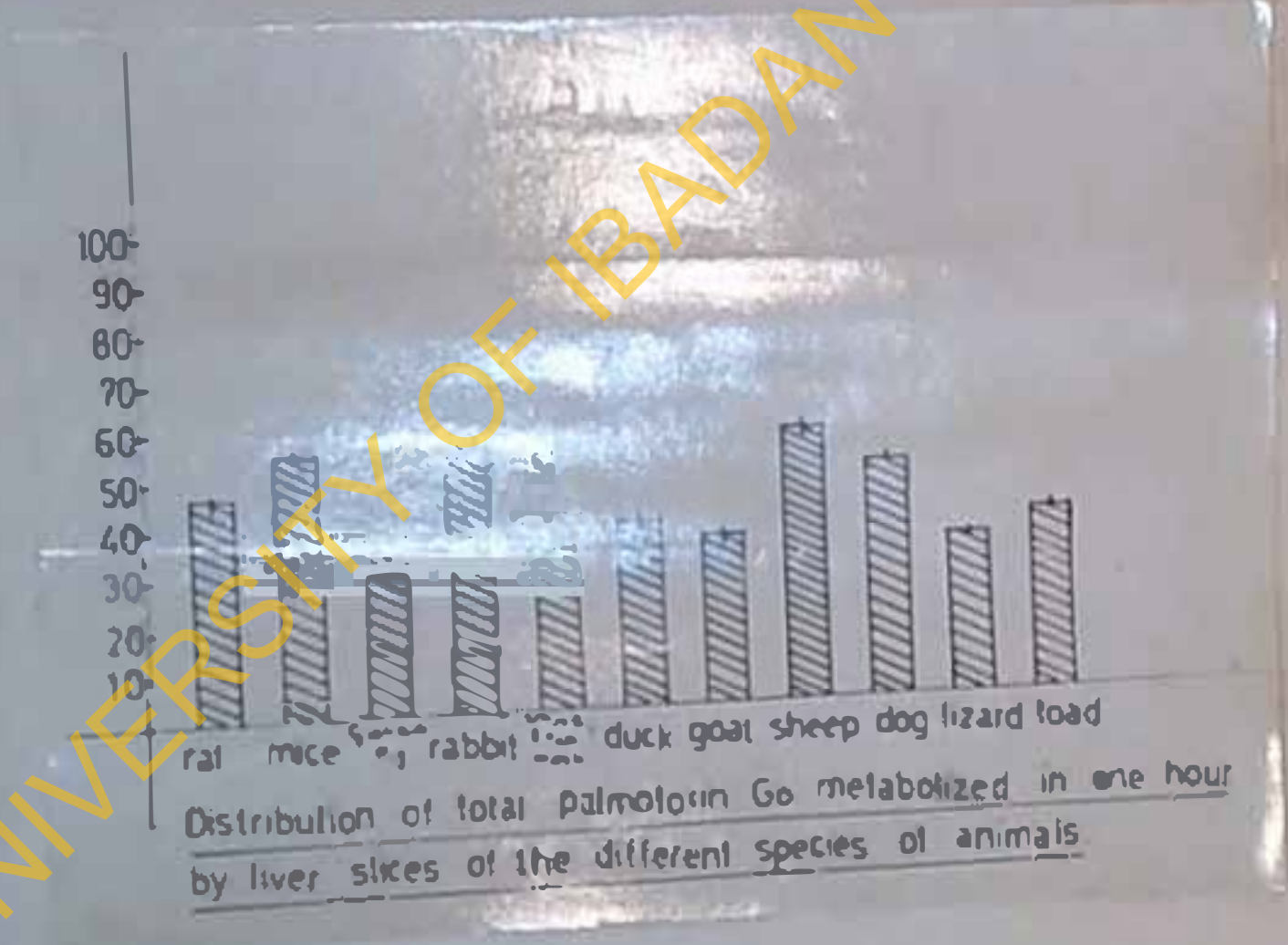
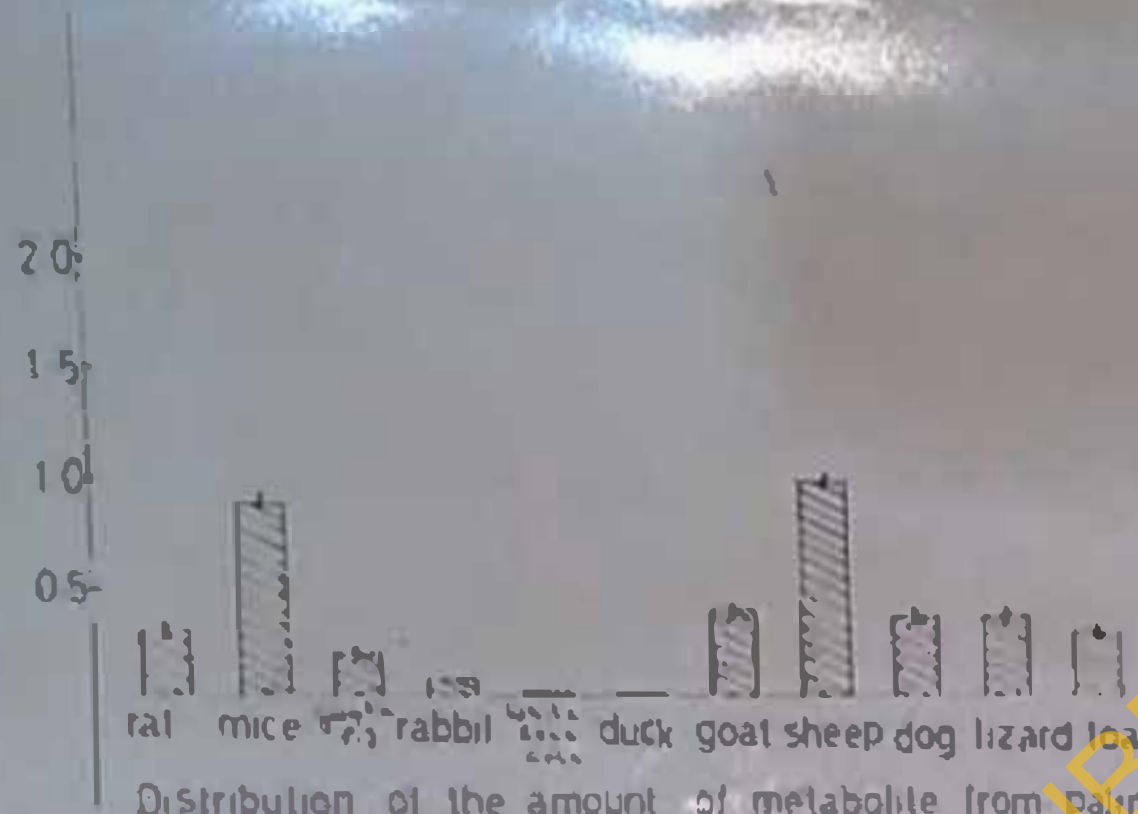
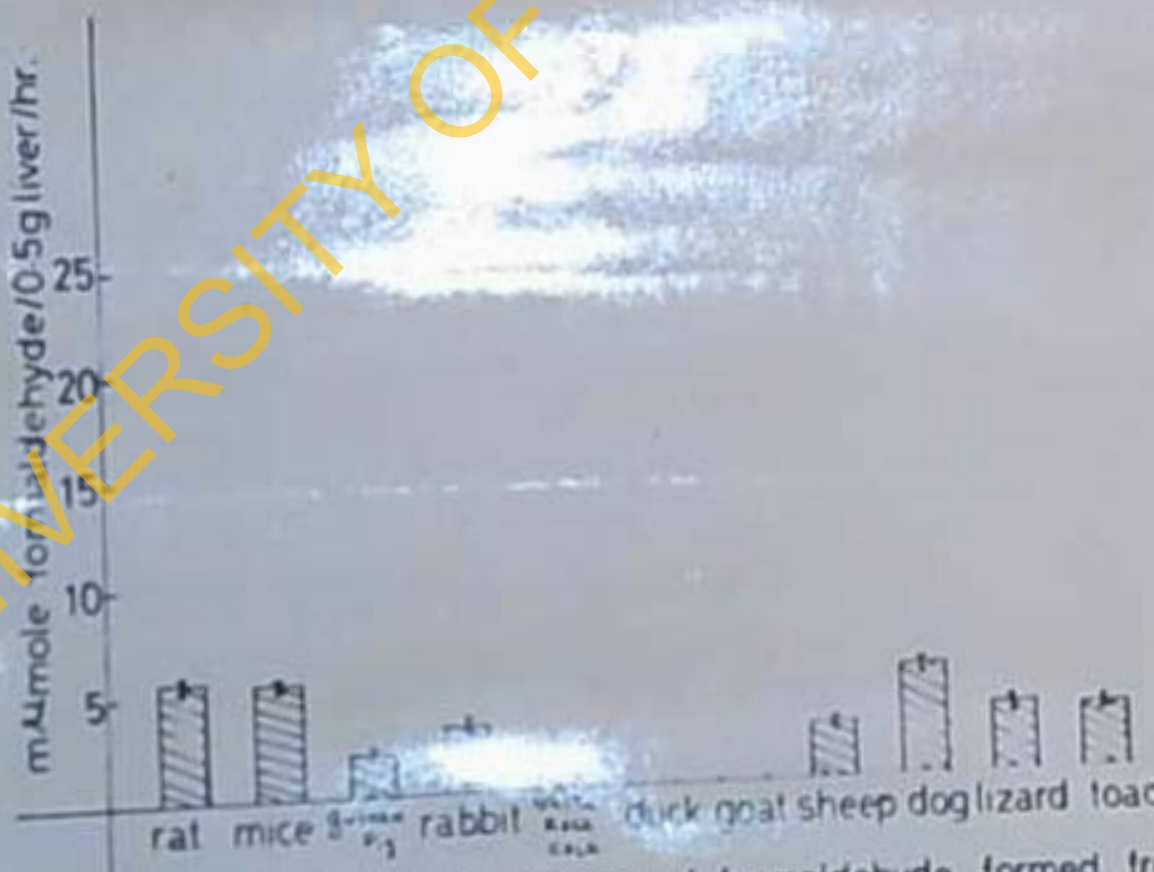


Fig. 40 a



Distribution of the amount of metabolite from palmotoxin Go formed by liver slices of the different species of animals

Fig 40b

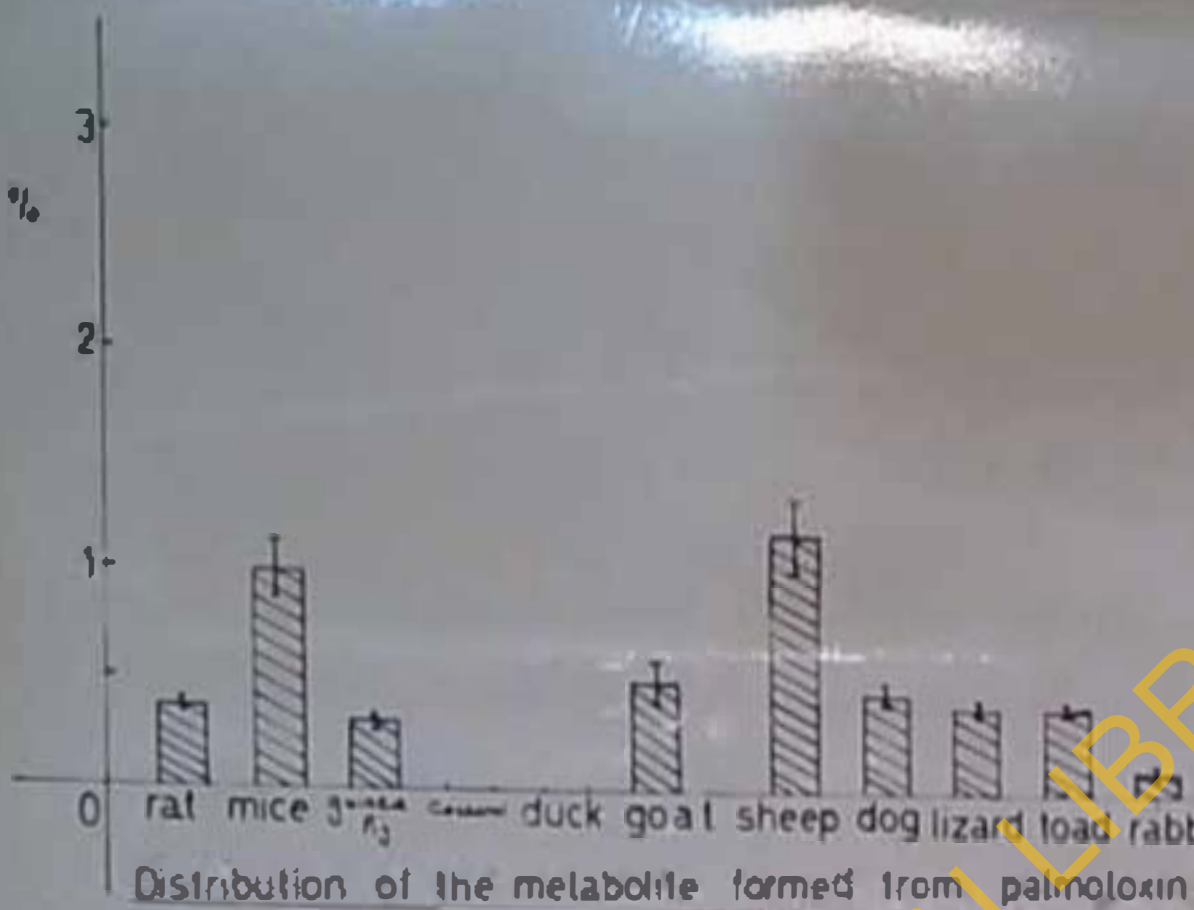


Distribution of the amount of formaldehyde formed from palmotoxin Go in one hour by the liver slices of the different species of animals

Fig. 40c

Distribution of the total palmotoxin [illegible] in one hour by liver microsomal fractions of the [illegible] animals

Fig. 41e.



Distribution of the metabolite formed from palmoloxin Go by the microsomal fraction of the different species of animals

Fig. 41b

Distribution of the metabolite formed from palmoloxin Go by the microsomal fraction of the different species of animals

Fig. 41c

than those for palmotoxin Bo (Figure 42).

Generally, values for incubation with liver slices were lower than the ones in which microsomal-plus-soluble fractions were used. This is believed to be due to the diffusion rate of the substances into the cell.

The formation of hydroxy products from the samples, was observed to show a greater variation than the total toxin metabolized. The white rock, cock and duck did not exhibit any ability to form the hydroxy products both with liver slices and the microsomal-plus-soluble fraction. This result was consistent in all the toxins studied. The sheep and dog were found to be the highest producers of aflatoxin M_1. The mouse also was found to be capable of producing aflatoxin M_1 in addition to some other fluorescent compounds. The rat, goat, lizard and toad were moderate producers while the lowest values were recorded in mouse, guinea pig and rabbit.

With palmotoxin Bo, the sheep, goat and guinea pig were more efficient producers of the suspected hydroxy products. The adult dog was moderate while the rat, mouse, rabbit, lizard, and toad produced very small amounts. Generally, the amount of these fluorescent

1581I

Comparison of total aflatoxin B1 palmolysin Bo and palmolysin Go metabolized in one hour by the different species of animals

Fig 42

products were very small in comparison with the total amount of toxin metabolized.

The mouse and sheep, were more efficient in the production of fluorescent metabolites from palmotoxin Go. The goat, lizard, dog, and toad were moderate while the rabbit, the guinea pig and the rat were poor producers of the metabolite.

Most of the animals appeared to demethylate the toxins. The duck, however, did not demethylate any of the toxins. The guinea pig, lizard and toad microsomal fractions did not demethylate the toxins, though formaldehyde was detectable when liver slices were used. It does appeared that the metabolizing enzymes in these animals may not be very stable or they may exist in so small a quantity as to looo their activity during preparation.

Adult dogs demethylate both aflatoxin B_1 and palmotoxins Bo and Go. In all cases, the rat and mice were found to demethylate the toxins faster than the other animals.

For the same amount of toxin, aflatoxin B_1 demethylase activity was higher than those of palmotoxin

Bo and Go, except in the dog. The goat was not able to demethylate palmotoxins Bo and Go. The demethylase activity for aflatoxin B_1 in the goat was found to be very small.

Conclusion:

1. Species differences in the metabolism of aflatoxin B_1, palmotoxin Bo and palmotoxin Go have been observed.
2. Comparatively, aflatoxin B_1 showed a higher rate of metabolism in most of the species than either palmotoxin Bo or palmotoxin Go.
3. Demethylation and possible hydroxylation of aflatoxin B_1, palmotoxin Bo and palmotoxin Go, appeared fairly well distributed within the species studied, though to a varying degree in each case.

EXPERIMENT B

~~Analysis of the conjugation products of the toxin in the different species:~~

It has earlier been mentioned that during the thin layer chromatography of supernatants from incubated liver slices or microsomal-plus-soluble fractions an intense fluorescence was observed at the point of application of the samples. This metabolite which did not move from the base line during chromatography with methanol-chloroform mixtures was present in all the species and with all the three toxins studied. This metabolite, suspected to be conjugates in view of their polarity (Bucfo, 1976) was examined further in an attempt to identify it.

Experimental Procedure:

The fluorescent spot at the origin of each thin layer chromatogram was scraped off and eluted with methanol: chloroform: water (5:2:2 v/v). The eluate was concentrated with the rotary film evaporator. The concentrates were run on thin layer plates of silica gel G (E. Merck, AG. Darmstadt, Germany) and developed

in n-butanol: glacial acetic acid: water (10:1:1 v/v). The isolates were tested for possible hydrolysis as described in the method, with 0.3N hydrochloric acid. Sulphate, mercapturic acid, amino acid and glucuronide conjugation tests were applied to the samples from each toxin. Glucuronide conjugation was further tested by the modified Fishman and Green method and hydrolysis with β-glucuronidase (E. Merck, Dermotadt, Germany). All these procedures have been described in pages 96-98.

Results:

Two fluorescent spots were identified on thin layer when the isolates from aflatoxin B_1 were run in n-butanol-glacial acetic acid: water (10:1:1 v/v). The first with an Rf. value of 0.023 was present in goat, sheep, rat, mouse and dog; while the second with Rf. of 0.5 was present in rabbit guinea pig, duck, white rock cock, lizard and toad. The later conjugate did not give any positive result for any of the tests applied. It was, however, hydrolysed by 0.3N hydrochloric acid. The conjugate with Rf. of 0.023 did not give any positive results for mercapturic acid; amino acid and

sulphate conjugation. It gave positive results with naphthoresorcinol. On incubation with β-gluouronidase (ketodase) for 48 hours, it yielded a substance which on thin layer had an Rf. value in 3% methanol in chloroform (v/v), similar to the authentic aflatoxin B_1.

For palmotoxins Bo and Go, only single fluorescent spots were observed in each case. The Bo isolate had an Rf. value of 0.42 and that of Go had an Rf. value of 0.32 all in Butanol:glacial acetic acid: water (10:1:1 v/v). These substances were not hydrolyzed by 0.3N hydrochloric acid as fluorescence remained at the base line still, when the mixture was chromatographed in 5% methanol in chloroform (v/v). The fluorescent spots were subjected to the same tests as aflatoxin B_1 and on the basis of their response to the tests, they have been grouped into two: those animals whose isolates gave positive results for glucuronide only were grouped as A, while those animals whose isolates did not respond to any of tests were grouped as B.

Hydrolysis of group A compounds with β-glucuronidase (E. Merck, Darmstadt, Germany) and separation of the

TABLE 21 (a)

Conjugation of Aflatoxin B_1 by Different Species

Species with conjugate Rf 0.023 (A).	Species with conjugate Rf 0.5 (B)
Goat	Rabbit
Sheep	Guinea pig
Rat	Duck
Dog	White rock cockerel
Mouse	Toad
	Lizard

Aflatoxin B_1 - Characterization of Conjugates

Test	Reagent	Conjugate A	Conjugate B
Acid Hydrolysis	0.2N HCl	No hydrolysis	Hydrolyzed Brown colour (-ve)
Glucuronide conjugation	(i) Naphthoresorcinol spray	Violet-blue colour (+ve)	
	(ii) Naphthoresorcinol reaction (Fishman & Green 1955)	Violet colour (+ve)	Pink colour (-ve)
	β- glucuronidase (Kotodase)	hydrolysed (+ve)	Not hydrolysed (-ve)
Amino acid conjugation	Ninhydrin reagent	Pink colour (-ve)	Pink colour (-ve)
Sulphate conjugation	Potassium Hodisonate	Orange-pink (-ve)	Orange-Pink (-ve)
Mercapturic acid conjugation	Potassium dichromate + Silver nitrate	Reddish brown colour (-ve)	Reddish brown colour (-ve)

(-ve) - Negative
(+ve) - Positive

TABLE 21 (b)

Conjugation of Palmotoxin Bo by the different species

Species with conjugate Rf 0.42 (Group A)	Species with conjugates Rf 0.42 (Group B)
Goat	Guinea pig
Sheep	Duck
Rat	White rock cockerel
Mouse	Lizard
Dog	Toad
Rabbit	

Palmotoxin Bo - Characterization of Conjugate

Test	Reagent	Conjugate (Group A)	Conjugate (Group B)
Acid Hydrolysis	0.3N HCl	No hydrolysis	No hydrolysis
Glucuronide conjugation	(1) Naphthoresoreinol spray	Violet-blue colour (+ve)	Brown colour (-ve)
	Naphthoresorcinol reaction (Fishman & Green 1955)	Violet colour (+ve)	Yellowish pink (-ve)
	β - glucuronidase (ketodase)	Hydrolyzed (+ve)	No hydrolysis (-ve)
Amino acid conjugation	Ninhydrin reagent	Pink colour (-ve)	Pink colour (-ve)
Sulphate conjugation	Potassium rhodizonate	Orange-pink colour (-ve)	(-ve) Brown
Mercapturic acid conjugation	Potassium dichromate + Silver Nitrate	Reddish Brown Colour (-ve)	Reddish Brown colour (-ve)

(-ve) - Negative

TABLE 21(c)

Conjugation of Palmotoxin Bo by the different species

Species with conjugate Rf. 0.34 (Group A)	Species with conjugate Rf. 0.34 (Group B)
Goat	Guinea pig
Sheep	Duck
Rat	White rock cockerel
Mouse	Lizard
Dog	Toad
Rabbit	

Palmotoxin Go - Characterization of Conjugate

Test	Reagent	Conjugate (Group A)	Conjugate (Group B)
Acid hydrolysis	0.3N HCl	No hydrolysis	No hydrolysis
Glucuronide Conjugation	(i) Naphthoresorcinol spray	Violet-blue colour (+ve)	Brown colour (-ve)
	Naphthoresorcinol reaction (Fishman & Green 1955)	Violet colour (+ve)	Yellowish pink colour (-ve)
	β - glucuronidase (ketodase)	hydrolysed (+ve)	No hydrolysis (-ve)
Amino acid conjugation	Ninhydrin reagent	Pink colour (-ve)	Pink colour (-ve)
Sulphate Conjugation	Potassium rhodizonate	Orange-pink colour (-ve)	Orange-pink colour (-ve)
Mercapturic acid conjugation	Potassium dichromate + Silver nitrate.	Reddish Brown colour (-ve)	Reddish Brown colour (-ve)

(-ve) - Negative result
(+ve) - Positive

mixture by thin layer chromatography gave fluorescent spots with the same Rf. values, as the original palmotoxins Bo and Go. This appears to be in accordance with the suggestion of the presence of a hydroxyl group in palmotoxins Bo and Go as this could readily be a conjugation point. The results have been tabulated and presented in Tables 21a, 21b, and 21c.

Conclusion:

1. Both liver slices and microsomal fractions of all the species form conjugation products with aflatoxin B_1, palmotoxin Bo and palmotoxin Go, though only a few of these could be identified.

2. Glucuronide conjugation was confirmed in a number of species both for aflatoxin B_1, palmotoxin Bo and palmotoxin Go.

INVESTIGATION 7

Influence of phenobarbitone treatment and carbon monoxide aeration on the demethylation and hydroxylation of palmotoxin Bo, palmotoxin Go and aflatoxin B_1 by rat liver microsomal-plus-soluble fractions of rat:

Several polycyclic hydrocarbons, notably amino pyrine, hexobarbitone, phenobarbitone and chlopromazine, have been known to enhance the activity of the drug metabolizing enzymes. The phenomenon has been widely reported (Conney, Miller and Miller, 1956; Gillette, 1962, and Orrenius, 1965).

The mechanism of the induction is believed to involve an increase in the cytochrome P450 content in the haem as well as the protein moiety and also results in the increased synthesis of several enzyme protein (Ernster and Orrenius, 1965, Kato, et al. 1966, and Remmer. 1969). The metabolism of drugs and foreign compounds, involve the carbon monoxide sensitive cytochrome P450 generally. However, some studies have revealed also that cytochrome P450 is not required for

the metabolism of all substances (Harnandez, Mazel and Gillette, 1966; Gillette, 1966). It has been shown that reactions like microsomal sulphoxidation of diaminodiphenyl sulphide, N-hydroxylation of aniline and N-ethyl aniline and metabolism of azo-dyes could proceed in other ways other than through the cytochrome P450 pathway. Carbon monoxide aeration, therefore, does not inhibit such reactions and increase in cytochrome P450 levels arising from the administration of inducers, will not lead to increased microsomal activities for such substrates.

The present experiment was, therefore, designed to test

(a) the effect of phenobarbitone on the microsomal enzymes responsible for the '*in vitro*' metabolism of aflatoxin B_1, and the palmotoxins Ba and Oa, and (b) the effect of carbon monoxide on the '*in vitro*' metabolism of these compounds.

Experimental Procedure:

Litter mates of rats (wistar strain 100 - 105g-wt.) were weighed and arranged in two groups, in such a way that their average weights were equal. The first

group was injected with a dose of phenobarbitone sodium salt equivalent to 75mg/kg body weight in a minimal volume of normal saline. The second group, which served as controls received equivalent doses of the normal saline only. All injections were given intraperitoneally and daily for 5 days. An hour after the last injection, the animals were sacrificed, their livers excised, weighed and pooled together in their respective groups.

Microsomal-plus-soluble fractions were obtained as before for each group.

50 mμ mole of each toxin was incubated with 2ml of the liver fraction and 50 μ mole magnesium chloride, 50 μ mole nicotinamide, 50 μ mole glucose-6-phosphate, 0.52 μ mole NADP and 25 μ mole semi-carbazide hydrochloride (pH 7.6) in a total volume of 5ml and in 50ml Erlenmeyer flasks.

For each toxin, the flasks were distributed into three groups of four.. Two flasks in each group were used as controls - one containing liver extracts but no toxin and the other containing deactivated liver extracts and the toxin in addition to the co-factors.

The first group of flasks contained liver extracts

from rats pre-treated with phenobarbitone and incubated in air.

The second group, contained liver extracts from pre-treated rats and incubated in an atmosphere of carbon-monoxide (Christensen and Wiseing, 1972).

The last group contained liver extracts from the control rats, and these were incubated in air.

Incubations were for one hour at 37.0 ± 0.5°C. Supernatants were obtained at the end of incubation by precipitating the proteins and centrifuging the extracts. The metabolites, aflatoxin M_1, Bo(m) or Go(m) in the reaction mixtures were estimated. Demethylase activity was also determined by the amount of formaldehyde produced during the reaction.

Results:

The results are given in Table 22. Aflatoxin M_1 formed in phenobarbitone treated rats was about twice the amount in the control. The formaldehyde was about 1.5 times greater than the control.

The de-methylation in palmotoxins Bo and Go was about twice the amount in the control, while the

TABLE 22(a)

Metabolism of aflatoxin B_1, Palmotoxins Bo and Go by Microsomal-plus-soluble fractions from rats pretreated with phenobarbitone

	Percentage of Fluorescent Metabolites (M_1, Bo(m), Go(m) formed		
Toxin	Incubation with rats pretreated with phenobarbitone.	Liver fraction from pretreated rats under carbon monoxide	Normal rat liver fractions under air
M_1	4.1 ± 0.2	0.00	1.86 ± 0.1
Bo(m)	1.95 ± 0.3	0.00	0.60 ± 0.05
Go(m)	1.1 ± 0.06	0.00	0.4 ± 0.02

M_1 = Aflatoxin M_1, Go(m) = metabolite of Palmotoxin Go
Bo(m) = metabolite of Palmotoxin Bo

TABLE 22(b)

Quantity of Formaldehyde formed (n mole)

Toxin	Liver fractions from pretreated rats incubated in air	Liver fractions from pretreated rats incubated in Carbonmonoxide	Liver fractions from normal rats incubated in air
B_1	38.4 ± 2.0	0.00	24.5 ± 1.5
Bo	40.8 ± 3.0	0.00	19.91 ± 1.0
Go	21.1 ± 0.9	0.00	9.81 ± 0.5

± = standard error for 5 determinations

hydroxylation products were about three times the value obtained for the control. There was neither demethylation nor hydroxylation in the carbon monoxide aerated flasks.

Conclusion:

1. Phenobarbitone treatment of rats enhanced the hydroxylation and demethylation of aflatoxin B_1, palmotoxins Bo and Go.

2. Carbon-monoxide inhibited the hydroxylation and demethylation of palmoxin Bo, palmotoxin Go and aflatoxin B_1.

CHAPTER V

DISCUSSION

Production of toxins in palm oil and yeast extract sucrose media:

Yeast extract sucrose medium has been found to support better growth of *Aspergillus flavus* and more toxin production than the palm cop medium (Figs 12, 13 and 14). A similar trend has been observed by Emafo (1970) using the yeast-extract-sucrose medium, the Czapek dox medium and the aerobic sucrose medium. There seems to be, therefore, a relationship between the growth of the fungus and the attendant toxin production in view of the fact that the media that supported better growth of fungus, also gave better yield of toxins. However, since the production of toxin is dependent, to a large measure, on the carbon and nitrogen sources, (de Iongh, Vles and de Vogel, 1965; Matolee and Adye, 1965, Davis, Diener and Eldridge, 1966), the disparity in the case of the palm cop, might be due to the differences in sucrose or

carbohydrate content of the media. Bassir (1962), Paparusi (1966) and Adekunle (1969) have shown that the palm cap contains only 2% sucrose.

The toxin concentration of 3mg/100ml of medium obtained with the yeast extract sucrose medium is close to the value obtained by Uwaifo (1971) but differs from the value reported by Enaifo (1970). However, Armbrecht, et al.(1963); de Vogel, et al. (1965) and Kulik and Holaday (1967) have shown that repeated transfers of A. flavus strains on nutrient media usually led to either a decrease in or a total loss of aflatoxin producing ability. This might explain the disparity in yields obtained from the same strain of fungus on the same medium.

The pH of the medium was lowest at the period of maximum toxin production in both the palm cap and the yeast extract sucrose media (Fig 13). This has also been observed by Davis, Diener and Eldridge (1966) and Enaifo (1970) using only the yeast-extract sucrose medium. The decrease in pH might be due to some acids released during the secondary metabolism of the fungi (Buntley and Campbell, 1968).

Physical characteristics of palmotoxins Bo and Go:

Table 6 summarises most of the spectral data obtained in this work. Reports from literature have been included for comparison.

The Ultraviolet spectra of palmotoxins Bo and Go (Figures 6 and 7) show a strong absorption near 200nm and a weaker one at a higher wavelength. This end absorption could be due to $n - \sigma^*$ transition arising from the presence of oxygen in the molecule since elemental analysis has shown that the compounds contain hydrogen, carbon and oxygen only (Assoir and Adekunle, 1968). Dyer (1965) and Dyke, et al. (1971) have attributed this type of absorption to a carbonyl chromophore. This is also supported by the spectral shift observed in water and methanol (Figs 15 and 16). Dyer, (1965) has shown that polar solvents generally shift the $n - \sigma^*$ bands to shorter wave lengths and that absorptions involving non-bonding electrons of the $n - \sigma^*$ or $n - \pi^*$ type are sensitive to the polarity of the solvent in which the spectrum is determined. This bathochromic shift observed in the aqueous solutions relative to the peak in methanol

is also shown to characterize $\pi - \pi^*$ transitions found in unsaturated chromophores. The existence of a similar spectral pattern in aflatoxins B_1 and G_1 and the consistence with which the absorption peak around 265nm appears in all the compounds, seem to give support to this speculation and point to the possible existence of a common chromophore responsible for this absorption in all the compounds.

The infra-red absorption peak of palmotoxins Bo and Go (Figs 17 and 18) around 1750 cm^{-1} confirm the presence of a carbonyl group. This absorption peak in addition to weaker ones around 1630 cm^{-1}, 1595 cm^{-1} and 1545 cm^{-1} which are also present in the aflatoxins (Table 6) have been attributed to the stretching vibration system of coumarin moiety and ketone carbonyl groups arising from an $\alpha-\beta$ unsaturated lactone function, (Asao, et al. 1963 and 1965; Hartley, Nesbitt and O'Kelly, 1963). The absence of peaks around 3.7 - 4.0 μ (2700 - 2500 cm^{-1}) due to bonded O - H stretching of a carboxylic acid is suggestive of the absence of a carboxylic acid in the molecule. Dyer (1965) has shown that this is a diagnostic feature of the carboxylic acid

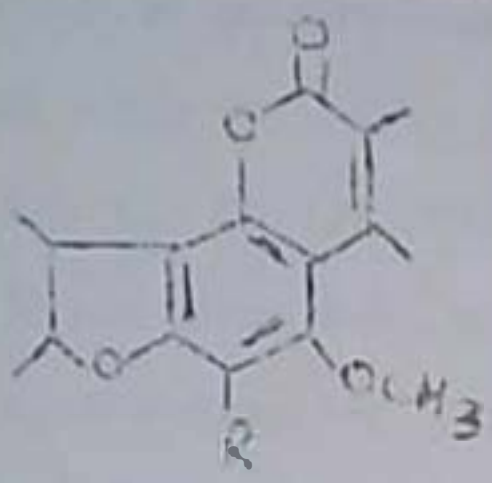
functional group.

The peaks at 3400 cm^{-1} in palmotoxin Bo and 3450 cm^{-1} in palmotoxin Go confirm the presence of free hydroxyl groups and this is in accordance with the increased polarity of the substances as observed on thin layer plates.

Not much was revealed in the nuclear magnetic resonance due probably to the small quantity of the substance available and poor resolution of the peaks. However, chemical shifts deciphered from palmotoxin Bo are at d = 1.25; 2.05; 2.6; 3.7 and 4.5; palmotoxin Go gave peaks at d= 1.25; 2.03; 2.6; 3.65 and 3.85. The peaks at d = 3.7 and 4.5 for palmotoxin Bo and d = 3.65 and 3.85 for Go are not distinct but there is a definite indication to the presence of these peaks. Aflatoxin B_1, however, gave peaks at d = 1.25, 2.61, 3.4 and 6.5 (Umaifo, 1971).

The prominence of the peaks at 1.25 in palmotoxins B_o and Go may indicate the presence of an alkyl chain grouping. There is, however, a resemblance of the chemical shifts here with those of aflatoxin B_1. The absence of the aflatoxin B_1 aromatic chemical shift at 6.5 in palmotoxins Bo and Go might be due to the replacement

of the single free aromatic hydrogen by another group thus;



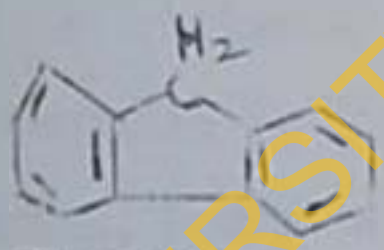
R - Substituent group.

Moreover, fluorescence in the aflatoxins has been attributed to the presence of the coumarin ring system.

The similarity in the fluorescence spectra of the palmotoxins with the aflatoxins (Table 6) indicates a probable presence of this system in the palmotoxins also. Though there is no straight forward correlation between structure and fluorescence (Udenfriend, 1962), the similarity in this case does not appear coincidental.

The iodine values (Table 7) obtained in this study indicate the possible presence of unsaturated groupings already noted in the Ultra-violet spectra of the compounds. The values are a little higher than those of the four aflatoxins cited in Table 7. This might indicate that the compounds are more unsaturated than the aflatoxins.

Nevertheless, the values appear too low to arise from a straight chain unsaturated fatty acid. This view is in agreement with the disparity in iodine values obtained by McClean and Thomas (1921), between some cyclic compounds and some straight chain compounds too. For instance, fluorene and phenanthrene have iodine values of 5.7 - 6.2 and 10.8 - 11.5, respectively, as compared with the values the authors obtained for crotonaldehyde and citral - 334.2 and 268. - 281.0 respectively.



Fluorene

$CH_3.CH = CH.CHO$

Crotonaldehyde

The apparent insolubility of the palmotoxins in diethyl ether does not appear to be in keeping with a long chain fatty acid property.

There appears to be a striking similarity between the aflatoxins and the palmotoxins. It is possible that the palmotoxins are a modified form of the aflatoxin group of compounds, though the extent of the modification

cannot be specified.

Toxicity of palmotoxins Bo and Go to 20-day old rats:

Palmotoxin Bo has been found to induce losses in body weight and a reduction in liver size in relation to the body weight (Table 8). Chang, et al. (1963) has demonstrated also that aflatoxin B_2 induced such abnormalities in the white perking-lucklings. Palmotoxin Go did not exhibit similar potency at the dose levels studied. Increase in the levels of serum-glutamic oxaloacetic acid transaminase and alkaline phosphatase was also observed in palmotoxin Bo treated rats (Table 10). Alterations in the activities of these enzymes are known to indicate onset of necrosis of hepatic cells and myocardial infarction (Bessey, Lowry and Brock (1946), Lalue, Wroblewski and Karmen (1954) and Reitman and Frankel (1957). It does appear, therefore, that palmotoxin Bo might be carcinogenic to the rat in a similar manner as aflatoxin B_1. Rao and Oberling (1971) have observed similar increases in glutamic oxalo acetic transaminase; glutamic-pyruvic transaminase, alkaline phosphatase and isocitric dehydrogenase in the serum of aflatoxin B_1 poisoned monkeys. It is pertinent to note that enzyme activity

values obtained with 50 µg of palmotoxin Bo were in the same range as with 15 µg of aflatoxin B_1. This probably indicates a difference in toxicity with reference to the rat and does not seem to be in line with earlier findings of Bassir and Alokunle (1969). In this work, it has been established that even at a dosage of 6.6 mg/kg body weight, no deaths were recorded. Barnes (1967) has given the LD_{50} of aflatoxin B_1 in a 21-day old male rat as 5.5 mg/kg body weight. It does appear, therefore, that the LD_{50} of palmotoxins Bo and Go would be higher than 6.6 mg/kg body weight. Hence the toxin may not be of comparable toxicity as aflatoxin B_1 in the rat.

Palmotoxin Go did not appear toxic to the 20-day old rat. Serum glutamic oxaloacetic acid transaminase and alkaline phosphatase levels were basically unaltered. This finding is in keeping with earlier reports of Bassir and Alokunle (1969 and 1970b). It may be that the hydroxylation of this compound reduces its toxicity. Dutton and Heathcote (1966) have found that some hydroxylated metabolites of *Aspergillus flavus*, notably, aflatoxins B_{2a} and G_{2a} exhibited little or no toxic properties.

Histological examination of the liver cells did not reveal any morphological changes between normal and the palmotoxins Bo and Go treated rats. There was a normal lobular pattern except for occasional ill-defined cells which showed signs of peripheral necrosis. The aflatoxin B_1 treated cells showed a greater sign of damage than the other liver cells. Butler (1964); Barnes and Butler (1964) have demonstrated that the onset of carcinogenesis in the rat arising from aflatoxin poisoning is slow. It appears, therefore, that despite the increase in the enzyme levels characteristics of liver and heart diseases, a time lag is required before the effects are manifest on the general morphology of the cells.

Metabolism of aflatoxins B_1 and G_1 by the rat liver microsomal plus soluble fraction:

The results obtained in this work indicate that the differences in the disappearance of aflatoxins B_1 and G_1 from the incubation media, did not appear significant. Nevertheless, a higher value was obtained for aflatoxin G_1 than aflatoxin B_1 (Table 12). The similarity in values might be due to equal uptake of the toxins by the

liver tissues (Portman, Plowman and Campbell, 1968). However, the hydroxylation and demothylation of the toxins differ substantially. Aflatoxin B_1 was more readily hydroxylated and demothylated than aflatoxin G_1. Allcroft, et al. (1966), has also observed a similar pattern 'in vivo' in the sheep. Since the LD_{50}s of aflatoxins B_1 and M_1, have been found to be in the same order - 12 µg and 16.6 µg respectively, for day-old ducklings (Holzapfel, Steyn and Purchase, 1966), it would appear that a higher rate of metabolism, may not lead to greater loss in activity. The metabolites, therefore, appear to be implicated in the general potency of the compounds. This view has been shared also by Portman, Plowman and Campbell (1968); Sohnbort and Storm (1969), and Patterson (1970).

Besides, Schoental (1970) had proposed the possibility of an 'activated species' - the epoxides of aflatoxins, main the proximal carcinogens. Thus it would appear that the accumulation of the hydroxy products might depend on the rate of formation of the epoxides, assuming that the epoxides are intermediates in the formation of

the hydroxy aflatoxins in the media. Daudel and Daudel (1966) have also proposed the epoxides pathway for the formation of hydroxy derivatives of most carcinogenic hydrocarbons.

If the epoxides were intermediates in the formation of the hydroxy derivatives of aflatoxins B_1 and G_1, and Schoental's theory for aflatoxins B_1 and G_1 carcinogenesis holds, the values of aflatoxins M_1 and 'GM' would be a measure of the epoxides formed at a particular time and thus could form a clue to their differences in carcinogenic effects.

Finally, the substitution of the terminal lactone ring in aflatoxin G_1 for the pentanone ring in aflatoxin B_1, appears to have had a remarkable effect on the enzymic reactivity of the toxins. This feature has also been regarded as a contributory factor to the difference in toxicity (Carnaghan, Hartley and O'Kelly (1963).

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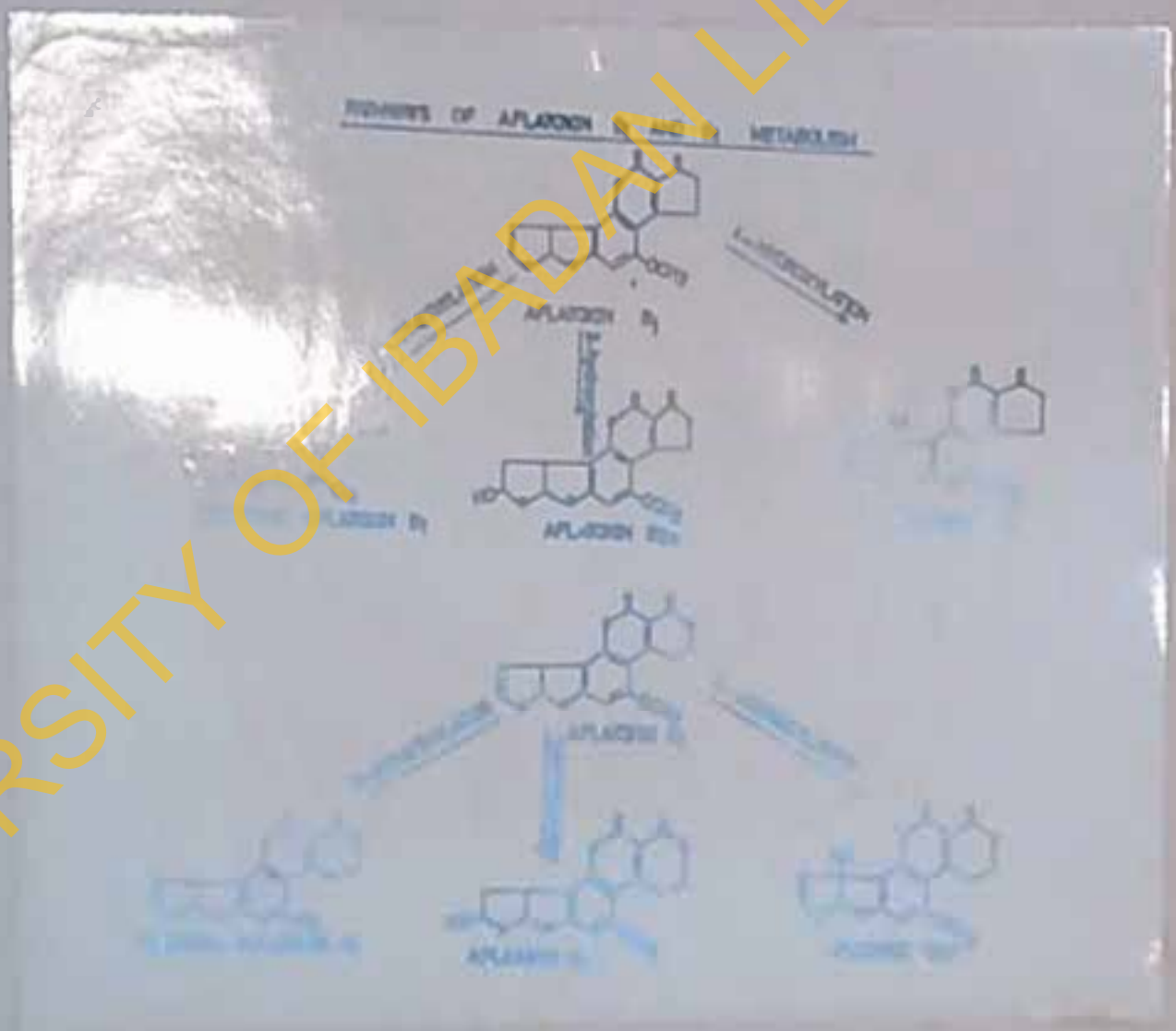

Epoxide formation might proceed thus:-

aflatoxin G_1 — aflatoxin G_1 epoxide.

Metabolism of palmotoxins Bo and Go:

The metabolism of palmotoxins Bo and Go appear to follow a pattern similar to the aflatoxins and involving hydroxylation and demethylation. In the rat, two fluorescent metabolites are discernible after the incubation of either aflatoxin B_1 and palmotoxin Bo. Both the Rf. values and their reactivity tend to indicate that these metabolites are more polar than the parent compounds and probably contain hydroxyl groups.

The trends in spectral shifts are similar. Palmotoxin Bo metabolite showed a shift in the Ultraviolet spectrum from 203nm in the original compound to 215nm; the palmotoxin Go metabolite also showed a shift from 204nm in the original compound to 212nm. Transformation of aflatoxin B_1 to M_1 also caused a shift from 223nm to 226nm. And in the

transformation of G_1 to 'G', a shift from 218nm to 232nm was observed. This is a further evidence for the increase in polarity of the metabolites (Dyer, 1965) and tends to support the view that the fluorescent metabolites of palmotoxins Bo and Go might be their hydroxy derivatives.

The similarity in the fluorescence properties of the palmotoxins Bo and Go, (Figures 20 and 21) and their metabolites (Figures 31 and 32) could indicate that the fluorescing chromophore remained intact all through the biotransformation.

In previous reports on co-factors involved in the metabolism of compounds, microsomal preparations have been shown to require both the soluble fraction and an extreneous supply of NADPH for the restoration of activity (Axelrod, 1955 and 1956). Emafo (1970) has demonstrated that rat liver microsomal-plus-soluble fractions, required the inclusion of a reduced pyridine nucleotide generating system for the restoration of the activity of the aflatoxin demethylating and hydroxylating enzyme. Our present results (Table 14) confirm this and show also that the metabolism of palmotoxins Bo and Go, requires a similar system. This system, does not appear to require an addition of glucose-6-phosphate dehydrogenase, contrary

to the indication of Portman, Plowman and Campbell (1968).

The concentration of NADP in the NADPH generating system appeared to be a limiting factor in the manifestation of the enzyme activity, for a fixed quantity of substrate and glucose-6-phosphate. The demethylation of palmetoxins Bo and Go showed an increase with an increase in NADP concentration up to a maximum of 0.4 µ mole (Fig 33), when there was no more increase due to the concentration of the co-factor. Orronius (1965) has also recorded similar increases in the 'in vitro' metabolism of amino pyrine due to increase in NADP concentration. Optimal pH found for the metabolism of palmetoxins Bo and Go (Fig 35) were within the range of values described for the metabolism of aflatoxin B_1 in the same system (Enafo, 1970).

Influence of species variation on the metabolism of aflatoxin B_1, palmotoxins Bo and Go:

Aflatoxin B_1 appears generally well metabolized in all the species studied (Table 15 and Figure 36). It is, however, not certain whether the values obtained represent metabolism per se or uptake of the toxin from the incubation medium by the liver tissues. The control tube

with inactivated liver was included to compensate for this. However, it still has the defect that denaturation due to heat might also destroy the binding sites and thus may not exhibit comparable binding ability as the fresh tissues. However, the figures for total metabolism given in Table 15, are taken to represent the sum total of all the processes taking place and leading to the removal of the original substance from the medium. Patterson (1970) working with 9 avian and mammalian species has also demonstrated that aflatoxin B_1 was almost completely metabolized by the species used. Our results agree with this and those of Smafo (1970).

The sheep liver slices showed the minimum ability to metabolise aflatoxin B_1, while the toad with a recorded value of 99.5% was most active in this respect. A similar trend was obtained with the microsomal-plus-soluble fractions. The rat, lizard, dog, and guinea pig liver slices metabolized between 80 - 90% of aflatoxin B_1, while the mouse, rabbit, white rock cock, duck, goat and toad metabolized well over 90% of the substance. The rat, sheep and dog liver microsomal-plus-soluble fractions did not metabolize aflatoxin B_1 as well as liver fractions from other species. However,

there was a general increase over the values obtained with the liver slices.

The total palmotoxins Bo and Go metabolized were far less than that of aflatoxin B_1 under the same conditions. Except in the cases of the sheep, goat, rabbit and mouse, only about 50% of palmotoxin Bo were metabolized (Figs 17 and 18). The lowest value was recorded with the guinea pig while the highest value was obtained in the sheep. Palmotoxin Go showed a similar pattern, with the highest value in the sheep (Figs 19 and 20). It is remarkable that the sheep that was the poorest in aflatoxin B_1 metabolism showed the greatestactivity for palmotoxins Bo and Go metabolism. There appears to be no reasonable explanation for this except a possibility that the largely toxic aflatoxin B_1 might be inhibiting its own metabolism in the sheep while the less toxic palmotoxins may not possess this characteristic. Such reversals have also been noted in the toad, duck, cock, and goat but in these cases, the reverse was the case. Those animals that metabolized aflatoxin B_1 very well showed poor activity for palmotoxin Go metabolism. There is

a possibility that the enzymes involved for the metabolism of these compounds might not be the same and since the structures of the compounds are different, the enzymes might be acting at different sites and their distribution may vary from speciesto species. This suggestion appears to be in line with the postulate of Posner, Mitoma and Udenfriend (1961) that a family of hydroxylases are available in the liver microsomes.

Generally, the metabolism of palm toxins Bo and Go, appears to involve a hydroxylation and a demethylation process similar to that of aflatoxin B_1. The enzyme systems responsible for these transformations are NADP-dependent and require magnesium chloride for activation. These enzymes appear widely distributed in the species studied. However, aflatoxin B_1 is much more rapidly metabolized than the palmtoxins Bo and Go (Fig 43). The differences might be due to differences in polarity arising from structural modifications. Gaudette and Brodie (1959) have proposed a model that the microsomal enzymes were protected by a lipoidal layer which can only be penetrated by lipid soluble materials. Mcmahon (1961) working with six aryl - alkyl

dimethylamines, had shown that there was a positive correlation between lipid solubility and demethylation of the substances. Thus, the more polar amines were less demethylated '*in vitro*' and '*in vivo*'. Our present results with aflatoxin B_1 and palmotoxins Bo and Go appear to conform to this general pattern.

In all the substances studied, despite the high values of total substances metabolized, only very small quantities of the fluorescent derivatives were formed. It is either that most other processes operating in the species yield non-fluorescent metabolites or that the fluorescent metabolites can be further decomposed to yield non-fluorescent products (Ennfo, 1970 and Patterson, 1970). A great species variation has been found in the formation of these fluorescent products. Creaven, Parke and Williams (1965) have observed species differences also in the hydroxylation of coumarin by liver microsomes.

The white rock cock and duck appeared not to form any fluorescent derivatives from any of the three compounds we have studied. This might imply either a general absence of the hydroxylase enzyme or a subdued activity. It might also be that hydroxy products from

these species decompose spontaneously to non-fluorescent substances. The sheep showed the greatest ability to form the various derivatives. Similar observation have been reported for aflatoxin B_1 by Allcroft, et al. (1966) and Emafo (1970). The mouse produces aflatoxin M_1 in addition to a yellowish green fluorescent metabolite. This observation has also been reported by Portman, Plowman and Campbell (1968) and Steyn, Pitout and Purchase (1971). However, this differs from the findings of Bassir and Emafo (1970) and Patterson and Allcroft (1970).

Demethylation appeared to constitute a major pathway in the metabolism of the compounds in the rat, mouse and dog. Appreciable demethylation of aflatoxin B_1 also occurred in the white rock cock and in the rabbit. The absence of any detectable formaldehyde in the duck might be due to the absence of the demethylase enzyme or to reduced activity or to the presence of inhibitors of the enzyme in the system. However, these illustrate species differences in the activity of the demethylating enzymes. In the guinea pig, lizard and toad, evidence of demethylation was obtained only with the liver slices but not with the microsome-plus-soluble fractions. This might be due to the instability

of the enzymes. It could also be that the enzyme systems required a different co-factor or possibly some inhibitors might be acting in the enzyme preparation. However, this trend was observed with the three compounds and has also been reported by Ranfo (1970) with respect to aflatoxin B_1.

Aflatoxin B_1 is more toxic than the palmotoxins especially in the rat. It is also more readily transformed in most species of animals than the palmotoxins. A similar situation, operative in the aflatoxins (page 184) could also be playing a leading role in determining the total potency of these substances.

All the animals used in this study appeared to conjugate aflatoxin B_1 and the palmotoxins Bo and Go (Tables 21a, b, and c). Only the glucuronide conjugate has been identified in most of the species. The inability to detect the particular conjugates in other species might be due to the small quantities formed and to the sensitivity of the reactions employed in the identification.

Hydroxylation and demethylation of aflatoxin B_1 and palmotoxins Bo and Go, by the rat liver microsomal-plus-soluble fractions of the rat are enhanced by

Phenobarbitone pretreatment of the rats. (Table 22a and b). Schabort and Steyn (1969) have reported the induction of aflatoxin 4-hydroxylase in the rat arising from phenobarbitone treatment. Our present findings are in line with this report. Similar increases recorded for both the hydroxylation and the demethylation of palmotoxins Bo and Go might also show the induction of the hydroxylases and demethylases responsible for this transformation. Since carbon monoxide inhibited the hydroxylation and demethylation of aflatoxin B_1 and palmotoxins Bo and Go, it does appear that the metabolism of these compounds might be proceeding through a carbon monoxide sensitive pathway almost exclusively.

SUMMARY

The production of toxins by Aspergillus flavus in the yeast extract-sucrose and palm sap medium has been compared. The yeast extract-sucrose medium has been found to be superior to the palm sap medium both in supporting the growth of Aspergillus flavus and in the attendant production of the toxins. A time course of the production of toxins has revealed maximum production on the 6th and 5th days respectively for the yeast extract-sucrose medium and the palmsap medium.

Investigation into the physical characteristic of palmotoxins Bo and Go revealed a striking similarity between the aflatoxins and the palmotoxins (Fig 7). It is thus felt that they could be structurally related.

Toxicity of palmotoxins Bo and Go to rats have been investigated and compared with aflatoxin B_1 - induced toxic effects. Both substances did not exhibit comparable toxic effects with aflatoxin B_1. Palmotoxin Bo was, however, found to be more toxic than palmotoxin Go in the rat.

A comparison of the 'in vitro' metabolism of aflatoxins B_1 and G_1, revealed that aflatoxin B_1 was more easily hydroxylated and demethylated by rat-liver

microsomal fractions. 'In vitro' studies, similar to those used in aflatoxin B_1 studies, have shown that the palmotoxins Bo and Go could possibly be further hydroxylated. Optimal pH, co-factor requirement and the time course of the demethylation, have also been found to be identical in the metabolism of both aflatoxin B_1 and the palmotoxins Bo and Go.

Species differences have been observed in the 'in vitro' metabolism of aflatoxin B_1 and palmotoxins Bo and Go. The rat was shown to produce two fluorescent products suspected to be hydroxy derivatives, from each of palmotoxin Bo and aflatoxin B_1 while only one such derivative was observed from palmotoxin Go.

The mouse was found to produce aflatoxin M_1 in addition to a yellowish green fluorescent metabolite from aflatoxin B_1. Both the duck and white rock cock, did not demonstrate any ability to hydroxylate any of the toxins. The other species hydroxylated the toxins to varying extents with the sheep showing more hydroxylating activity than the rest.

Species differences have also been observed in the demethylation of the toxins. The duck did not exhibit any demethylase activity in any of the substances. The goat did not demethylate palmotoxin Bo or palmotoxin Go

either. The lizard, toad and guinea pig liver slices only showed demethylase activity while their cell-free fractions did not. Varying degrees of demethylation were shown by the other species. In all the species, aflatoxin B_1 was much more rapidly transformed, than palmotoxin Bo or palmotoxin Go.

Both the hydroxylation and demethylation of the three toxins were subject to phenobarbitone induction and could be inhibited by carbon monoxide.

CONTRIBUTION TO KNOWLEDGE

1. Evidence has been put forward to indicate a possible structural similarity between the aflatoxins and the palmotoxins.

2. 20-day old rats have been shown to respond more to palmotoxin Bo toxicity than to palmotoxin Go toxicity. The biochemical changes were similar to those induced by aflatoxin B_1. Aflatoxin B_1 was, however, more toxic to the rat than either palmotoxins Bo or Go.

3. It has been established that aflatoxins B_1 and G_1 can be hydroxylated and demethylated by the rat liver microsomal-fractions. Aflatoxin B_1 was, however, much more rapidly hydroxylated and demethylated than aflatoxin G_1.

4. There is evidence that palmotoxins Bo and Go may be hydroxylated and demethylated. Optimal conditions for these reactions have been shown to be similar to those of aflatoxin B_1.

5. The following species differences have been shown to exist in the metabolism of aflatoxin B_1 and the palmotoxins, Bo and Go:-

(a) The duck neither demethylates nor hydroxylates any of the three toxins.

(b) The white rock cock does not appear to hydroxylate any of the toxins.

(c) The mouse produces aflatoxin M_1 in addition to a yellowish green fluorescent metabolite.

(d) The goat did not seem to be capable of demethylating palmotoxins Bo and Go.

6. Evidence is presented to the effect that hydroxylation of aflatoxin B_1 and palmotoxins Bo and Go in the rat are phenobarbitone inducible and respond to carbon monoxide inhibition.

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