AN INVESTIGATION OF THE MECHANISM OF ANTICOAGULANT ACTION OF AFLATOXIN IN SOME ANIMAL SPECIES

A

THESIS

PRESENTED BY

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ABSTRACT

It has been reported that aflatoxin B_1, isolated from foodstuff contaminated with a toxic strain of Aspergillus flavus produces in rats a lesion similar to the "sweet clover disease". Biochemical, kinetic and microscopic evidence has been produced to show that aflatoxin lengthens blood clotting time in rat in a way similar to 4-hydroxycoumarin.

"In vitro" experiments with liver slices confirm that the inhibition of the synthesis of blood clotting factors by aflatoxin is a result of a competition with vitamin K for the apoenzyme involved in the production of prothrombin in the liver. When the interaction of aflatoxin with blood clotting enzymes is compared with that of carbon tetrachloride (a known hepatocarcinogen) and of 4-hydroxycoumarin it is found that aflatoxin decreases the level of prothrombin, in a way similar to 4-hydroxycoumarin and unlike carbon tetrachloride.

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Sensitive hepatic function tests followed by light and electron microscopy confirm that at the time when [illegible] lengthened blood clotting time maximally, the entire ultrastructure of the parenchymal cell was intact.

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CHAPTER ONE.

INTRODUCTION.

(i) "Sweet Clover Disease"

Haemorrhagic septicaemia, a disease associated with excessive bleeding and a long whole blood clotting time, was recognised in Canadian cattle early in this century. Schofield (1924) traced this "sweet clover disease" to ingestion of improperly cured hay made from common types of sweet clover (Melilotus alba). Roderick (1931) was unable to prepare prothrombin from the blood of affected animals and also found that when prothrombin was added to the plasma of the diseased animals, the clotting time was reduced.

A bio-assay using rabbits was later developed by Campbell, Smith, Roberts and Link (1941) in order to identify the haemorrhagic agent in the spoiled sweet clover. Quick's (1937) one stage prothrombin technique was used. This culminated in the isolation and synthesis of the haemorrhagic agent dicoumarol. Campbell, Roberts and Link (1941) then

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suggested that the biological synthesis of this substance from coumarin during spoilage of the hay might have been due to oxidation of coumarin to 4-hydroxycoumarin, which coupled with formaldehyde to give dicoumarol.

Recently Bye and King (1970) showed that a strain of Aspergillus fumigatus Fresenius, isolated from spoiled hay, will convert melilotic acid (o-hydroxyphenylpropionic acid) and o-coumaric acid into 4-hydroxycoumarin and dicoumarol (Figure 1). The conversion of o-coumaric acid into 4-hydroxycoumarin has been observed several times (Becks, 1967; Bellis, Spring and Stoker, 1967; Shieh and Blackwood, 1967) and it seemed that the mechanism involved in this reaction is that of a β-oxidation (Bye, Acton and King, 1968; Spring and Stoker, 1969). In the presence of semicarbazide, o-coumaraldehyde is formed from o-coumaric acid; however, there is no evidence that this lies on the normal metabolic pathway (Bye and King, 1970).

Although the biosynthesis of 4-hydroxycoumarin from melilotic acid involves several enzymic steps, the coupling of 4-hydroxycoumarin with formaldehyde to form dicoumarol is a non-enzymic reaction (Spring and Stoker, 1968). Bye and King (1970) has stated that the isolation of the enzyme(s)

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involved in the biosynthesis of 4-hydroxycoumarin, during the spoilage of sweet clover, is necessary to show the relation, if any, between the enzyme(s) involved in this reaction and those of the fatty acid β-oxidation.

(ii) Recognition of "Turkey X Disease"

Several decades after the "sweet clover disease" problem, another obscure disease described as "turkey X disease" was discovered in 1960 in England as a result of deaths of about 100,000 turkeys in poultry farms. Soon after, a similar condition was observed in ducklings. No specific poisonous agent was identified in samples of their meals but Blount (1961) and Asplin and Carnaghan (1961) showed that the common factors in these tragic incidents were (a) the presence of Brazillian groundnut meal in the feed and (b) certain liver lesions in poultry farm animals and ducklings. The earliest indication that a groundnut meal other than that from Brazil could cause the disease was given by Asplin and Carnaghan (1961) who showed that ducklings which had fed on a groundnut meal grown and processed in Uganda and Tanganyika (Tanzania), both East African

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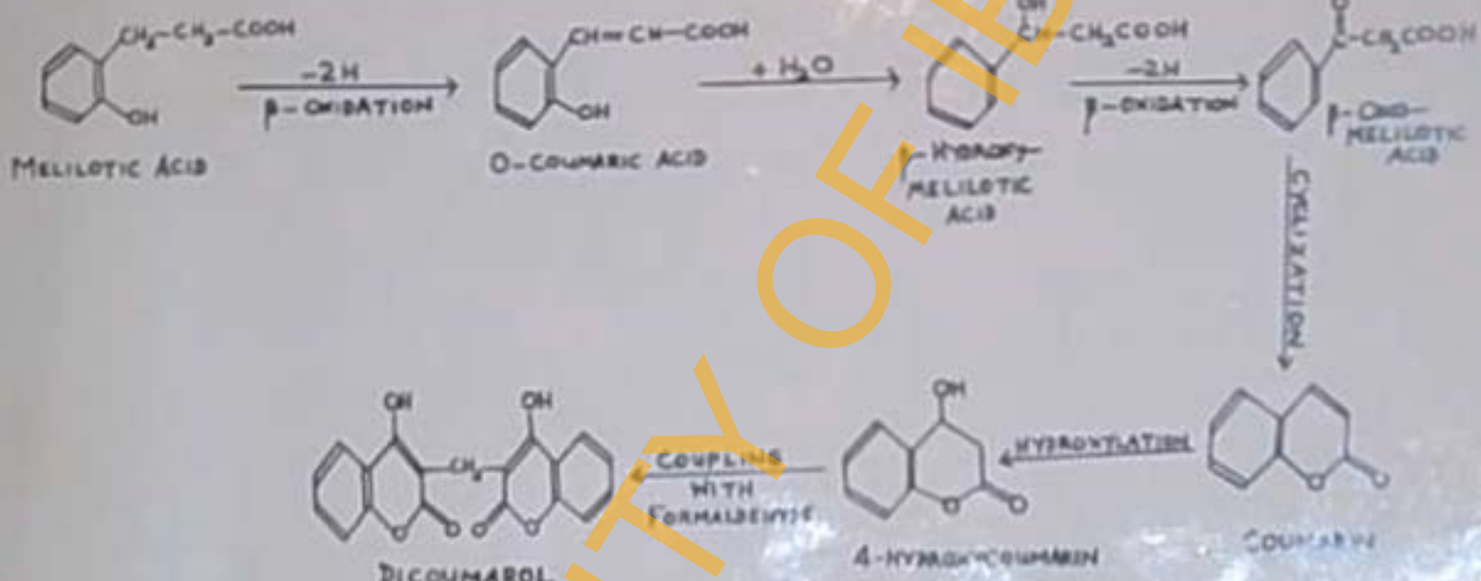

THE BIOSYNTHESIS OF 4-HYDROXYCOUMARIN AND DICOUMAROL

Figure 1: The biosynthesis of 4-hydroxycoumarin and dicoumarol by Aspergillus fumigatus (Bye and King, 1970).

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countries, had liver lesions similar to those produced by the Brazilian groundnut meal. Simultaneously other workers (Loosmore and Harding, 1961; Loosmore and Harrison, 1961) established that some unknown disease in pigs and calves was caused by feeding on Brazilian groundnut meal.

Using ducklings as experimental animals, Sargeant, O'Kelly, Carnaghan and Allcroft (1961) carried out an extraction of the toxic substance from some samples of the Brazilian groundnut meal. No alkaloids were present in the extract (Allcroft, Carnaghan, Sargeant and O'Kelly, 1961).

(a) Discovery of Aflatoxin.

There were speculations with regard to the nature of the toxic substance. Sargeant, Sheridan, O'Kelly and Carnaghan (1961) showed that an isolate of the common mould, Aspergillus flavus was the poisonous material. When this isolate was grown on a sterilised non-toxic groundnut sample, it produced the toxic principle. In view of the origin of this toxin, it was named "aflatoxin" (Interdepartmental Working Party on Groundnut Toxicity Research, 1962).

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The discovery of aflatoxin as a contaminant in human and animal feeds has aroused the interest of scientists in all countries which produce or consume groundnuts because of the potential public health hazards and the ultimate economic consequences. Nigeria is one of the world's major exporter of groundnuts.

(b) Susceptibility of Various Animal Species.

Feeding trials, using toxic groundnut meal of a known aflatoxin content, have indicated a species variation in susceptibility of animals. Table 1 shows some results of LD_{50} studies for aflatoxin in different animals. In the rat the toxicity of aflatoxin decreases rapidly with age and weight of the animal (Butler, 1964). This may be due to the relative lack of microsomal enzymes including cytochrome P-450 (Parke, 1968) which oxidatively metabolise drugs and foreign compounds. These enzymes tend to appear a few days after birth and to reach a maximum after about a month in rats (Kato, Vassanelli, Frontino and Chiesara, 1964).

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Animal	Age (or weight)	Sex	Route* of Administration	LD_{50} (mg/kg)	Reference
Duckling	1 day	M	O	0.37	Allcroft and Carnaghan (1963)
Rat	Neonate	M/F	O	0.56	Asao, et al. (1965)
	21 days	M	O	5.5	Asao, et al. (1965)
	21 days	F	O	7.2	Butler, (1964)
	150 g	F	O	17.9	Butler, (1964)
	100 g	M	i.p.	6.0	Butler, (1964)
Hamster	30 days	M	O	10.2	Wogan, (1966)
Guinea-pig	Adult	M/F	i.p.	ca 1.0	Butler, (1966)
Mouse	Adult	M/F	O	ca 9.0	Newberne and Butler, (1969)
Rabbit	Weanling	M/F	i.p.	ca 0.3	Newberne and Butler, (1969)
Dog	Adult	M/F	i.p.	ca 1.0	Newberne et al. (1966)
	Adult	M/F	O	ca 0.5	Newberne et al. (1966)
Pig	6-7 kg	M/F	O	0.62	Newberne and Butler (1969)
Cat	Adult	M/F	i.p.	0.55	Newberne and Butler (1969)

Table 1: LD_{50} Values for Aflatoxin B_1

*(O = Oral, i.p. = Intraperitoneal.)

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(iii) Structures of Toxins Isolated from Aspergillus Flavus Cultures.

(a) Aflatoxins B_1, B_2, G_1 and G_2.

With the use of thin layer chromatography on silica gel (Kieselgel G, Merck) Smith and McKernan (1962) separated extracts of cultures of toxic strains of Aspergillus flavus and obtained at least twelve fluorescent components. Five of these components damaged the livers of ducklings. van der Zijden, Koelensmid, Boldingh, Barrett, Ord and Philip (1962) then reported the isolation of a crystalline form of a toxin which caused a lesion similar to the "turkey X disease". Also in 1962, de Iongh, Doorenbos, Vles, Barrett, and Ord obtained a concentrate of the toxin after treatment of a chloroform extract with Girard T reagent. Two dimensional chromatography on silica gel G showed two spots; the material from both spots were toxic to ducklings. The one material with a blue-violet fluorescence was assigned the molecular formula $C_{17}H_{12}O_6$ after chemical and mass spectrometric analyses and the presence of an OCH_3 group was identified using nuclear magnetic resonance spectra.

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Substance	Molecular Weight (Mass Spec.)	Molecular formula	UV max.	E	I.R. ν^{cm-1}	Reference
Aflatoxin B_1	312	$C_{17}H_{12}O_6$	223 265 362	25,600 13,400 21,800	1760-1684 1632 1562	Asao et al. (1963, 1965)
Aflatoxin B_2	314	$C_{17}H_{14}O_6$	220 265 362	19,600 9,200 14,700	1760 1685 1600	Chang et al. (1963)
Aflatoxin G_1	328	$C_{17}H_{12}O_7$	243 257 264 362	11,500 9,900 10,000 16,100	1760 1695 1630 1595	Asao et al. (1963, 1965)
Aflatoxin G_2	330	$C_{17}H_{14}O_7$	217 245 265 363	28,000 12,900 11,200 19,300		Asao et al. (1963, 1965)

Table 2: Physicochemical properties of aflatoxins B_1, B_2, G_1 and G_2.

UV = Ultraviolet absorption; I.R. = Infrared absorption;
E = Extinction coefficient.

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Hartley, Nesbitt and O'Kelly (1963) were the first to report the isolation and characterization of four closely related toxins. These toxins separated on silica gel chromatoplates using chloroform/methanol (98:2, v/v) as developing solvent and were designated aflatoxins B_1, B_2, G_1 and G_2 in order of decreasing R_f value. Aflatoxin B_1 was identical with the substance isolated by van der Zijden et al (1962) and by de Iongh et al (1962). Table 2 shows some physicochemical properties of these toxins. With interpretation of ultraviolet, infrared, nuclear magnetic resonance and mass spectral data, the complete structures (Asao, Buchi, Abdel-Kader, Chang, Wick and Wogan, 1963, 1965) and the absolute configuration (Brechbuhler, Buchi and Milne, 1967) of these aflatoxins have been established. The total synthesis of a racemic aflatoxin B_1 (Buchi, Foulkes, Kurono and Mitchell, 1966; Buchi, Foulkes, Kurono, Mitchell and Schneider, 1967) has been described. The structures of these four closely related aflatoxins are shown in Figure 3. The molecules of aflatoxins B_1 and G_1 possess an α,β-unsaturated lactone ring, and they are difuranocoumarin derivatives. In the case of aflatoxins B_2 and G_2 the terminal furano ring in aflatoxins B_1 and G_1 is reduced by addition of two hydrogen atoms. The double bond

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in the α, β-position to the carbonyl group could account for the pharmacological activities of these toxins (Haynes, 1948). Since these substances are lactones, their biological activity could be related to their high reactivity to electrophilic agents and their potential action as alkylating agents under physiological conditions (Dickens and Jones, 1963; Dickens, 1964).

(b) Aflatoxins M_1 and M_2.

Allcroft and Carnaghan (1962, 1963) discovered that the milk of cattle which had fed on aflatoxin-containing meal contained a substance which was toxic to ducklings, producing a lesion similar to aflatoxicosis. Later, de Iongh, Vles and van Pelt (1964) showed by thin layer chromatography on silica gel (Kieselgel G) that the toxin ("milk toxin") is a blue-violet fluorescent substance with an R_f value much lower than that of aflatoxin B_1. Two hours after an administration of a single dose of mixed aflatoxins, Allcroft, Rogers, Lewis, Mabney and Best (1966) identified a substance identical with the milk toxin in the liver, kidney and urine of sheep. Allcroft and her collaborators then suggested that the milk toxin be assigned aflatoxin M to indicate its original isolation from milk. Aflatoxin M was, afterwards, resolved

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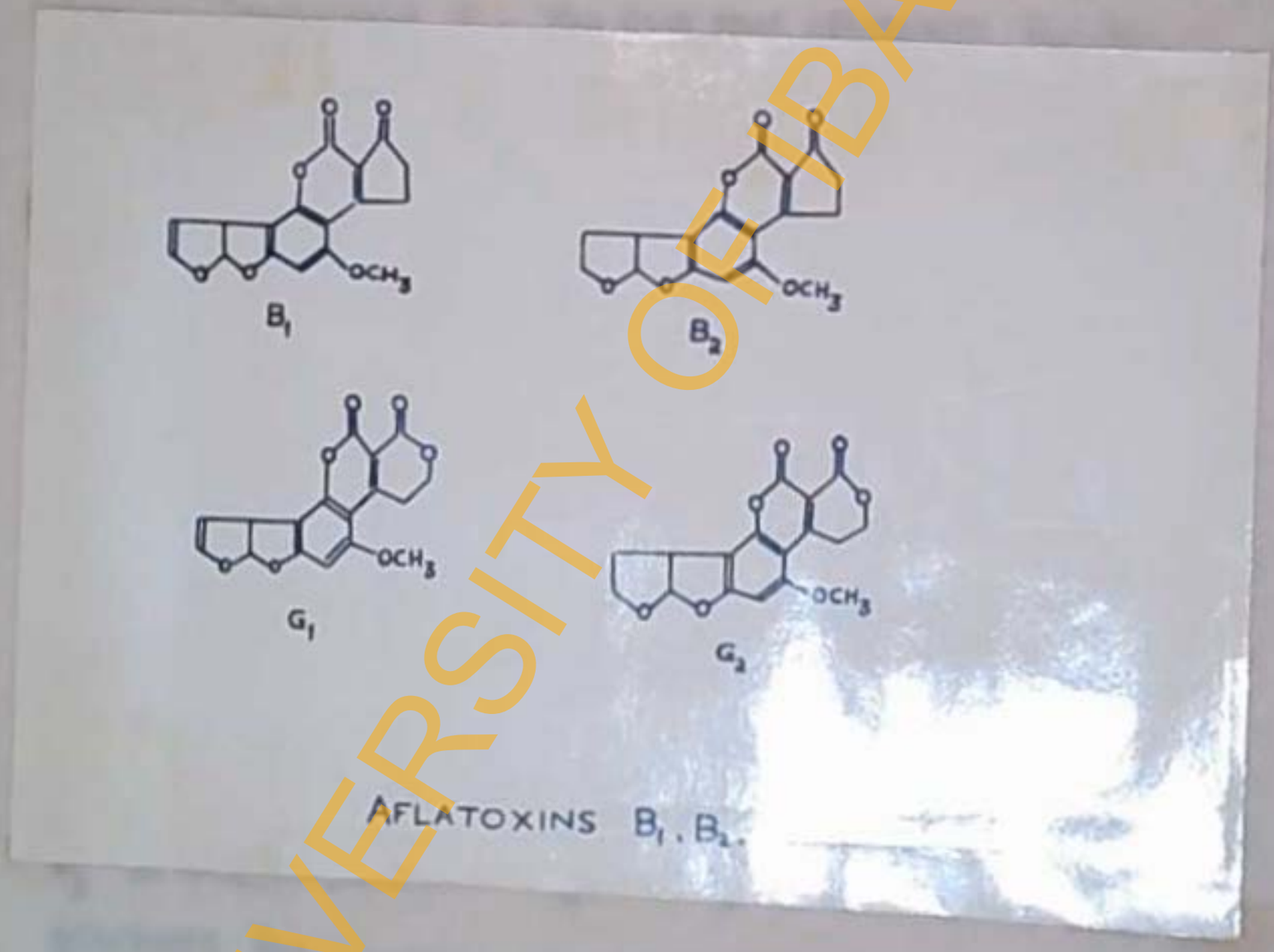

Figure 2: The structures of aflatoxins B_1, B_2, G_1 and G_2 (Asao *et al*, 1963, 1965; Brechbuhler *et al*, 1967).

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into two components with the use of paper chromatography (Allcroft et al, 1966; Holzapfel, Steyn and Purchase, 1966). The spot with the blueviolet fluorescence was designated M_1 whilst the other violet fluorescent spot with a lower R_f value was designated M_2. The fact that aflatoxins B_1, B_2, G_1 and G_2 would separate on silica gel but not on paper whereas M_1 and M_2 would separate on paper and not silica gel is a definite evidence that they are distinctly different substances. Holzapfel and his group were also able to isolate aflatoxins M_1 and M_2 in addition of aflatoxins B_1, B_2, G_1 and G_2 from mouldy groundnuts. On the basis of ultraviolet, infrared, nuclear magnetic resonance and mass spectral data, confirmed by appropriate chemical reactions, the complete structures of aflatoxins M_1 and M_2 which are illustrated in Figure 3 have been reported by Holzapfel et al (1966) and Nasuti, Lamkin, Page and Garcia (1967). Aflatoxin M_1 is 4-hydroxy-aflatoxin B_1 and M_2 is 4-hydroxy aflatoxin B_2.

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Figure 3: The structure of aflatoxins M_1, M_2, B_{2a} and G_{2a}.

(Holzapfel et al, 1966; Nasri et al, 1967; Dutton and Heathcote, 1966).

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(c) Aflatoxin B_{2a} and G_{2a}.

The isolation of aflatoxins B_{2a} and G_{2a} from cultures of Aspergillus flavus was reported by Dutton and Heathcote (1966). B_{2a} fluoresces blue whilst G_{2a} has a greenish fluorescence. Dutton and Heathcote confirmed the structures (see Figure 3) as 2-hydroxy derivatives of aflatoxins B_2 and G_2 and were accordingly named aflatoxins B_{2a} and G_{2a} respectively. These structures were formulated on the basis of ultraviolet, infrared, mass and nuclear magnetic resonance spectra, supported by selected chemical reactions. These two aflatoxins, however, were relatively non-toxic to ducklings but the possibility that aflatoxins B_{2a} and G_{2a} could give rise to the highly toxic B_1 and G_1 by dehydration is highly speculative.

(d) Aspertoxin.

Wilson Smith and McKerman (1962) obtained from cultures of Aspergillus flavus five fluorescent compounds by thin layer chromatography using silica gel, they speculated that there might exist even more complex phytotoxins. Rodricks, Hesseltine, Campbell, Stoloff and Verrett

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(1968) confirmed this by reporting the isolation of another toxic metabolite from cultures of Aspergillus flavus with the trivial name "aspertoxin" and a molecular formula $C_{19}H_{14}O_7$. The structure of aspertoxin illustrated in Figure 4 was established almost simultaneously by two different groups (Rodricks, Lustig, Campbell, and Stoloff (1968); Waiss, Jr., Wiley, Black and Lundin (1968). Aspertoxin is 3-hydroxy-6, 7-dimethoxybisfuroxanthone.

(e) Palmetoxins B_o and G_o.

Isolation of two additional fluorescent toxins from cultures of Aspergillus flavus on palm sap obtained from a variety of Elaeis guineensis has been reported by Bassir and Adekunle (1968). The substances were designated palmetoxins B_o and G_o to indicate their original isolation from an unfermented palm sap. The substances ran near the origin when they were separated and purified by thin layer chromatography in kieselgel G using 50:2 (v/v) chloroform in methanol as the developing system. R_f values of B_o and G_o were 0.20 and 0.13, respectively. On the basis of ultraviolet, infrared, nuclear magnetic resonance and mass spectra supported by specific chemical investigations, Adekunle (1969) suggested the structures as long chain

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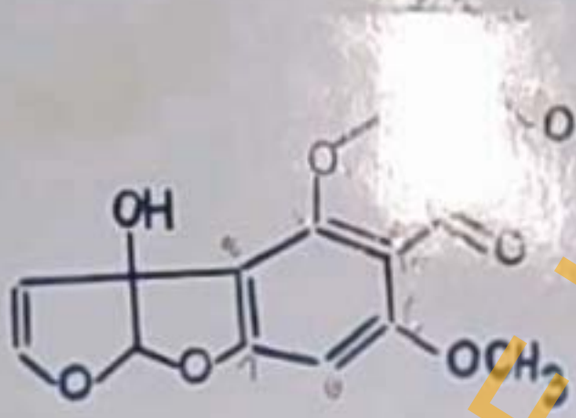

ASPERTOXIN

Figure 4: The structure of aspertoxin (Roderick et al, 1968; Wains, Jr., et al, 1968).

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unsaturated fatty acids (dienes) which may be spatial isomers of each other. Each of them exhibited a mass peak corresponding to $M^+ = 380$ an ion for which the molecular formula $C_{24}H_{44}O_3$ has been proposed.

Toxicity titrations of palmotoxins B_o and G_o on 6-day old White Rock chick embryos showed that B_o is as toxic as aflatoxin B_1. Palmotoxin G_o is relatively non-toxic, being as toxic as aflatoxin G_2 (Ansari and Adekunle, 1969).

(iv) Biochemical Effects of Aflatoxin.

Most of the investigators on the biochemical mode of action of the aflatoxins have utilised aflatoxin B_1 because this is the most potent aflatoxin, with regard to its toxicity to animals. Toxicity and carcinogenicity of aflatoxin in animals and its cytotoxicity in cell cultures are a result of interaction of the drug with cellular constituents.

(a) Interaction with DNA.

Aflatoxin B_1 binds to native, double-stranded helical calf thymus DNA and in this respect a maximal hypochromicity at 355 mμ and hyperchromicity at 385 mμ were

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demonstrated by Clifford and Rees (1966, 1967). Sporn, Dingman, Phelps and Wogan (1966), however, reported a shift in absorption maximum (from 363 mμ to 366-368 mμ) upon binding of aflatoxin B_1 to calf-thymus DNA when the two compounds were equilibrated in phosphate buffer solutions: this shift was accompanied by a marked hyperchromism at 362 mμ. The toxicity of the aflatoxins was proportional to the magnitude of spectral shift induced by DNA binding. The spectral shift of aflatoxin B_1 was similar to the difference in absorption spectrum given by actinomycin D in the presence of calf-thymus DNA (Reich and Goldberg, 1964; Bernhard, Fraysseinet, LaFarge and Le Breton, 1965).

Using equilibrium dialysis, Black and Jirgensons (1967) showed that aflatoxin B_1 binds to DNA and also to two highly purified histones. This interaction results in gross conformational changes in the lysine-rich histone and DNA.

The binding of aflatoxin with DNA gives rise to inhibition of DNA and RNA polymerases resulting in the impairment of the synthesis of both DNA and RNA. The blockage of messenger RNA (m-RNA) formation results ultimately in the inhibition of protein synthesis. Although binding of DNA and aflatoxin B_1 has been demonstrated "in vitro" there is no evidence to support such an interaction "in vivo".

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(b) Carcinogenesis.

With very few exceptions, the effect of an acute dose of aflatoxin is tissue-specific affecting only the liver (see Wogan, 1966). The sequential histological alterations caused by aflatoxin B_1 in rat liver has been described by Butler (1964). A single dose of aflatoxin B_1 to rat or other animals causes parenchymal cell necrosis, bile duct proliferation and other histological changes in the liver. Indeed, aflatoxin B_1 is a potent hepatocarcinogen for several animal species (Wogan, 1966). The alteration caused by aflatoxin B_1 in nucleolar morphology may be specific to the aflatoxins since similar changes have been reported after administration of actinomycin D (Bernhard et al, 1965) and also by certain pyrrolizidine alkaloids (Svoboda and Sogn, 1966). Comparative studies of the nucleoli (Umuma, Morris, and Butler, 1967), however, suggest that the lesion appears to be reversible and does not appear after multiple and repeated doses of the compound.

(c) Cytotoxicity.

Several workers (Juhasz and Greesi, 1964; Gabliks, Schaeffer, Friedman and Wogan, 1965; Daniel, 1965) have demonstrated cytotoxic effects of aflatoxins in "in vitro"

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cultures of a wide variety of animal cell types. Legator and Withrow (1964) and Legator et al (1965) reported that aflatoxin inhibited the synthesis of DNA and protein in heteroploid human embryonic lung cells at concentrations of 0.1 to 1.0 p.p.m. Cytotoxicity of aflatoxin B_1 in cultures of human embryonic liver cells has also been reported by Zuckerman, Rees, Inman, and Betts (1967). The toxic response involved the loss of cytoplasmic RNA and marked changes in nucleolar morphology.

(d) Response in Plant Tissues.

The nature of the effects of the aflatoxins in plants is similar to those caused in animal systems. Schoental and White (1965) showed that aflatoxin inhibited seed germination when Lepidium sativum (watercress) seeds are exposed to the action of a concentration of 25 p.p.m. of the toxin. Lower levels of the toxin will inhibit the synthesis of chlorophyll in the leaves of young plant. Lilly (1965) has reported that mixed aflatoxins induce chromosomal abberations in roots of Vicia faba seedlings. These findings may imply that aflatoxins alter genetic expression possibly through binding with DNA. So, there is an obvious similarity in the mechanism of action in both plant and animal systems.

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(v) Pharmacological Action of Aflatoxin.

In a review by Schoental (1967) emphasis was placed on the need for studies on purely pharmacological properties of aflatoxin in doses which are not acutely toxic. It has, hitherto, not been established whether aflatoxin can be photosensitising like methoxypsoralens or oestrogenic like coumestrol.

Anticoagulant Activity.

Several coumarin derivatives have been studied from the point of view of their anticoagulant action (Arora and Mathur, 1963). It has been established by Bababunmi (1967), Bababunmi and Bassir (1969) that aflatoxin B_1 prolongs blood clotting time of rats and that this compound was effective as an anticoagulant in much smaller doses than 4-hydroxycoumarin.

In this thesis is presented:

(a) the variation of the anticoagulant action of aflatoxin B_1 in eleven different animal species. Both the young and adult animals have been compared because the activity of some drug metabolising enzymes vary with age in animals of

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many species (Kato, Vassanelli, Frontino, and Chiesara, 1964; Parke and Williams, 1969). However, only male animals have been used because, in general, they metabolise drugs and foreign compounds more rapidly than females (Kato and Gillette, 1965; Parke, 1968; Williams, 1969).

(b) Studies which were undertaken in an attempt to elucidate the intimate mechanism of the anticoagulant action of aflatoxin B_1.

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CHAPTER TWO

MATERIALS AND METHODS.

Fungal Isolates.

(a) Aspergillus flavus (UIC 81) isolate was supplied by Dr. S.O. Alasoadura, Botany Department, University of Ibadan. This was a strain of Aspergillus flavus Link ex Fries, originally obtained from mouldy groundnuts. A culture of this fungus was presented to the Mycology Laboratories (Botany Department) of the University of Ibadan by the Tropical Products Institute, London. It is commonly found in the microflora of stored products. The mycelium is septate and the conidia were green in colour. The spore diameter is 0.45 to 0.55 µ.

(b) Aspergillus flavus (NRRL 2999) isolate was supplied by Dr. C.W. Hesseltine, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois. This strain was isolated from mouldy Ugandan groundnuts in 1961 by Dr. P.M.C. Austwick, Weybridge, England, who designated it as V.3734/10 and deposited it in the Commonwealth

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Mycological Institute where it was assigned CMI 91619b. The strain is very stable and consistently yields high levels of aflatoxin, especially B_1, even after many transfers (Shotwell, Hesseltine, Stubblefield and Sorenson, 1966).

Composition of Balanced Diet.

A balanced diet was prepared according to the method of Bassir (1964) and used as growth medium for Aspergillus flavus. The diet was composed of: 1,000g guri flour (Manihot esculenta, Crantz) and 2,000g soyabean flour. This mixture was supplemented with 40.0g methionine, 2.0g lysine and 100.5g salt mixture. The salt mixture was made up with 22.0g sodium chloride, 250.0g calcium phosphate, 125.0g potassium citrate, 30.0g magnesium sulphate, 5.0g ferric citrate and 0.7g mixture of trace elements. This mixture of trace elements contained 12.0g potassium iodide, 10.0g sodium fluoride, 2.0g manganese sulphate, 1.0g potash alum and 1.0g zinc sulphate.

Aflatoxin Working Standard.

5.0 ml. of a chloroform solution of aflatoxin was supplied by the United States Department of Agriculture,

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New Orleans, Louisiana. It contained:

(a) 38×10^{-4} µg per µl aflatoxin B_1

(b) 10×10^{-4} µg per µl aflatoxin B_2

(c) 32×10^{-4} µg per µl aflatoxin G_1

(d) 5×10^{-4} µg per µl aflatoxin G_2

This solution was analysed on silica gel G thin-layer plate, developed in 3 per cent methanol in chloroform. When the plate was examined in ultraviolet light, aflatoxin B_1 gave intense bluish fluorescent spot at R_f, 0.48; B_2 gave a very faint bluish spot at R_f, 0.43. An intense greenish spot at R_f 0.38 and a faint greenish spot at R_f, 0.34 indicated the presence of aflatoxins G_1 and G_2 respectively.

Animals.

(a) Rat and mouse: These were collected from the animal house of our Department. They were fed with a commercial diet purchased from Livestock Feed Limited, Ikeja, Nigeria. The diet was composed of crude protein (21.0%); fibre (4.0%) and oil (3.5%).

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(b) Rabbit, Guinea-Pig and Hamster: The animals were also collected from the animal house. Their diet was composed of crude protein (20.0%); fibre (3.4%) and oil (3.7%), purchased from Livestock Feed Limited, Ibadan, Nigeria.

(c) Duck and Chicken: These birds were supplied by the University of Ibadan Poultry Farm. Their diet was compounded in the farm as follows: maize (yellow or white) 72.5%; crude protein (26.5%) and palm oil (1.0%).

(d) Cat and Dog: These were purchased from a local market in Ibadan and they fed mainly on meat and bones.

(e) Goat: This was also purchased from a local market and had access to green leaves and various bruised vegetables.

(f) Monkey: This was supplied by the Zoological Garden of Ibadan University. It was fed with all kinds of foodstuff ranging from vegetables to meat.

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Chemicals and Reagents.

(a) 4-Hydroxycoumarin (m.p. 212-214°C). It was used as purchased from Hopkin and Williams, Essex, England.

(b) Carbon Tetrachloride. It was used as purchased from Hopkin and Williams, Essex, England.

(c) Indocyanine Green. The green powder (Hynson, Westcott and Dunning, Inc., Baltimore, Md., 21201, U.S.A.) which was supplied in 50 mg size vials was a gift from Dr. C.M. Leevy, Division of Hepatic Metabolism and Nutrition, Veterans Administration Hospital, East Orange, N.J., U.S.A.

(d) Thrombotest Reagent. The reagent (Nyegaard and Co., Norway) was purchased from Duncan, Flockhard and Evans, Limited, London, England. It was supplied as a freeze-dried substance in vacuum-sealed ampoules of 2.2 ml. each.

(e) Russell Viper Venom. This fibrinogen coagulase was purchased from Koch-Light Laboratories, Limited, Colnbrook, Buckinghamshire, England. 0.1 mg of the venom was dissolved in 1.0 ml. of distilled water immediately before use.

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(f) Ribonucleic Acid (Hopkin and Williams, Essex, England). It was used (as purchased) in the construction of a standard curve for the assay of ribonucleic acid in liver homogenate.

Buffers.

(a) Imidazole Buffer (pH 7.3) (Hurts and Owen, 1940). This was prepared by dissolving 1.72g imidazole (glyoxaline) (BDH 127, Koch-Light Laboratories, Limited, Colnbrook, Buckinghamshire, England) in 30.0 ml. of 0.1 N hydrochloric acid and diluting the solution with distilled water to 100 ml.

(b) Michaelis Buffer (pH 7.4) Michaelis, 1931). This was made by mixing 5.0 ml. of veronal acetate solution, 5.0 ml. of 0.1N hydrochloric acid and 15.0 ml. of distilled water. The solution of veronal acetate was prepared by dissolving 1.94g of anhydrous sodium acetate (Hopkin and Williams, Essex, England) in 100 ml distilled water.

(c) Owen's Buffer (pH 7.35) (see Biggs and MacFarlane, 1962). This was prepared by dissolving 5.88g of sodium barbitone (sodium diethylbarbiturate) and 7.34g of sodium

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chloride in a mixture of 785.0 ml. distilled water and 215.0 ml. of 0.1N hydrochloric acid.

(d) Phosphate Buffer (pH 7.4) (see Wooton, 1964). This was made by dissolving 7.55g of dry anhydrous disodium hydrogen phosphate and 1.81g of dry anhydrous potassium dihydrogen phosphate in a litre of distilled water.

(e) Bicarbonate Buffer (pH 10.0) (see Wooton, 1964). 6.3g of anhydrous sodium carbonate and 3.36g of sodium bicarbonate were dissolved in a litre of water.

(f) Bicarbonate-Buffered Balanced Salt Medium (Peters and Anfinsen, 1950). This was an aqueous solution containing 10 mM of calcium chloride, 30 mM sodium bicarbonate, 105 mM sodium chloride and 10 mM potassium bicarbonate.

All the chemicals used in the preparation of Curen's, Phosphate, Bicarbonate buffers and Bicarbonate-buffered balanced salt medium were products of Hopkin and Williams, Essex, England.

All buffers were kept at 4°C.

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(f) Bicarbonate-Buffered Balanced Salt Medium (Peters and Anfinsen, 1950). This was an aqueous solution containing 10 mM of calcium chloride, 30 mM sodium bicarbonate, 105 mM sodium chloride and 10 mM potassium bicarbonate.

All the chemicals used in the preparation of Ouren's, Phosphate, Bicarbonate buffers and Bicarbonate-buffered balanced salt medium were products of Hopkin and Williams, Essex, England.

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Culture Techniques.

Among the three species of fungi, Aspergillus flavus, Penicillium rubertulum, and Aspergillus parasiticus which are known to produce aflatoxin, Aspergillus flavus seems to be the most widely distributed both in wild and cultivated strains (Hesseltine, Shotwell, Ellis and Stubblefield, 1966). It has also been shown that growth can occur on any agricultural commodity provided that temperature, moisture and aeration are adequate (Austwick and Ayerst, 1963). Bassir (1964) demonstrated that a balanced diet containing in part a mixture of gari flour and soyabean flour is an ideal substrate for the growth of Aspergillus flavus. However, several strains of this fungus have been used to produce aflatoxin on a wide variety of agricultural commodities. Hesseltine et al (1966) using three strains of Aspergillus flavus, NRRL 3000, NRRL 2999 and NRRL A-11,613 reported that the amount of aflatoxin produced varied greatly depending on (a) the commodity used, and (b) the type of strain used. In the survey NRRL 2999 was the best.

Therefore, as a preliminary experiment we investigated the growth of NRRL 2999 and compared it with that of the local strain UIC 81 on a mixture of gari flour

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and soya bean flour. The strain which grew faster and produced higher amounts of aflatoxins was then used throughout the experiments to culture the substrate in the attempt to produce sufficient quantities of the toxin for use in the present pharmacological and biochemical investigations.

(i) Preparation of Inoculum.

NRRL 2999. The method of Shotwell et al (1966) was used. The inoculum was prepared by inoculating tubes (1.5 cm x 15.0 cm) of potato-dextrose-agar with spores of NRRL 2999. The potato-dextrose-agar was prepared as follows: Flask 1 contained: distilled water, 100.0 ml; dextrose, 20.0g; calcium carbonate, 0.1g; and magnesium sulphate crystals, 0.2g. Flask 2 contained: 400.0 ml of distilled water and 15.0g of agar. Flask 3 contained: 200.0g of potatoes (peeled and sliced) and 500.0 ml of distilled water. Contents of flask 3 were brought momentarily to 120°C in an autoclave and filtered through sterile cheesecloth. The solution was brought up to the original volume. Simultaneously, the agar in flask 2 was melted and the solution in flask 1 was heated to boiling. Contents of the three flasks were mixed, but the pH was not adjusted.

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Inoculated slants were incubated for 7 to 21 days at 28°C. By 7 days, the cultures had a heavy crop of green conidia, removed by adding 3.0 ml of 0.05% Triton X-100 per slant. Spores were scraped loose with a loop, the slant was shaken to give a uniform suspension of spores, and the spore suspension was used to inoculate the substrate.

<u>UIC 81</u>. The method described by Ogiyemi (1968) was used in the preparation of UIC 81 inoculum. Fungal spores were scraped loose from cultures on agar slant with a platinum loop and transferred into 10.0 ml of sterile distilled water. A drop of Teepol was added and then mixed thoroughly to give a uniform distribution of spores. In order to free the suspension from the mycelium, the preparation was filtered through sterile absorbent cotton wool. Counting of the conidia present in the filtrate was carried out with the aid of a haemocytometer. The size of inoculum was then adjusted to 2×10^6 conidia per 1.0 ml by adding sterile distilled water to the suspension.

(ii) <u>Growth of Aspergillus flavus Cultured on a Balanced Diet</u>.

Growth of the two fungal isolates, NRRL 2999 and UIC 81 were compared under the same conditions in culture

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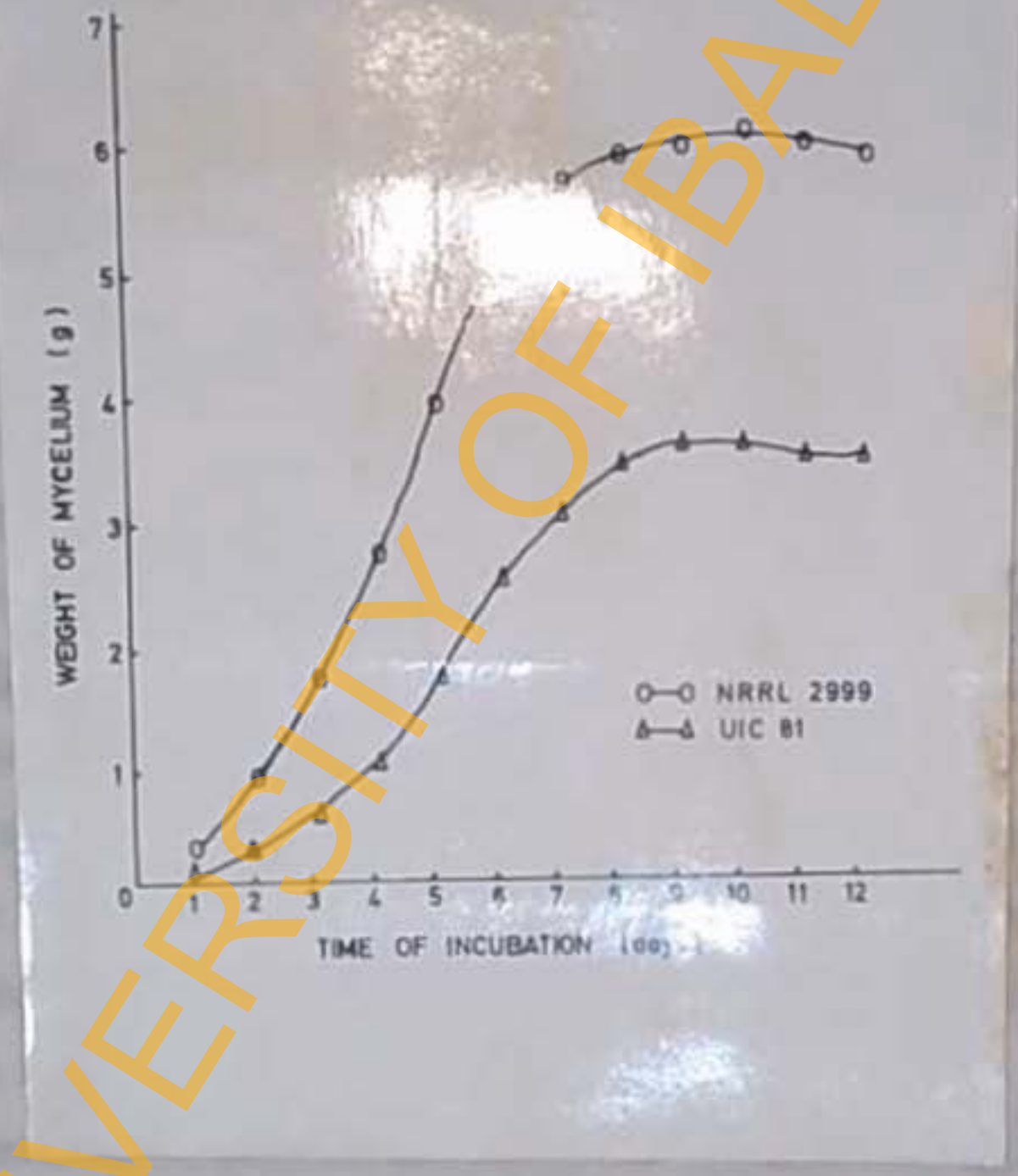

Figure 2: Growth rates of fungal isolates NRRL 2999 and UIC 81 on soybean-suri diet.

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(1) Extraction.

The procedure of an exhaustive soxhlet extraction of the aflatoxins, from mouldy substrates, with methanol was first described by Cosmos and Saunders (1965). While aflatoxins were apparently completely removed in a 6-hour methanol extraction, more than 10 per cent of aflatoxin B_1 would be decomposed using this technique.

In this thesis, a slightly modified technique of Robertson, Penn and Goldblatt (1967) has been employed for the extraction of the aflatoxins.

After five days of incubation, when the strains of Aspergillus flavus had grown sufficiently, aflatoxins were extracted and the mould spores killed by refluxing the mouldy diet with 150 ml. of chloroform in a soxhlet extractor, for six hours. Using chloroform extraction the amount of aflatoxin B_1 which would have decomposed has been shown to be less than 1 per cent (Penn, Robertson and Goldblatt, 1966). After cooling to room temperature, the extract was filtered successively through a double thickness of Whatman No. 1 filter paper and through 400 g of anhydrous sodium sulphate in a sintered-glass funnel. Then the chloroform extract was evaporated "in vacuo" to approximately 10 ml.

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(ii) Thin-layer Chromatography.

Separation of the aflatoxins by thin-layer chromatography were simultaneously reported by Coomes and Saunders (1963) and Broadbent, Cornelius and Shone (1963).

(a) One-Dimensional.

For the preparation of chromatoplates, 30 g of "Chromalay" silica gel (May and Baker, Limited, Dagenham, England) was used to coat five glass plates (20 cm x 20 cm) to a thickness of 250µ using Shandon's thin-layer chromatographic equipment according to the procedure of Stahl (1965). Activation of the chromatoplates was carried out in a drying oven at 110°C for 90 minutes. The prepared plates were then stored in a cabinet for use.

10 µl aliquots of the chloroform extract of aflatoxins were then spotted on an activated thin-layer chromatoplate along with a standard containing all four aflatoxins. The plates were immediately developed in a solution of chloroform in acetone (85:15; v/v) in unlined and unequilibrated chambers (Pons, Robertson and Goldblatt, 1966). The plates were then examined for fluorescence under ultraviolet light at 365 mµ. The aflatoxin content

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of each chloroform extract was then determined by visual comparison of the fluorescent intensity of each individual aflatoxin with that of the aflatoxin standards.

(b) Two-Dimensional.

Although the preceding one-dimensional method provides a generally satisfactory separation of aflatoxins B_1, B_2, G_1 and G_2, visual discrimination of the spots is somewhat difficult, because the R_f values for B_2 and G_1 are similar. Therefore, the thin-layer chromatoplates were run two-dimensionally using the method of Peterson and Ciegler (1967). By this procedure the four aflatoxins were distinguishable and separated from other fluorescent impurities.

10 µl of a crude chloroform extract was spotted approximately 1 in from the corner of the plate, and developed with acetone-chloroform (1:9, v/v) for the first direction. The solvent system for the second direction (the plate turned 90°) was ethyl acetate-isopropanol-water (10:2:1, v/v). The plate was also inspected for fluorescence under ultraviolet light at 365 mµ. Figure 6 shows thin-layer chromatogram of a chloroform aflatoxin extract using the two-dimensional method.

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(iii) Null-Fluorescence Technique.

In the attempt to estimate the production of aflatoxin by each strain of Aspergillus flavus and thence compare the relative amounts of aflatoxin per known quantity of substrate, the null-fluorescence dilution technique (Coomes and Saunders, 1963) was used.

A set of dilutions of test sample extract in chloroform was prepared such that the dilution factor ranged from 2 to 2^{10}. 0.2 ml portions of these solutions were transferred onto 'Chromalay' silica gel thin-layer plates. The diameter of each spot being less than 1 cm. The chromatogram was run in 2 per cent methanol in chloroform (v/v) and viewed in ultraviolet light at 365 mμ. The dilution factor of the solution with just visible fluorescence was noted. The smallest quantity of aflatoxins B_1 and G_1 observable were 0.004 μg and 0.003 μg respectively (Coomes, Crowther, Francis and Stevens, 1965). By adopting a similar experimental procedure the minimum quantities of aflatoxins B_2 and G_2 observable were determined. A photorecord of the fluorescent spots on chromatoplates is shown in Figure 7.

The concentration of aflatoxin present in the extract was calculated according to the method of Coomes et al (1965).

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If

i = visual limit for detection of aflatoxin in µg

h = dilution factor

K = volume applied to plate (0.2 ml)

V = volume of test sample extract (10 ml)

W = dry weight of material extracted (g)

C = concentration of aflatoxin in µg per litre

then $C = i \times \frac{V}{K} \times \frac{1}{W} \times \frac{1}{10^6} \times h$

$= \underline{i \times 5 \times 10^{-5} \text{ µg per litre.}}$

The relative production of aflatoxin by the two fungal isolates is shown in Table 3.

Using NRRL 2999 isolate the yield of the aflatoxins was double over that obtained from the local strain UIC 81 when they grew on the same amount of a gari-soyabean diet under the same conditions. This production represents at least a tenfold increase over the largest amount reported by Hesseltine et al (1966) when they used soyabean alone as

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substrate for the growth of Aspergillus flavus. The ratio 1:2 of soya-gari seems to be the best substrate when compared with other mixtures (Bassir and Bababunmi, 1969).

(iv) Crystallisation

After thin-layer chromatography, the silica gel containing aflatoxin B_1 was scraped from the plate and eluted with chloroform using Whatman No.1 filter paper until the silica gel was non-fluorescent. The solvent was evaporated using a rotary evaporator to approximately 1 ml. Then n-hexane was added dropwise to incipient turbidity. Crystallisation was induced by allowing the solution to stand overnight at room temperature. The crystals were collected by decanting the mother liquor through a sintered-glass funnel under atmospheric pressure. Recrystallization from chloroform was repeated three times. The crystals were considered pure when the material exhibited a single spot when analysed by thin-layer chromatography.

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Figure 6: Two-dimensional thin-layer chromatogram of aflatoxin chloroform extract. Adsorbent: 'chromagram' silica gel. Solvent: first direction - acetone/chloroform (1:9, v/v), second direction - ethyl acetate/isopropanol/water (10:2:1, v/v).

B_1, aflatoxin B_1; B_2, aflatoxin B_2;

G_1, aflatoxin G_1; G_2, aflatoxin G_2

I, impurities.

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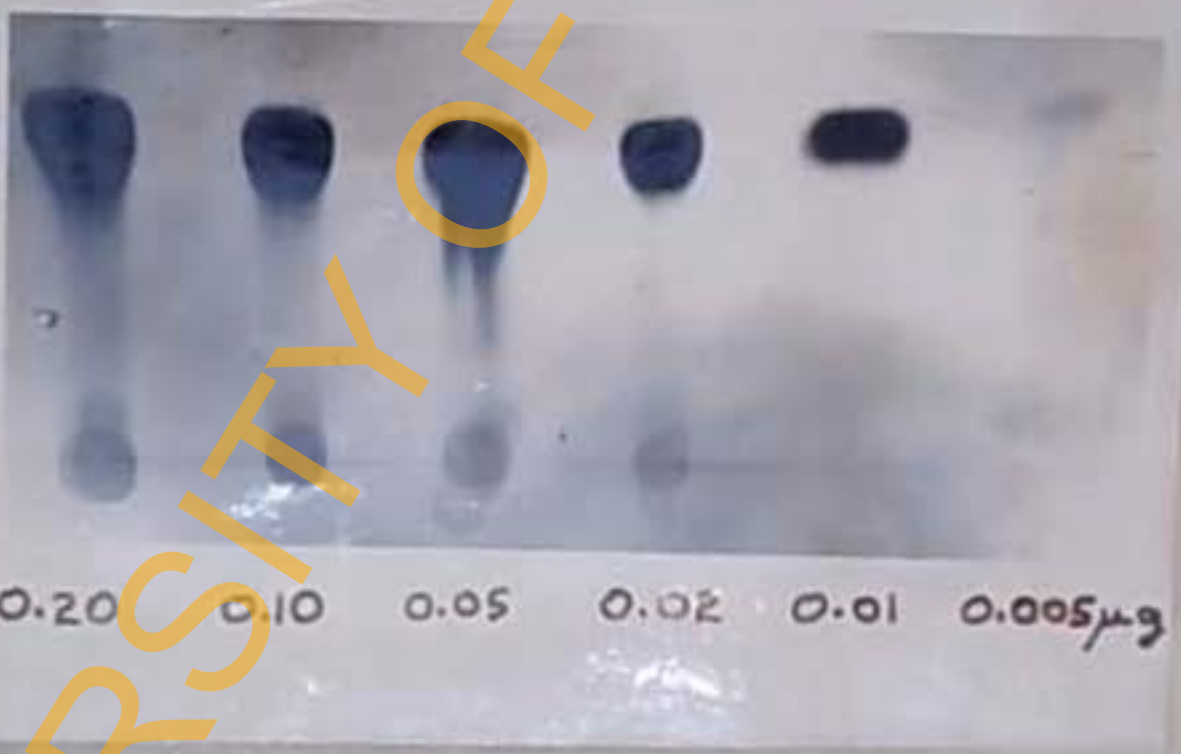

Figure 7: Photorecord of fluorescence of aflatoxin B_1 on thin-layer of 'chromalay' silica gel. A serial dilution of aflatoxin B_1 in chloroform was prepared. Equal volume (0.2 ml) of each was spotted on the chromatogram.

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Isolate	Mycelium wt. (g/30g substrate/120 hours)	Aflatoxin (µg per 100g substrate)		
		B	G	Total B+G
NRRL 2999	3.8	131.0	100.0	231.0
UIC 81	1.5	69.8	52.5	122.3

Table 3: Production of aflatoxin by Aspergillus flavus isolates growing on a suri-soya diet (page 37). Weights of mycelium were determined after 120 hours of growth in a petridish containing about 30g of the diet and incubating at 27° ± 1°C. Weight values are the mean of 6 runs.

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and propylene glycol) injected slowly intraperitoneally. The anaesthesia was given in doses of 0.6 to 1.0 ml per kg body weight.

The left femoral vein was cannulated with the appropriate size polyethylene tubing, and 0.2 ml of heparin (4,000 units/ml) was injected and flushed in with saline.

Blood was collected after 3 hours in the aflatoxin-treated animals (Bababunmi and Bassir, 1969) whilst it was collected after 48 hours in the 4-hydroxycoumarin-treated animals (Arora and Mathur, 1963; Bababunmi and Bassir, 1969).

Measurement of Thrombotest Time.

(i) Preparation of Reagent.

The freeze-dried thrombotest reagent was dissolved in 2.2 ml of 3.2 mM solution of calcium chloride. 0.25 ml of the reagent was pipetted off into a small test tube and was placed in a water bath at 37°C for a few minutes to attain the working temperature.

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(ii) Collection of Blood.

Blood from each animal was collected in a different plastic tube (11 cm x 1.5 cm) containing 0.5 ml of 3.13 per cent (w/v) aqueous solution of sodium citrate to a mark of 5.0 ml to stop the blood from clotting.

(iii) Clotting Time Determination.

0.05 ml of the citrated blood was pipetted off and blown into the thrombotest reagent immediately (see Owren, 1959), holding the top of pipette just above the surface of the reagent and against the inner wall of the test tube, and starting the chronometer simultaneously. The test-tube containing the citrated blood and the reagent was flicked once and the mixture was then left in the water for at least 30 seconds. At short intervals, afterwards, the test-tube was taken out of the water bath and tilted gently and observed. The time between the commencement of the reaction and the moment of coagulation of the mixture in the tube was recorded. All reactions were carried out at 37°C ± 0.1°C.

"In Vitro" Synthesis of Prothrombin by Liver Slices.

(i) Incubation.

A slightly modified technique of Pool and Robinson

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Species	Body Weight (kg)		Dose of Hem-butal (ml/kg body weight)		Diameter of Cannulae (mm)			
					Young		Adult	
	Young	Adult	Young	Adult	Internal	External	Internal	External
Dog (familiaris L)	8 ± 1.1	9.1 ± 0.3	0.5	0.6	0.56	0.80	1.21	1.44
Cat (domestica)	0.5±0.2	2.9 ± 0.6	0.6	0.6	0.56	0.80	0.65	0.93
Mouse (albino, musculus)	0.02±0.002	0.04±0.003	-	-	-	-	-	-
Hamster (golden)	0.32±0.01	0.8±0.03	-	-	-	-	-	-
Rat (albino, wistar)	0.18±0.01	0.30±0.03	-	-	-	-	-	-
Rabbit (New Zealand White)	0.43±0.05	1.37±0.04	0.6	0.6	0.69	0.92	0.89	1.20
Goat (domestica)	1.6 ± 0.4	8.9 ± 1.05	0.6	0.6	0.56	0.80	0.92	1.30
Guinea Pig (English)	0.107±0.006	0.26±.003	-	-	-	-	-	-
Monkey (Papio, West African)	-	8.4 ± 0.7	-	0.8	-	-	1.21	1.44
Chicken (White rocks domestica)	0.056±.002	0.80±0.04	-	0.5	-	-	1.15	1.40
Duck (Muscovy)	0.45±0.07	1.21±0.02	-	0.5	-	-	1.15	1.40

Table 4: Species, body weights, dose of anaesthetic and size of cannulae.

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(ii) Sampling.

1.0 ml of 3.13% (w/v) aqueous solution of sodium citrate was added to the medium in each of the four flasks which were incubating to stop any possible coagulation, prior to assaying for prothrombin.

After incubation, the liver slices were removed from the flask and thoroughly macerated in the incubation medium in a homogeniser and assayed for prothrombin as follows:-

(iii) Assay of Prothrombin.

The liver homogenate and the incubation medium were severally assayed for prothrombin using the technique of Page Russell (1940). 0.2 ml of either the medium or the homogenate was withdrawn and immediately mixed with 0.2 ml of imidazole buffer (pH 7.3) to stop further synthesis of the coagulation factors.

0.1 ml of homogenate (or medium) was transferred into the bottom of a small test-tube in a water bath at 37°C. 0.1 ml of Russell viper venom was added and the tube twirled to mix the contents. The tube was allowed to stand in the water bath for at least 30 seconds to come to the working temperature at 37°C. 0.1 ml of 0.02M calcium chloride was then blown forcibly and directly into the mixture

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in the tube and the chronometer was started simultaneously. The test-tube was shaken quickly and held in the bath without agitation. At second intervals, the tube was tilted to the horisontal position and was observed for a formation of clot. At this point the chronometer was stopped and the time was recorded to a tenth of a second.

The clotting time in this system depended on the concentration of prothrombin in the test-tube. The clotting time was transferred to per cent of normal activity by using a correlation graph.

(iv) Construction of Correlation Graph.

The method of Owren (1951) was used. Normal blood from the animal was collected into 3.13% (w/v) aqueous solution of sodium citrate in the proportion of one part of citrate solution to nine parts of blood. The citrated blood was centrifuged for ten minutes at 1,500 r.p.m. in order to obtain plasma.

0.2 ml of the plasma was diluted with 1.8 ml of the dilution solution, giving 1 in 10 dilution. The dilution solution was made of Owren's buffer, pH 7.35 (200 ml) (the preparation of which has been described on page 33) 0.9% saline (600 ml) and solution A (200 ml). Solution A

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Figure 6: Standard curve for normal rat plasma coagulation.

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was made of 3.13% (w/v) aqueous solution of sodium citrate (240 ml) and distilled water (760 ml).

0.1 ml of the diluted plasma was then transferred into a small test-tube in a water bath at 37°C and assayed for prothrombin following the procedure described above in this section of Methods.

The dilution curve (the correlation graph) of normal plasma was obtained by plotting the concentration of normal plasma (taking the 1 in 10 dilution as 100%) against the clotting time of the plasma (in seconds) on a double-logarithmic paper. Campbell and Link (1941) had demonstrated that there is an exponential relationship between the clotting time of plasma and the concentration of the plasma clotting factors.

Figure 8 shows the standard curve for a normal rat plasma coagulation.

Measurement of Indocyanine Green Clearance.

(i) Preparation of Indocyanine Green Solution.

The indocyanine green powder (50 mg) was dissolved with 10 ml of the aqueous solvent made available by the manufacturers, giving a concentration of 5 mg of the dye per ml of solution.

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(ii) Administration and Dosage of the Dye.

Each animal was weighed and the dosage was calculated on the basis of 0.5 mg per kg body weight. Prior to the injection of the dye, 5.0 ml of venous blood was removed from the femoral vein of each animal into a plastic tube containing 0.5 ml of sodium citrate solution (3.13%, w/v) for a plasma blank and standard curve construction. Through the same needle the correct amount of dye was injected into the lumen of the vein very rapidly.

(iii) Calibration of the Standard Curve.

Using the citrated blood obtained previously from the animal, the dye was added to the plasma in dilutions of 100 µg per ml. The optical density readings for various dye concentrations were obtained by reading in a S.P. 500 spectrophotometer (Unicam, Cambridge, England) at 805 mµ. The difference between the plasma blank reading and samples containing dye gave the optical density due to indocyanine green. All readings were taken at the same temperature. The standard curve which is linear was obtained by plotting the optical density readings against the various dye concentrations on a double-linear graph paper.

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(iv) Determination of the Clearance of the Dye.

Determination of the rate at which indocyanine green was removed in the aflatoxin-treated animals took place three hours after the administration of aflatoxin B_1 whilst in animals which were treated with carbon tetrachloride the time of determination was after 72 hours. At the start of the experiment, each animal was anaesthetised with sodium pentobarbitone (B.P. Abbott's Veterinary Nembutal, containing 60 mg per ml of Nembutal in a mixture of alcohol and propylene glycol) injected slowly intraperitoneally. The anaesthetic was given in doses of 0.6-1.0 ml per kg body weight. After the injection of the dye, blood samples were obtained at 5, 10, 15 and 20 minutes in order to calculate the removal rate of the dye. 5.0 ml of blood was drawn from a femoral vein in the opposite leg to that injected and placed in a test-tube containing 0.5 ml sodium citrate solution (3.13%, w/v). The citrated blood was centrifuged and the plasma pipetted into a cuvette. The optical density was obtained by reading in a S.P. 500 spectrophotometer (Unicam, Cambridge, England) at 805 mμ. The difference between the plasma blank reading and samples containing dye gave the optical density due to the indocyanine green. Concentrations were then read from the standard curves

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constructed for each experiment. All readings were taken at the same temperature.

Techniques Used in the Measurement of Serum Enzymes.

(i) Lactic Dehydrogenase.

The method of Wroblewski and LaDue (1955) was used to measure the activity of lactic dehydrogenase in serum.

2.7 ml of phosphate buffer (pH 7.4) was mixed with 0.1 ml of serum and 0.1 ml of NAD solution and the test-tube containing the mixture was allowed to stand at room temperature (28°C) for 30 minutes to destroy the endogenous substrate. 0.1 ml of buffered 0.7 M sodium pyruvate (substrate) was then added, mixed well and the optical density of the solution was read at 340 mμ in an S.P. 600 spectrophotometer (Unicam, Cambridge). Readings were taken every minute for ten minutes and a linear graph was obtained by plotting change in optical density against time (in minutes). The change in optical density per minute was calculated from the graph.

The activity of the enzyme was expressed in International units of enzyme activity (μ mole per min. per litre).

In a litre of serum,

$$\text{Activity} = \text{O.D. change/min} \times 10^3 \times 4.8.$$

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(ii) Glutamic-Oxaloacetic Transaminase.

The procedure described by Karmen (1955) was employed to determine the activity of serum glutamic-oxaloacetic transaminase.

1.4 ml of phosphate buffer (pH 7.4) was mixed with 0.2 ml serum and 0.2 ml of $NADH_2$ solution in a test-tube. 0.5 ml of 0.2M L-aspartic acid and 0.5 ml of malic dehydrogenase were added to the mixture and the test-tube was allowed to equilibrate at room temperature (28°C) for 30 minutes. 0.2 ml of α-ketoglutarate was then added to the test solution and mixed.

Change in optical density was observed in the S.P. 600 spectrophotometer at 340 mμ. Readings were taken every minute for ten minutes when a linear decrease in optical density was recorded. The change in optical density per minute was calculated from the graph obtained by plotting change against time (in minutes).

The activity of the enzyme was expressed in International units of enzyme activity (μ mole per minute per litre).

In a litre of serum,

$$\text{Activity} = \text{O.D change/min} \times 10^3 \times 2.4.$$

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(iii) Alkaline Phosphatase.

For this assay, the method described by Wooton (1964) was used.

For test (T), 1.0 ml of bicarbonate buffer (pH, 10.0) and 1.0 ml of 0.01M di-sodium phenyl phosphate (substrate) were mixed in a test-tube. The tube was allowed to remain in a water bath at 37°C for three minutes. 0.1 ml of serum was added to the buffer-substrate solution in the tube and mixed gently. The tube was stoppered and allowed to remain in the bath for exactly 15 minutes. The reaction was stopped by adding 0.8 ml of 0.5N sodium hydroxide solution.

The control (C) was prepared by mixing 1.0 ml of the substrate and 0.8 ml of 0.5N sodium hydroxide solution followed by 0.1 ml of normal serum.

The standard (S) was prepared in a separate tube by mixing 1.1 ml of buffer with 1.0 ml of a phenol standard solution (containing 1 mg phenol in 100 ml distilled water) and 0.8 ml of 0.5N sodium hydroxide solution.

To each of the test, control and standard solutions 1.2 ml of 0.5N sodium bicarbonate aqueous solution followed by 1.0 ml of aminoantipyrine solution (which contains 6.0g 4-aminoantipyrine in 1 litre of distilled water) and

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1.0 ml of potassium ferricyanide solution (containing 24.0g of potassium ferricyanide in 1 litre of distilled water) were added.

The standard, control and test solutions were then read in the S.P. 600 Spectrophotometer against a blank (B) at 510 mμ. The blank was made of 1.1 ml buffer, 1.0 ml distilled water, 0.8 ml of 0.5N sodium hydroxide solution, 1.2 ml of 0.5N sodium bicarbonate aqueous solution and 1.0 ml of the potassium ferricyanide solution.

The activity of the enzyme was expressed in King-Armstrong units per 1,000 ml of serum.

$$\text{Activity} = \frac{T - C}{S - B} \times 100 \quad \text{(K.A. units per 1,000 ml).}$$

Methods Used in the Evaluation of the Structure of Liver.

(i) Light Microscopy.

Samples of liver (5 mm thick) were fixed in ice-cold Bouin's fluid for 24 hours and then embedded in paraffin.

6μ sections were cut by a microtome, mounted on glass microscope slides and stained with haematoxylin and eosin.

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Sections were examined in a Patman light microscope (Watson and Sons Limited, London) and photographed using Watson half-plate camera with an Agfa film 25 ASA or 15 DIN.

(ii) Electron Microscopy.

Liver tissue samples were minced very gently into 1 mm cubes on a plate of wax, immersed in 1 per cent cold osmium tetroxide buffered with veronal acetate at pH 7.4 (Palade, 1952) with 0.045g of sucrose per ml of solution added (Caulfield, 1957) and allowed to fix for 60-90 minutes at refrigerator temperature of 4°C. The tissues were dehydrated in a graded ethanol series ending with acetone, every ten minutes. Then, the tissues were embedded in an epoxy resin (EPON 812) according to the method of Luft (1961) and incubated in three successive temperatures, 35°C (overnight), 45°C (24 hours) and 60°C (overnight).

Thin sections were cut on a Porter-Blum MT-2 microtome equipped with a diamond knife. Contrast was enhanced by double staining with uranyl acetate followed by lead citrate (Reynolds, 1963).

Specimens were examined in a Philips EM 200 electron microscope and photographed using LEICA M1 camera (Ernst Leitz Gmbh, Wetzlar, Germany), with Recordak Micro-file film Type 5669.

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Sections were examined in a Patmos light microscope (Watson and Sons Limited, London) and photographed using Watson half-plate camera with an Agfa film 25 ASA or 15 DIN.

(ii) Electron Microscopy.

Liver tissue samples were minced very gently into 1 mm cubes on a plate of wax, immersed in 1 per cent cold osmium tetroxide buffered with veronal acetate at pH 7.4 (Palade, 1952) with 0.045g of sucrose per ml of solution added (Caulfield, 1957) and allowed to fix for 60-90 minutes at refrigerator temperature of 4°C. The tissues were dehydrated in a graded ethanol series ending with acetone, every ten minutes. Then, the tissues were embedded in an epoxy resin (EPON 812) according to the method of Luft (1961) and incubated in three successive temperatures, 35°C (overnight), 45°C (24 hours) and 60°C (overnight).

Thin sections were cut on a Porter-Blum MT-2 microtome equipped with a diamond knife. Contrast was enhanced by double staining with uranyl acetate followed by lead citrate (Reynolds, 1963).

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(iii) Estimation of Ribonucleic Acid in Liver Homogenate.

(a) Chemical Fractionation of Liver.

About 3g of liver was weighed and cut into pieces finely with scissors and homogenised in 0.25M sucrose for 20 minutes, keeping the homogeniser surrounded by crushed ice. The final concentration of homogenate was a 1:10 suspension in ice-cold 0.25M sucrose (1g wet weight of liver to 9.0 ml of sucrose solution). The suspension was kept at 0°C.

The homogenate was fractionated chemically according to the method of Schneider (1945). 2 ml homogenate was put in a centrifuge tube kept at 0°C, mixed thoroughly with an equal volume of 1M-perchloric acid cooled to 0°C and left for ten minutes at 0°C. The mixture was spun for five minutes at 3,000 r.p.m. in an MSE angle 13 refrigerated centrifuge. The supernatant was rejected. The residue was resuspended in 5 ml 0.5M-perchloric acid, and centrifuged at 3,000 r.p.m. The supernatant which contained acid soluble nucleotides and sugar phosphates was rejected.

The residue in the tube was then washed with 5 ml of cold acetone and the supernatant was rejected. The residue was extracted three times with 5 ml ethanol-ether (3:1, v/v) at 60°C in a water bath. The residue was then extracted twice

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with 5 ml ether, rejecting the supernatant but keeping the residue; the supernatant contained phospholipids.

The resulting residue was suspended in 5 ml of 0.5N-perchloric acid, warmed cautiously to remove excess ether and heated at 70°C for 30 minutes, covering the tube with non-absorbent cotton wool. It was then cooled and centrifuged at 3,000 r.p.m. for five minutes. The supernatant was completely removed, with care. 2.5 ml of 0.5N-perchloric acid was used to wash the final residue. The extract was made up to 10 ml with 0.5N-perchloric acid and used for the assay of ribonucleic acid.

(b) Assay of Ribonucleic Acid.

Ribonucleic acid level was measured using the orcinol-ferric chloride method of Greenbaum and Slater (1957).

1.0 ml aliquot was mixed with 2.0 ml distilled water, 3.0 ml of 0.1% ferric chloride in hydrochloric acid and 0.3 ml of 10% orcinol in 95% ethanol. The mixture was heated in a water bath at 100°C for 50 minutes. It was cooled and the optical density read at 670 mμ using Unicam S.P. 600 Spectrophotometer, using a 1.0 ml of distilled water treated similarly to set instrument to zero.

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The concentration of HMA was determined from a standard curve which was calibrated between 0-200 µg per ml.

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CHAPTER THREE

EXPERIMENTS AND RESULTS.

Experiment I: Species Differences in Anticoagulant Action of Aflatoxin B_1 and 4-Hydroxycoumarin.

Schoental (1967) reported that amongst the animal species which are very susceptible to the toxicity of aflatoxin B_1 (1 mg per kg body weight or less) are dog, rabbit, duckling and newly born rat. Those which are relatively less susceptible and require at least ten times higher doses include adult rat, monkey, hamster and chick, whilst mouse and sheep are quite resistant and will tolerate large doses of aflatoxin without ill effects.

The lengthening of thrombotest times in rat (Bababunmi and Bassir, 1969) by a sublethal dose (58.0 µg per kg body weight) of aflatoxin B_1 may not apply to other species. In this experiment, therefore, we examined the extent to which aflatoxin B_1 will affect the thrombotest times in eleven species of animals, namely, cat, dog, monkey, goat, chicken,

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duck, guinea-pig, mouse, hamster, rabbit and rat. Attempts were also made to compare these anticoagulant properties with those exhibited by 4-hydroxycoumarin in view of the similarities in the structures of the synthetic coumarins and the aflatoxins (see Asmo et al, 1963). Both the young and adult male animals were employed. Details of the body weights of the animals used, the dose level of anaesthetic and the sizes of the polyethylene cannulae used in this experiment are shown in Table 4.

Two separate groups of six, similar animals in each species (except for monkey, where two animals were used) were injected intraperitoneally with (a) aflatoxin B_1 (58.0 µg per kg body weight), (b) 4-hydroxycoumarin (50.0 mg per kg body weight). These substances were dissolved in distilled water, and administered in volumes of not more than 1.0 ml each. Another group of six animals of each species (except monkey) were kept as control and injected with 1.0 ml of pure distilled water.

4.5 ml of blood was obtained from each animal into a plastic tube containing 0.5 ml sodium citrate aqueous solution (3.13%, w/v) for experiment. The clotting times of these test samples of blood and their controls were determined using the thrombotest reagent as described on

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page 51. All reactions were carried out at 37°C ± 0.1°C.

Result.

In order to test whether or not the difference between the mean coagulation time of the control animals and that of each of the groups of experimental animals is significant, the student's t-test has been used. P values are given, as shown in Tables 5 and 6 for the comparison of each experimental group with their controls and are considered statistically significant if $P < 0.05$; where $P > 0.05$, the values are designated as N.S. (not significant).

Carnivorous Animals.

The extent of the anticoagulant action of aflatoxin on both young and adult animals are shown in Tables 5 and 6. With aflatoxin treatment there is a slight lengthening of blood clotting time in the young cats and dogs ($P > 0.1$). The effect of 4-hydroxycoumarin is somewhat similar to aflatoxin. In adult cats and dogs, also, the increase in clotting time due to aflatoxin treatment is not statistically significant. The results obtained from the adult monkey are somewhat similar to

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those of cat and dog. Furthermore, in the adult cat treated with 4-hydroxycoumarin, the lengthening of the blood clotting time is not statistically significant.

Omnivorous Mammals.

Aflatoxin and 4-hydroxycoumarin prolong markedly clotting time in rat, mouse and hamster. In both the young and adult hamster, aflatoxin seems to be more effective than 4-hydroxycoumarin, unlike in the rat. It is of interest to note that aflatoxin prolongs blood clotting time of both young and adult mice by at least a factor of 2, despite the reported resistance of this species to aflatoxin poisoning (Newberne and Butler, 1969). However, comparing the various t-values, the adult mouse seems to be more affected by the anticoagulant activities of 4-hydroxycoumarin and aflatoxin than the young mouse. This might be interpreted to mean that the acute toxicity of aflatoxin involves some haemorrhagic factor (amongst other factors) besides its carcinogenic action.

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Herbivorous Mammals.

The actions of these anticoagulants on goats, rabbits and guinea-pigs are very marked both in the young and the adult. Variation in age did not make any difference to the reaction of the herbivores to the drugs, except in the rabbit where the two drugs prolonged the blood clotting time of the adult more than that of the young.

Aves Species.

The normal blood clotting times of chickens and ducks are quite long and the extent of prolongation in the duck on treatment with each anticoagulant is also pronounced, in both the adult and in the young. In these birds, however, the anticoagulant effects of the two drugs seem to increase with the age of the animal.

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Species	Clotting Time (sec.) Mean ± S.E.M.							
	Control (A)	4-hydroxy-coumarin (B)	A versus B		Control (C)	Aflatoxin B_1 (D)	C versus D	
			t	P			t	P
Dog	23.5±5.9	34.5±5.9	1.8	N.S.	26.0±5.9	36.5±5.9	1.7	N.S.
Cat	30.5±7.1	46.5±7.1	2.1	N.S.	32.0±7.1	41.5±7.1	1.3	N.S.
House	15.8±0.3	46.0±0.3	94.4	<0.001	15.8±0.3	35.1±0.3	60.3	<0.001
Hamster	19.4±0.1	52.1±0.1	294.3	<0.001	19.4±0.1	67.2±0.1	430.2	<0.001
Rat	32.0±0.2	69.5±0.2	187.5	<0.001	32.0±0.2	45.5±0.2	67.5	<0.001
Rabbit	26.5±0.3	76.2±0.3	155.3	<0.001	22.0±0.3	49.9±0.3	87.2	<0.001
Goat	27.5±0.2	64.5±0.2	185.4	<0.001	25.0±0.2	46.5±0.2	107.7	<0.001
Guinea-Pig	29.6±0.1	81.1±0.1	463.7	<0.001	29.6±0.1	70.8±0.1	370.8	<0.001
Chicken	51.7±0.2	72.4±0.2	137.8	<0.001	51.7±0.2	70.2±0.2	92.7	<0.001
Duck	101.5±1.4	179.4±1.4	51.9	<0.001	101.5±1.4	194.5±1.4	62.0	<0.001

Table 5: Anticoagulant actions of 4-hydroxycoumarin and aflatoxin B_1 in the young of various species.

Males were used. The dose of 4-hydroxycoumarin was 50 mg/kg body weight, injected intraperitoneally in 1.0 ml. water. Blood was collected at 0 hour and after 48-hour period. The dose of aflatoxin B_1 was 58.0 µg/kg body weight injected intraperitoneally in 1.0 ml. water. Blood was collected at 0 hour and after 3-hour period. For determination of blood clotting time, thrombotest reagent was used. Results are expressed as mean values for six animals.

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Species	Clotting Time (sec.) Mean ± S.E.M.							
	Control (E)	4-hydroxycoumarin (F)	E versus F		Control (G)	Aflatoxin B_1 (H)	G versus H	
			t	P			t	P
Dog	14.0±3.8	21.5±3.8	1.9	N.S.	15.5±3.4	18.0±3.4	0.7	N.S.
Cat	28.0±5.5	40.5±5.5	2.2	N.S.	28.5±5.0	29.5±5.0	0.6	N.S.
Mouse	18.2±0.2	55.4±0.2	186.0	< 0.001	18.2±0.2	41.5±0.2	116.5	< 0.001
Hamster	19.3±0.2	46.2±0.2	134.8	< 0.001	19.3±0.2	76.8±0.2	288.1	< 0.001
Rat	28.5±0.3	79.6±0.3	159.7	< 0.001	28.5±0.3	47.1±0.3	58.1	< 0.001
Rabbit	29.5±0.2	80.6±0.2	255.5	< 0.001	28.1±0.2	59.3±0.2	156.3	< 0.001
Goat	32.5±0.3	68.5±0.3	112.6	< 0.001	29.0±0.3	52.5±0.3	73.4	< 0.001
Guinea-Pig	33.5±0.2	51.9±0.2	92.0	< 0.001	33.5±0.2	58.5±0.2	125.3	< 0.001
Monkey	42.0±2.1	44.5±2.1	1.1	N.S.	44.0±2.1	49.5±2.1	2.5	N.S.
Chicken	62.0±0.1	88.0±0.1	234.0	< 0.001	66.8±0.1	82.9±0.1	144.9	< 0.001
Duck	112.0±1.1	276.5±1.1	192.0	< 0.001	118.5±1.1	249.5±1.1	109.0	< 0.001

Table 6: Anticoagulant actions of 4-hydroxycoumarin and aflatoxin B_1 in adult animals of various species

Males were used. The dose of 4-hydroxycoumarin was 50 mg/kg body weight, injected intraperitoneally in 1.0 ml. water. Blood was collected at 0 hour and after 48-hour period. The dose of aflatoxin B_1 was 58.0 µg/kg body weight, injected intraperitoneally in 1.0 ml. water. Blood collected at 0 hour and 3-hour period. For determination of blood clotting time, thrombotest reagent was used. Results are expressed as mean values for six animals, except for monkeys where two animals were used.

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Experiment II: Indocyanine Green Clearance in Animals of Various Species Treated with Aflatoxin B_1.

The present investigations concern the clearance of indocyanine green in those animal species whose blood clotting times were prolonged significantly during aflatoxin administration. In this respect only one representative animal in each class was studied. By this method, it has been possible to assess the liver function of these animals at the time when a sublethal dose of aflatoxin B_1 prolongs blood clotting time maximally.

Sets of nine, male adult animals of the same strain in each species of goat, rat and duck were kept at 27°C ± 1°C. The animals were studied in a fasting, basal state. Two separate groups of three animals in each species were injected intraperitoneally with (a) 50.0 µg aflatoxin B_1 per kg body weight, (b) 1.0 ml carbon tetrachloride per kg body weight. The remaining three animals of each species were kept as controls. The aflatoxin was dissolved in distilled water and administered in volumes of not more than 1.0 ml each.

Using the method described on page 59, determination of the rate at which indocyanine green was removed in the aflatoxin-treated animals took place three hours after the administration of aflatoxin B_1 whilst in animals which were

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treated with carbon tetrachloride the time of determination was after 72 hours.

The removal rate is expressed as the percentage of remaining dye removed per minute and calculated using the method of Hunton, Bellmann and Hoffman (1949). The concentration of indocyanine green in each timed specimen was determined and expressed in mg per 100 ml plasma. These different values were plotted against time (in minutes) on a semilogarithmic paper. During the exponential period in each graph,

$$\text{Removal rate for dye} = \frac{100\ (\log_e N_o - \log_e N)}{t}$$

where N_o = dye concentration at time "0"

N = dye concentration at time "1"

and t = time "1" - time "0".

Results.

In order to test whether or not the difference between mean removal rate of the control animals and that of each of the groups of experimental animals is significant, the student's t-test has been used. P values are given as

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shown in Table 7 for the comparison of each experimental group with their controls; they are statistically significant if $P < 0.05$; where $P > 0.05$ the values are designated as N.S. (not significant).

Normal Plasma Removal Rate.

The results are summarised in Table 7 and the profiles can be seen in Figures 9, 10 and 11. With a dosage of 0.5 mg per kg body weight, the clearance of indocyanine green has been shown to be an accurate reflection of the state of the liver (Hounrd, Semyszyn and Leevy, 1965). The average plasma removal rates of the dye in goat, rat and duck are (4.9 ± 0.2) per cent per minute, (2.8 ± 0.2) per cent per minute and (6.7 ± 0.2) per cent per minute, respectively. For goat, the initial exponential period was at least 15 minutes whilst for rat and the duck the disappearance rates were exponential only for 10 minutes in each case. The results were, however, reproducible to within 5 per cent variation in all the experiments.

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Species	Body Weight (kg)	Removal rate (mg per 100 ml plasma per minute) Mean ± S.D.			
		Control	Aflatoxin B_1	Control	Carbon Tetrachloride
Herbivore (Goat)	2.14±0.25	4.9±0.2	4.6±0.2	4.9±0.2	1.0±0.05
		$P > 0.1$ (N.S.)		$P < 0.001$	
Omnivore (Rat)	0.31±0.02	2.8±0.2	2.5±0.2	2.8±0.2	0.6±0.05
		$P > 0.2$ (N.S.)		$P < 0.001$	
Bird (Duck)	1.20±0.03	6.7±0.2	7.2±0.2	6.7±0.2	2.0±0.1
		$P > 0.1$ (N.S.)		$P < 0.001$	

Table 2: Plasma removal rates for indocyanine green (ICG).

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Removal Rates in the Aflatoxin-Treated Animals.

At the end of a three-hour action of aflatoxin B_1, when the blood clotting time had been prolonged maximally in each of the species, the plasma removal rates had not changed significantly from the normal values. They were found to be (4.6 ± 0.2) per cent per minute, (2.3 ± 0.2) per cent per minute, (7.2 ± 0.2) per cent per minutes for goat, rat and duck, respectively. The results were quite reproducible to within 5 per cent variation.

Removal Rates in the Carbon Tetrachloride-Treated Animals.

72 hours after the injection of carbon tetrachloride, when the liver of each animal should have been definitely damaged (Seawright and McLean, 1966), the plasma removal rates of indocyanine green were considerably lowered in all the animals. The values obtained under these conditions were (1.0 ± 0.05) per cent per minute, (0.6 ± 0.05) per cent per minute and (2.0 ± 0.1) per cent per minute for goat, rat and the duck, respectively. These results were also reproducible to within 5 per cent variation.

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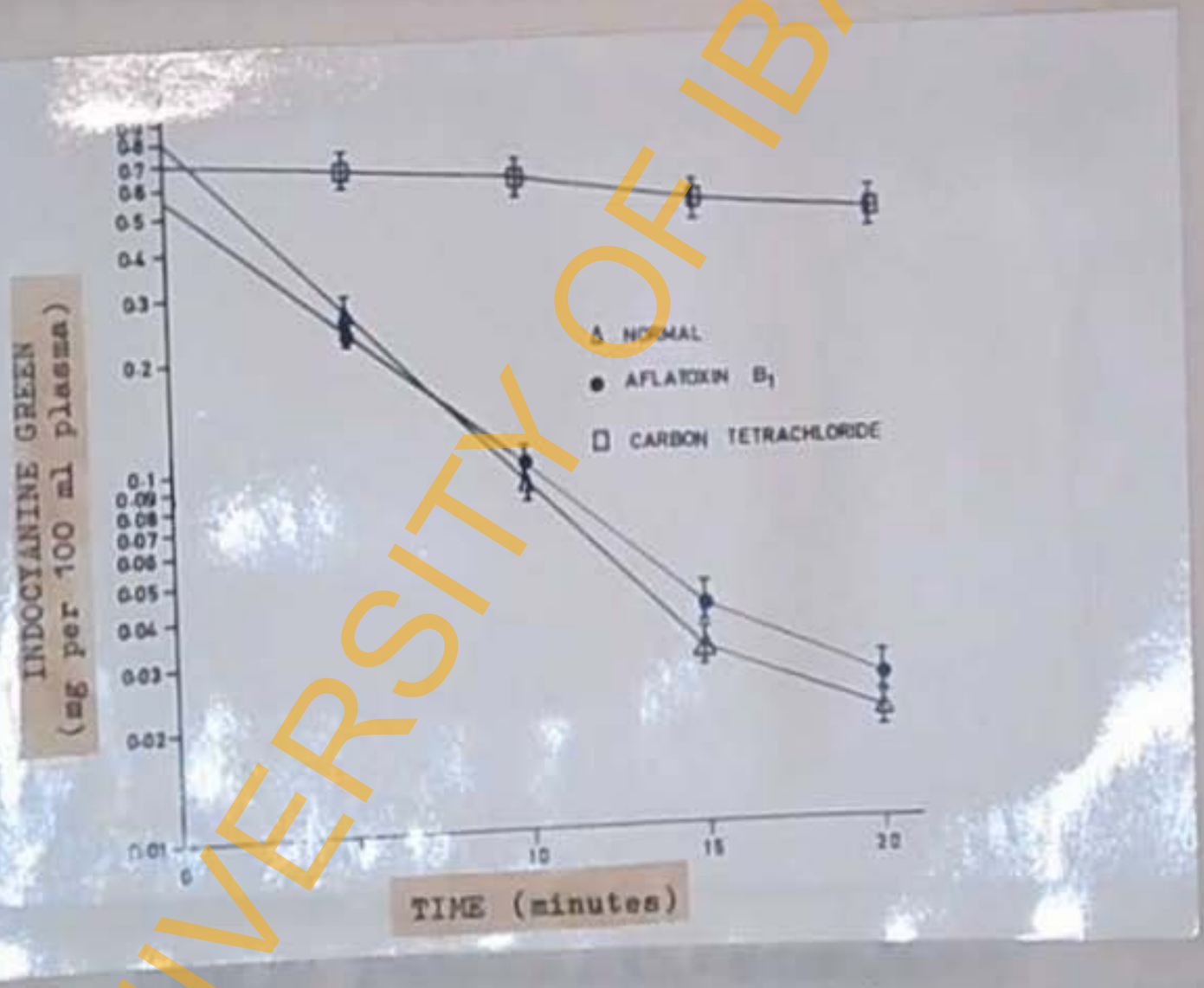

Figure 8: Indocyanine green clearance in goat.

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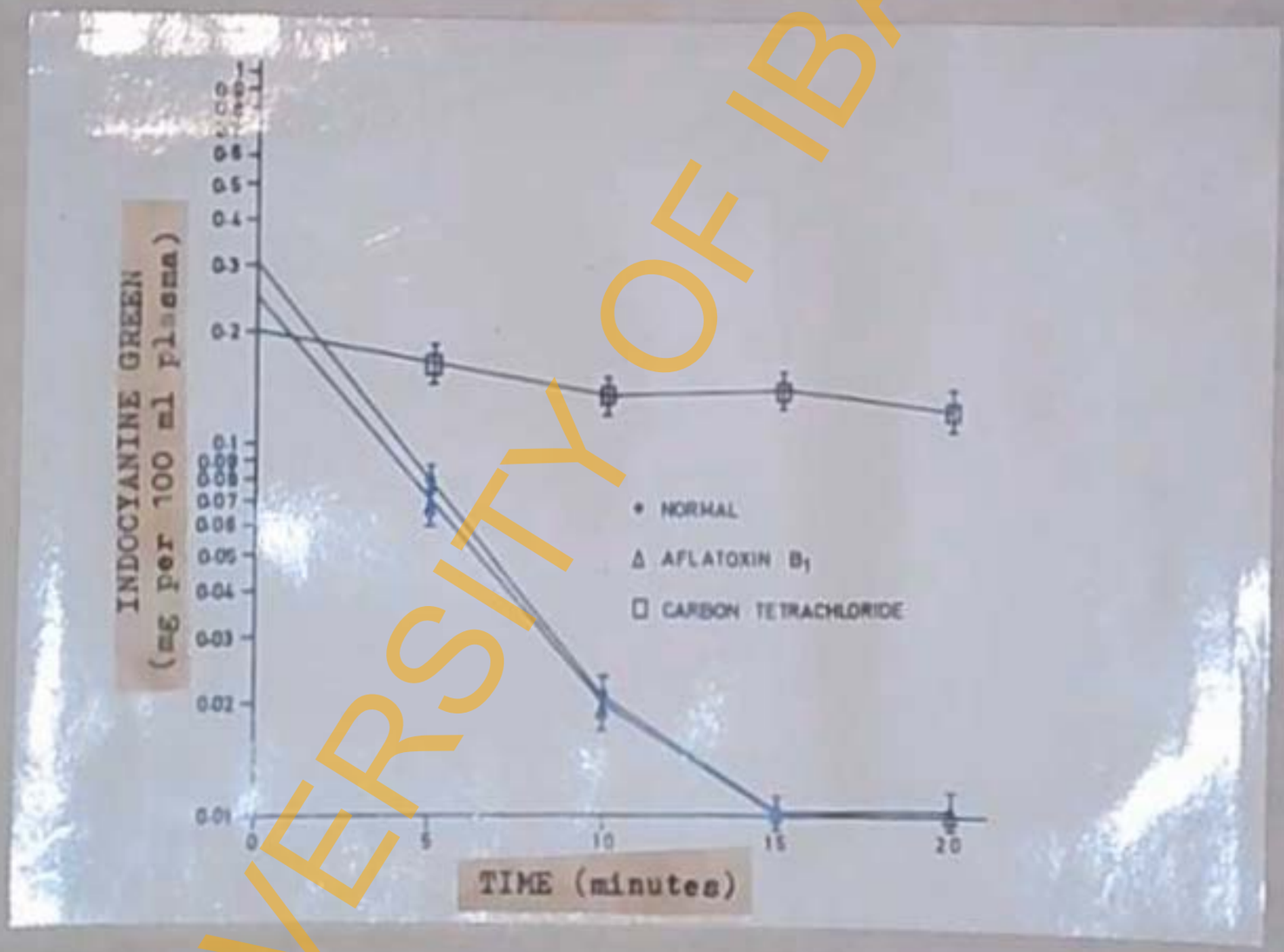

Figure 10: Indocyanine green clearance in rat.

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Figure 12: Indocyanine green clearance in the duck.

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Experiment III: Investigations on the Histology of the Livers of Various Animal Species During Anticoagulant Action of Aflatoxin B_1.

During the past decade several workers (Bassir, 1964; Newberne, Carlton and Wogan, 1964; Newberne and Wogan, 1968; Rogers and Newberne, 1969) have reported different parenchymal changes induced in some animals during aflatoxin poisoning.

It is the aim of this experiment to investigate the histology of the liver cells of the different animal species during the period when a sublethal dose of aflatoxin B_1 prolongs their blood clotting times, in the attempt to define the mechanism of anticoagulant action of this drug.

Adult male goats, rats and ducks, as described in Table 4 were used. Prior to administration of the drug they had free access to a balanced diet (Bassir, 1964) and were also given tap water *ad libitum*. After dosage, the animals were given water alone. All animals were decapitated three hours after an intraperitoneal injection of 58.0 µg aflatoxin B_1 per kg body weight. The toxin was dissolved in distilled water and administered in volumes of not more than 1.0 ml each. Control animals were injected with 1.0 ml of pure distilled water.

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For histology, samples of liver (5 mm thick) from both control and treated animals were fixed, embedded, cut, stained and examined using the method described in pages 63 and 64.

Result.

The sequence of histological changes in liver during aflatoxin B_1 carcinogenesis has been well documented (Newberne and Wogan, 1968). The general architecture of our control liver cells (Figures 12, a, b, c) was essentially similar to that described by other workers (Todd, Shalkpp, Dooley and Wiseman, 1968). Todd et al (1968) have shown that the main gross and microscopic changes in aflatoxicosis in the rat are transudation, haemorrhage, hyperplasia of bile ducts and hepatic cells, and megalocytosis of hepatocytes.

In the experimental liver cells described in this thesis, no significant abnormalities in the sizes of the nuclei were seen, neither was there any evidence of fibrosis of the central vein (Figure 13a). Also, after the three-hour period of action of aflatoxin B_1 (the period which has been shown to be required to prolong blood clotting time maximally - see Bababunmi and Bassir, 1969) there were no cellular changes and the typical hyperplastic nodules accompanied by bile duct

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Figure 12a: Centrilobular parenchymal cell in normal goat given distilled water. H and E: x 240.

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Figure 13a: Hepatic cell of goat given 58.0 µg aflatoxin B_1 per kg body weight. No significant abnormalities in the sizes of the nuclei are seen. There is no evidence of fibrosis of the central vein.

H and E: x 240.

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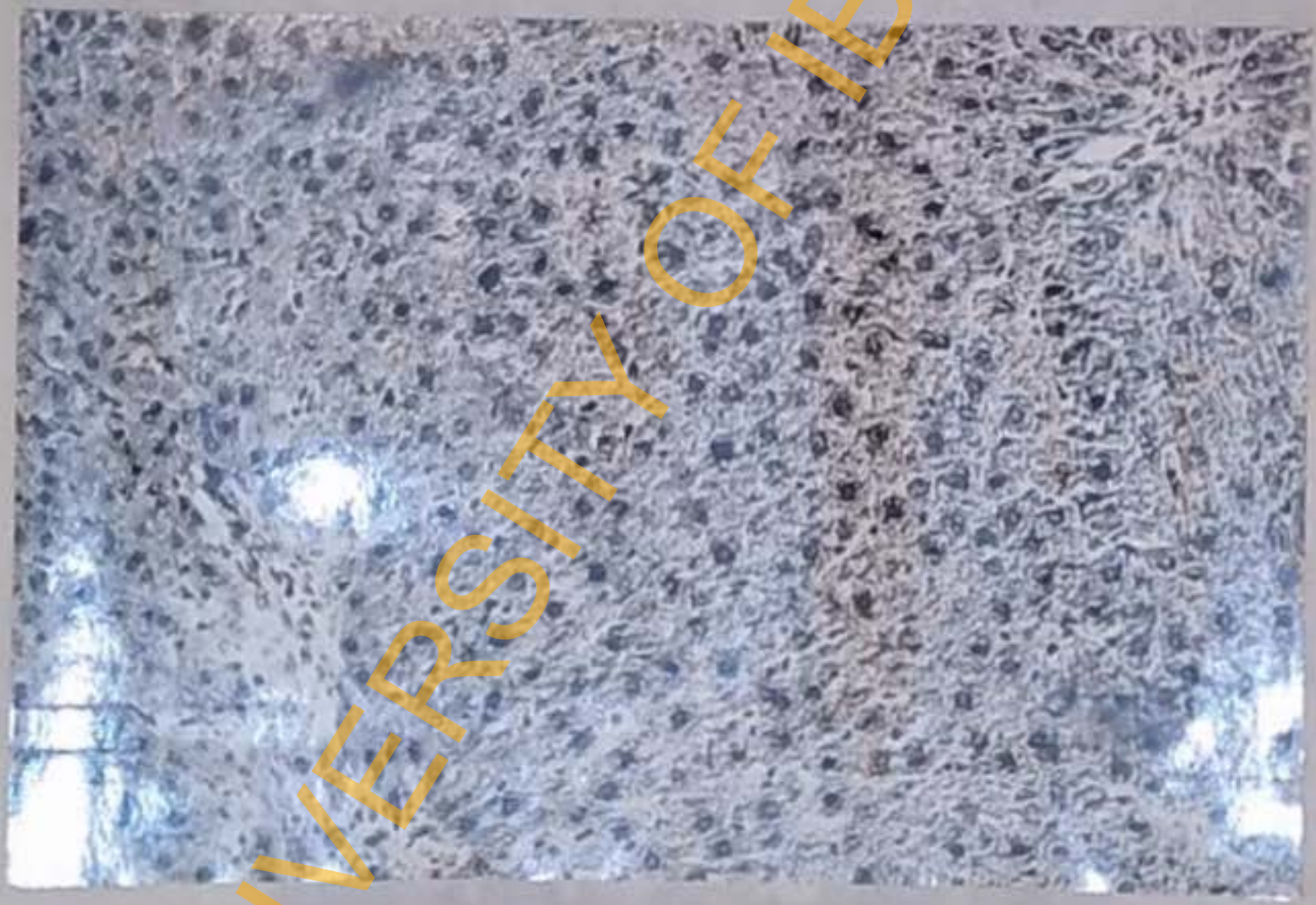

Figure 12b: Centrilobular parenchymal cell in normal rat given distilled water.

H and E: x 240.

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Figure 13b: Hepatic cell of rat given 58.0 µg aflatoxin B_1 per kg body weight. The nodular nature is comparable with the normal. Neither proliferating cells nor fatty infiltration were observed.

H and E; x 260.

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Figure 12a: Centrilobular parenchymal cell in normal duck given distilled water.

H and E: x 250.

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Figure 15a: Hepatic cell of duck given 58.0 µg aflatoxin B_1 per kg. body weight. There is no irregularity of parenchymal nuclear size. Biliary proliferation and fatty infiltration, characteristic of aflatoxin poisoning, were absent.

H and E: x 240.

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proliferation were not apparent (Figures 13a, b). There was no sign of irregularity of parenchymal nuclear size; and fatty infiltration which is characteristic of aflatoxin poisoning was absent in the hepatic cell of duck (Figure 13c).

Experiment IV: Effects of Aflatoxin B1 and 4-Hydroxycoumarin on the Synthesis of Prothrombin by Rat Liver Slices.

The rat is a common laboratory animal which has been recommended for toxicity studies (World Health Organization Technical Report Series, 1966). In this and subsequent experiments the rat has been used in the endeavour to accumulate evidence for the anticoagulant action of aflatoxin.

Pool and Robinson (1939) obtained evidence for the synthesis of blood clotting factors by the liver and inhibition of the synthesis of these factors by 3 (α-acetonylbenzyl) 4-hydroxycoumarin has also been demonstrated by Olson, Miller and Troup (1966). It is known that dicoumarol prolongs blood clotting time by competing with vitamin K for the apoenzyme in the production of prothrombin in the liver (Boyd and Warner, 1948; Mann and Hurn, 1950; Chmielewska and Cieslak, 1958).

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The purpose of this experiment was to make a comparable study of the effects of aflatoxin B_1 and 4-hydroxycoumarin on the *in vitro* synthesis of prothrombin by rat liver slices.

Each experimental animal was a male albino rat of approximately 300g weight. The procedure for the *in vitro* synthesis was a slightly modified form of the method of Pool and Robinson (1959) and described on page 51. Each rat received an intraperitoneal injection of either 58.0 µg aflatoxin B_1/Kg body weight or 50 mg 4-hydroxycoumarin/Kg body weight. The drugs were carried in distilled water and administered in volumes of not more than 1.0 ml each. Control animals were injected with 1.0 ml of pure distilled water. Twelve 1-g quantities of liver slices from (a) aflatoxin-treated rats, (b) 4-hydroxycoumarin-treated rats and (c) control rats were incubated under the conditions described on page 53 and allowed to synthesise prothrombin.

Four hours after start of incubation, 1.0 ml aqueous solution of vitamin K_1 (0.2 mg per ml) was added to each of six incubating flasks from the aflatoxin-treated and 4-hydroxycoumarin-treated groups. After the addition of vitamin K_1 synthesis of prothrombin was observed for a further period of six hours.

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Result.

The amount of prothrombin present in the liver homogenate (or in the incubating medium) at the end of every two hours were read off a standard dilution curve which had been constructed as described on page 55. The incubation medium withdrawn at every instance in the experiment did not clot when it was assayed for prothrombin, indicating that no measurable amount of the clotting factor had leaked across the liver cell membrane to the medium.

In Tables 8 and 9 it will be seen that the liver slices prepared both from the rats treated with aflatoxin B_1 and 4-hydroxycoumarin could not synthesise measurable quantities of prothrombin. But this condition was reversed when vitamin K_1 was added to the liver slices which had been incubating for four hours.

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Period of incubation (hours)	Clotting time of homogenate (sec.)			Concentration of prothrombin in homogenate (units)		
	Normal	Aflatoxin B_1	After the addition of vitamin K_1	Normal	Aflatoxin B_1	After the addition of vitamin K_1
0	200.0	709.0	-	0.95	0.1	-
2	106.0	618.0	-	3.5	0.1	-
4	78.0	410.0	-	6.4	0.23	-
6	52.0	242.0	159.0	15.0	0.65	1.2
8	-	-	64.0	-	-	9.2
10	-	-	54.0	-	-	14.4

Table 8: Effect of aflatoxin B_1 on the synthesis of prothrombin by rat liver slices.

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Period of Incubation (hours)	Clotting time of homogenate (sec.)			Concentration of prothrombin in homogenate (units)		
	Normal	4-hydroxycoumarin	After the addition of vitamin K_1	Normal	4-hydroxycoumarin	After the addition of vitamin K_1
0	201.0	704.0	-	0.95	0.1	-
2	105.0	627.0	-	0.3	0.1	-
4	79.0	460.0	-	0.4	0.18	-
6	53.0	287.0	121.0	-	0.48	2.2
8	-	-	60.0	-	-	10.9
10	-	-	54.5	-	-	13.7

Table 9: Effect of 4-Hydroxycoumarin on the synthesis of prothrombin by rat liver slices.

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Experiment V: Comparative Effects of Aflatoxin B_1, 4-Hydroxycoumarin and Carbon Tetrachloride on the Interaction of Blood Clotting Enzymes in Rat Plasma.

Kinetic studies (Hemker, Loeliger and Veltkamp, 1968) on the clotting reactions which occur with the use of thrombotest has led to the recognition of a protein entity which acts as a competitive inhibitor of thrombin formation. The original name of this protein was preprothrombin but it has recently been replaced by PIVKA (Protein Induced by Vitamin K Antagonists) in order not to confuse it with prothrombin. Relative amounts of PIVKA can be measured by means of a graph of clotting time against plasma dilution.

The use of the estimation of this inhibiter as a means of differentiating between vitamin K antagonism or absence and liver damage in clinical laboratory investigations is well established (Hemker, Van der Meer and Loeliger, 1965).

It is the aim of this experiment to make comparative kinetic studies with clotting tests on plasmas of rats treated with aflatoxin B_1, 4-hydroxycoumarin and carbon tetrachloride respectively, with a view to elucidate the intimate mechanism of the anticoagulant action of aflatoxin B_1.

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A set of sixty male albino rats of the same strain, each weighing approximately 300g were kept at 27°C ± 1°C. They were fed on a normal balanced diet (Bassir, 1964) throughout the experiment. They were also given tap water ad libitum.

Four separate groups, each of twelve rats, were injected intraperitoneally as follows:

(a) 17.5 µg of pure aflatoxin B_1; (b) 2.0 mg of pure aflatoxin B_1; (c) 15.0 mg of 4-hydroxycoumarin; (d) 1.0 ml of carbon tetrachloride. The remaining twelve rats were kept as controls. The aflatoxin and 4-hydroxycoumarin were dissolved in distilled water and administered in volumes of not more than 1.0 ml each.

After a period of three hours, when aflatoxin had prolonged the normal blood clotting time maximally, six of the rats from group (a) were decapitated and the plasmas were pooled (aflatoxin plasma); whilst the remaining six rats of that group were killed after 48 hours and their plasmas pooled (aflatoxin plasma). The plasmas of six rats from group (b) were also pooled (aflatoxin plasma) after a period of three hours; whilst the other six rats from the same group were killed after 48 hours and their plasmas pooled (aflatoxin plasma). The plasmas of the 4-hydroxycoumarin-treated rats in group (c) were

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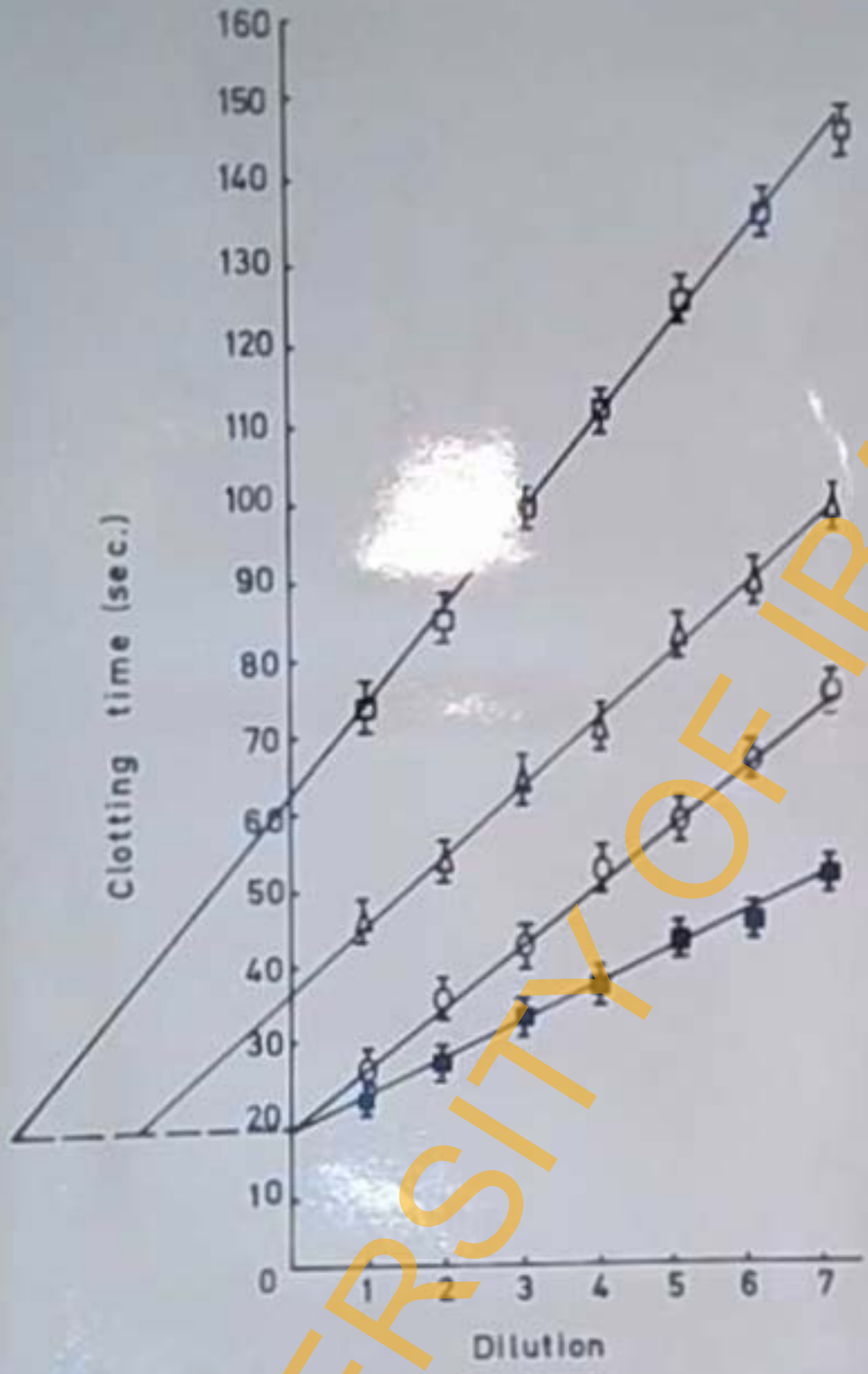

Figure 24: Thrombotest time-dilution plot.

■ Normal plasma.

○ Carbon tetrachloride plasma.

△ Aflatoxin (58.0 µg per kg body weight per 3 hours) plasma.

□ 4-Hydroxycoumarin (50 mg per kg body weight per 48 hours) plasma.

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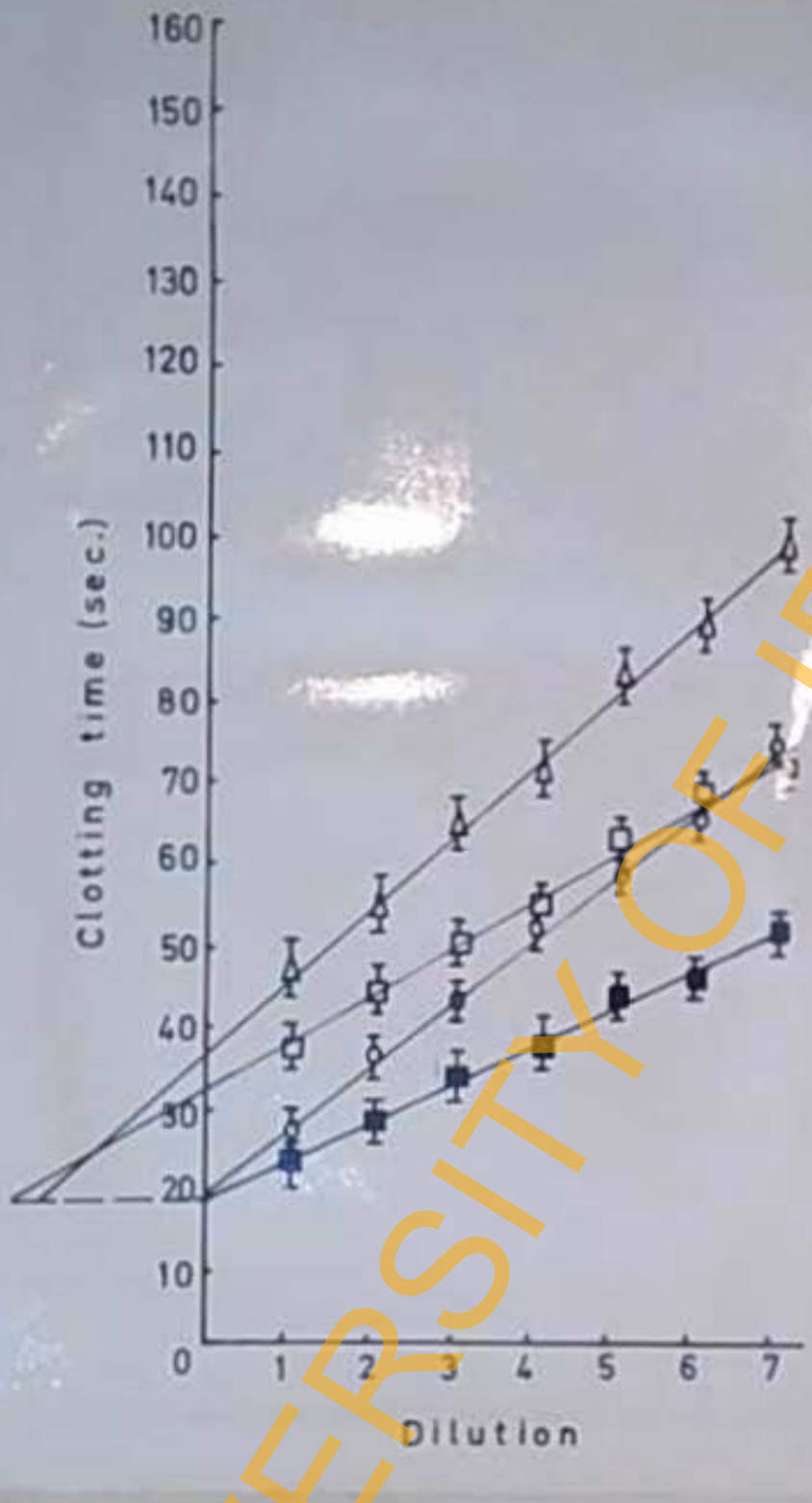

Figure 15: Thrombotest time-dilution plot.

■ Normal plasma.

○ Carbon tetrachloride plasma.

□ A mixture of equal parts of carbon tetrachloride plasma and aflatoxin (58.0 µg per kg body weight) plasma.

△ Aflatoxin (58.0 µg per kg body weight) plasma.

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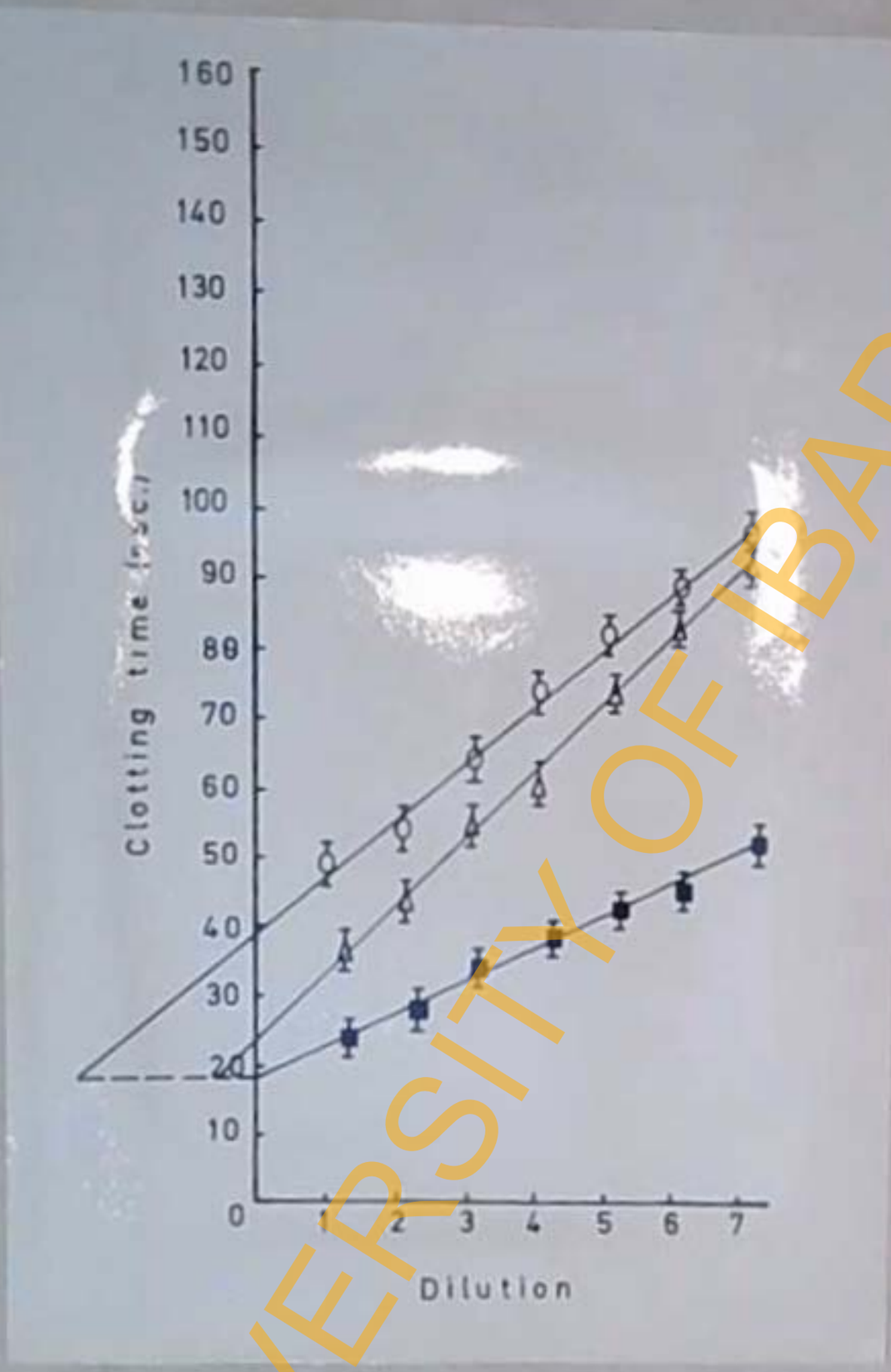

Figure 12: Prothrombotest time-dilution plot.

■ Normal plasma.

Δ Aflatoxin (2.0 mg per 300g body weight per 48 hours) plasma.

O Aflatoxin (2.0 mg per 300g body weight per 3 hours) plasma.

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Plasma	t_n (sec.)	"I" (units)
Normal	18.0	0
4-Hydroxycoumarin	62.0	3.4
Aflatoxin	36.0	2.0
Carbon Tetrachloride	18.5	0
Aflatoxin/Carbon Tetrachloride (1:1, v/v)	31.5	2.3
Aflatoxin/4-Hydroxycoumarin (1:1, v/v)	47.5	2.1

Table XO: Values of t_n and "I" from the clotting time-dilution plots shown in Figures 14, 15, and 16.

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Experiment VI: Effect of Aflatoxin B_1 on the Activities of Lactic Dehydrogenase, Glutamic-Oxaloacetic Transaminase and Alkaline Phosphatase in the Serum of Rat.

Serum levels of lactic dehydrogenase, glutamic-oxaloacetic transaminase and alkaline phosphatase have been determined as a measure of liver function.

Adult male albino Wistar rats weighing approximately 300g were used. They were maintained on the stock balanced diet and water *ad libitum*. Aflatoxin B_1 was administered intraperitoneally at a dose of 50.0 µg per kg body weight. The toxin was dissolved in distilled water and administered in volumes of not more than 1.0 ml each. Control animals received 1.0 ml of pure distilled water. All animals were fasted for 24 hours before sacrifice. Blood obtained from animals was run into clean dry test tubes and allowed to stand at room temperature (28°C) until it has clotted. It was then kept until the clot had retracted and the serum separated. Retraction of the clot was assisted by gently loosening it from the walls of the tube. The separated serum was carefully pipetted into a centrifuge tube and some contaminating suspended cells were removed by centrifuging using the ordinary MSE laboratory centrifuge.

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Experiment VI: Effect of Aflatoxin B_1 on the Activities of Lactic Dehydrogenase, Glutamic-Oxaloacetic Transaminase and Alkaline Phosphatase in the Serum of Rats.

Serum levels of lactic dehydrogenase, glutamic-oxaloacetic transaminase and alkaline phosphatase have been determined as a measure of liver function.

Adult male albino Wistar rats weighing approximately 300g were used. They were maintained on the stock balanced diet and water *ad libitum*. Aflatoxin B_1 was administered intraperitoneally at a dose of 18.0 µg per kg body weight. The toxin was dissolved in distilled water and administered in volumes of not more than 1.0 ml each. Control animals received 1.0 ml of pure distilled water. All animals were fasted for 24 hours before sacrifice. Blood obtained from animals was run into clean dry test tubes and allowed to stand at room temperature (28°C) until it has clotted. It was then kept until the clot had retracted and the serum separated. Retraction of the clot was assisted by gently loosening it from the walls of the tube. The separated serum was carefully pipetted into a centrifuge tube and some contaminating suspended cells were removed by centrifuging using the ordinary MSE laboratory centrifuge.

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Lactic dehydrogenase activity was measured by the method of Wroblewski and LaDue (1955). Glutamic-oxaloacetic transaminase activity was determined according to the procedure of Karmen (1955) and alkaline phosphatase activity was assayed by the method described by Wooten (1964). These procedures are described in pages 60-63.

Result.

Serum enzymes whose activities could be indicative of liver function include lactic dehydrogenase, glutamic-oxaloacetic transaminase and alkaline phosphatase (Wooten, 1964). The activities of these enzymes were determined in the sera obtained from our test rats and the control animals, respectively. The results are given in Table 11. P values are given, as above in the table for the comparison of each experimental group with their controls and are considered statistically significant if $P < 0.05$; where $P > 0.05$, the values are designated as N.S. (not significant). In all, there were no significant differences in the activities within the three-hour period of action of aflatoxin B_1.

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Experiment VII: Effect of the Anticoagulant Action of Aflatoxin B_1 on the Ultrastructure of Rat Liver Cell: (a) Electron Microscopic Studies; (b) Estimation of Ribonucleic Acid in Liver Homogenate.

Specific organelles have been identified in the attempt to correlate different biological action of aflatoxin B_1 with some of the hepatic ultrastructural changes induced in the rat during aflatoxin poisoning (Butler, 1964a; Butler, 1966; Todd et al, 1968; Svoboda and Hoggenson, 1968; Newberne and Butler, 1969).

The present experiments have been undertaken to investigate the effect of aflatoxin B_1 on organelles such as endoplasmic reticulum in the rat liver cells after a sublethal dose of aflatoxin B_1 has prolonged blood clotting. For this purpose morphological changes of liver cells have been observed by electron microscopy. The content of RNA in the liver has been estimated in order to be able to qualify any early structural change which might occur in the endoplasmic reticulum (Clifford and Rees, 1966a) with which most of the microsomal RNA and protein synthesis are associated.

In all the experiments, adult male Wistar rats, weighing approximately 300g each, were used. Prior to administration of the drug, they were fed on the stock balanced diet and were

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Result.

(a) Electron Microscopy. Figures 18 and 19 show sections of centrilobular parenchymal cells. The nucleus in the normal was similar in appearance to that in the experimental animal, and the two have identical structures as those described for a normal cell nucleus by Bruni and Porter (1965). The organelles distributed in the cytoplasm were essentially the same as those described by Fawcett (1955) and Bruni and Porter (1965). In no instance was there any abnormality of the cell membrane. The rough endoplasmic reticulum appeared as orderly stacks of cisternae but in between mitochondria there were only a few cisternae. The mitochondria in the test appeared more rounded with slightly less matrix whilst the mitochondria in the normal liver cell were more oval with an even, finely granular matrix and a few electron dense granules.

(b) RNA in Liver Homogenate. After a three-hour period of action of aflatoxin B_1 there was no increase in liver weight of the rat. After serial removal of acid-soluble phosphorus compounds and phospholipids, RNA was assayed in the supernatant obtained from the liver homogenate extract. There was no significant difference between the RNA content of liver removed

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from the test rats and that from the controls (see Table 11).

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Figure 18: This electron micrograph shows the ultrastructure of the normal rat liver cell. Opsium: X 28,000.

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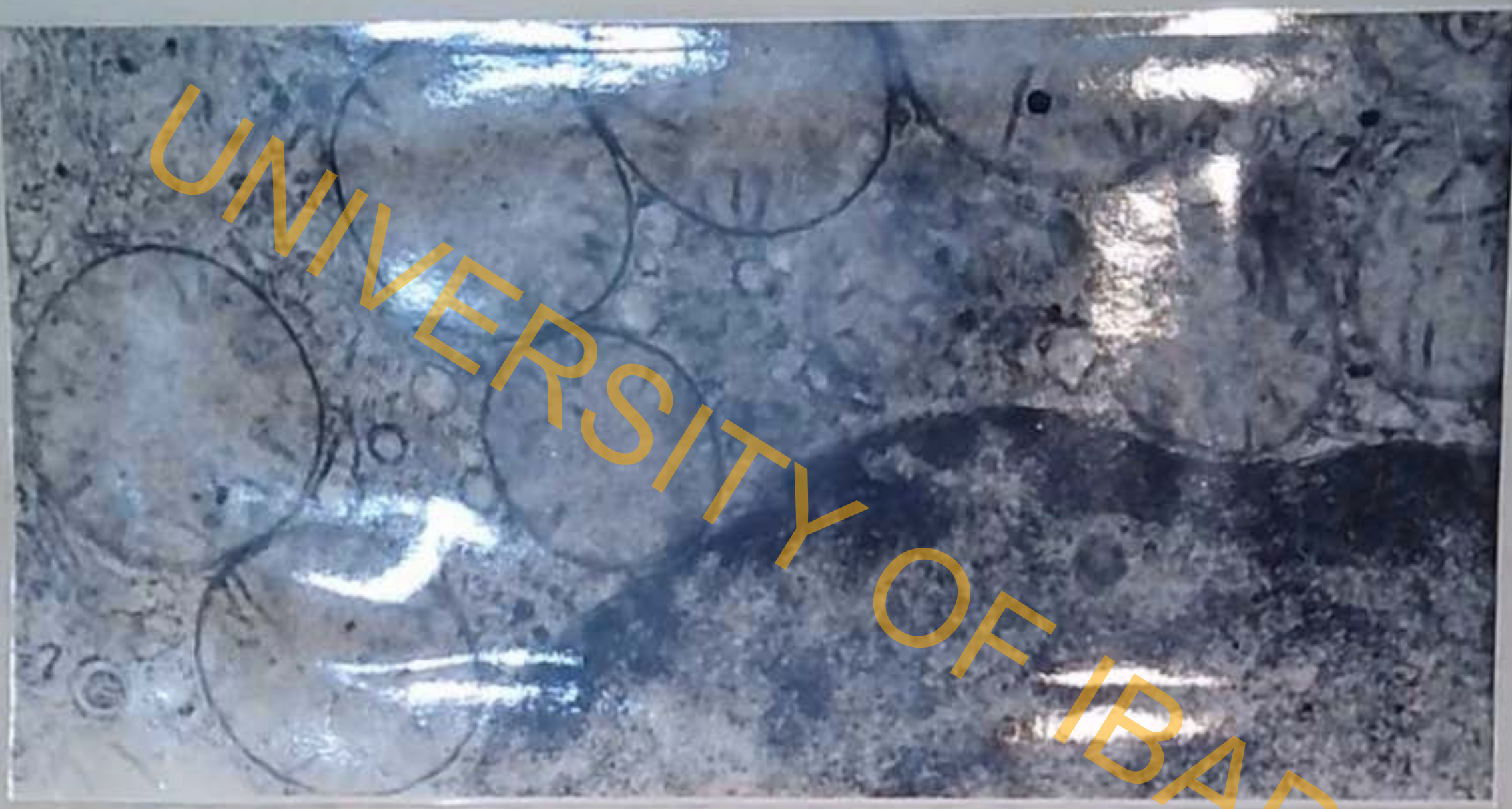

Figure 19: Electron micrograph of hepatocyte from a rat receiving 28.0 ug aflatoxin B_1 per kg body weight. The mitochondria are somewhat rounded. No evidence of any abnormality of the cell membrane. The rough endoplasmic reticulum appear as orderly stacks of cisternae. Osmium: X 28,000.

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CHAPTER FOUR

DISCUSSION

There are three reasons why an attempt has been made to investigate the species differences and the mechanism of anticoagulant action of aflatoxin.

Firstly, aflatoxin is elaborated by fungi, particularly those of the genus Aspergillus, which infest human foodstuffs as well as animal feeds. The anticoagulant activity of a balanced diet which was infected with a toxic strain of Aspergillus flavus had been shown to be due to the action of aflatoxin present in the mouldy diet on blood clotting factors of rats (Bababunmi and Bassir, 1969). Indeed, the presence of mould toxins is potentially the most serious problem which confronts producers and manufacturers of food and feed products.

Secondly, since aflatoxin can prolong blood clotting in the rat, there is need to know how various animal species, with different nutritional habits would react to this anticoagulant property of aflatoxin. The need for this investigation is strengthened by the observation of Joffe (1962, 1963)

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that humans who are undernourished are more severely attacked by haemorrhagic toxicosis - a disease caused by the ingestion of over-wintered cereals infested by the fungus Myrothecium sporotrichioides. This disease (sometimes described as alimentary toxic aleukia) resembles stachybotrytoxicosis caused by a toxin from certain strains of the fungus Stachybotrys atra, which is poisonous to horses and other animal species, including man (Forgacs and Carll, 1962). So far little or no evidence is available as to the susceptibility of man to aflatoxins, but a few instances of liver disease in humans have been correlated with the ingestion of fungal metabolites and plant materials (Selzer and Parker, 1951; Blank, Chin, Just, Meranze, Shimkin and Wieder, 1968). Bras, Selliffe and Stuart (1954) had reported that venous occlusive disease lesions were found in children in Jamaica where Crotalaria fulva L is often included in "bush teas". Future investigations will show whether or not there is a direct relationship between the incidence of kwashiokor, aflatoxicosis and anticoagulant activity of aflatoxin. Kwashiokor is considered as a purely nutritional deficiency syndrome and was first described as a deficiency disease by Williams (1933).

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A third reason why an investigation into the mechanism of anticoagulant action of aflatoxin was attempted is the fact that aflatoxin is probably the most potent carcinogen known (Butler and Barnes, 1964). And so, if we can establish that a minimal dose of aflatoxin (or a non-toxic derivative) prolongs blood clotting time without damage to the liver cell, aflatoxin might yet be therapeutically useful in the management of vascular diseases and, consequently, in the relief of human suffering.

The testing of the safety of potentially toxic substances is normally carried out on laboratory animals such as cats, dogs, mice, rabbits, hamsters and rats because these species are more readily available than human subjects and also they have generally been regarded as cognate substitutes for humans (Parke, 1968). In some experiments described in this thesis, birds (ducks and chickens) were also included because of the association of aflatoxicosis with poultry farms. Studies of species differences are of value in the prediction of the selective toxicity of a chemical substance and also in the consideration of any compound used in the service of mankind in such forms as pesticides and medicines (Williams, 1965; Parke and Williams, 1969).

Williams (1938) had remarked that the composition of diet may influence the metabolism of foreign compounds. In the dog

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Williams (1938) had remarked that the composition of diet may influence the metabolism of foreign compounds. In the dog

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and other animals glucuronic acid could be derived from proteins, or synthesized from carbohydrates or amino acids, and made use of in the detoxication of drugs.

Dingell, Joiner and Hurwitz (1966) observed that the action of foreign compounds can vary with the nutritional status of the animal. And in 1968, Caivem reported that rats maintained on a diet deficient in protein showed a diminished rate of aflatoxin metabolism.

In this thesis, the results obtained on the species variation of the anticoagulant actions of aflatoxin and 4-hydroxycoumarin seem to fit into a dietary classification of the mammalian species, that is, carnivores, omnivores, herbivores. The birds form a separate class. The carnivores have the shortest clotting times, next is the omnivores which are followed by the herbivores. The birds have the longest clotting times. The results obtained from the treatments with aflatoxin and 4-hydroxycoumarin are also somewhat comparable (Tables 5 and 6), except for mouse and the birds which react in a comparable manner to the anticoagulant action of aflatoxin, although these two species have different dietary habits. Also, hamster which has the shortest normal clotting time (with the exception of mouse) shows the greatest reaction to the anticlotting

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effect of aflatoxin. This would suggest that the normal clotting time and the anticoagulant effect of aflatoxin on the different animal species might be due to a number of factors amongst which dietary habits is of importance.

Foreign compounds undergo metabolism in the gastrointestinal tract by the action of the gut microflora (Dacre and Williams, 1968; Renwick and Williams, 1969). The microflora of the gut also give rise to the formation of toxic compounds such as amines and aromatic hydrocarbons (see Parke and Williams, 1969).

Many toxic chemicals, including aflatoxin B_1, are excreted in the bile (Bakir and Gaiyemi, 1967) mostly as glucuronides and other conjugates. Biliary excretion appears to be dependent on the size of the compound excreted; excretion through bile is usually the major route of elimination of a compound with a molecular weight greater than 400 (Millburn, Smith and Williams, 1967). Parke and Williams (1969) reported that conjugates excreted in the bile may be hydrolysed by the action of intestinal micro-organisms to give the original toxins or may be decomposed to give new toxic substances which are subsequently reabsorbed from the gut (Williams, Millburn and Smith, 1965).

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In the investigation described in this thesis the drugs were administered intraperitoneally and not orally. Therefore, the species variation of the anticoagulant effect of aflatoxin might be due to the enterohepatic circulation of aflatoxin and also to the different activities of the gut microflora in each animal species, in the metabolism of the drug in the gastrointestinal tract.

Lupton (1947) had shown conclusively that the liver was the site of action of the anticoagulant dicoumarol and confirmed that the mechanism of the anticoagulant action of coumarin or allied drugs is by vitamin K antagonism. In this respect, Koller, Koeliger and Duckert (1951); Douglas and Nair (1958) had observed that plasma obtained after a therapy of a coumarin compound such as ethyl biscoumacetate was deficient in the vitamin K-dependent clotting factors viz., II, VII, IX and X. Using thrombotest technique in our clotting time measurements indicates that any, or all, of these four factors could have been affected during aflatoxin-induced prolongation of blood clotting time.

Liver is the major site of action of aflatoxin (Clifford and Rees, 1966) and in view of the fact that aflatoxin is a potent liver carcinogen, it was considered necessary to investigate the functional state of the livers

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in different species, the differences in blood clotting times of rat, goat and duck follow the same pattern. Species differences in the metabolism of foreign compounds occur frequently and some of their enzymic bases have been discussed by Williams (1967) and Parke (1968). This study of the clearance of indocyanine green suggests that the liver parenchymal cell of each experimental animal is intact at the time when aflatoxin prolongs blood clotting time maximally.

Wooton (1964) had stated that serum enzymes whose activities could be indicative of liver function include lactic dehydrogenase, glutamic-oxaloacetic transaminase and alkaline phosphatase. As shown in Table 12, the results of tests for these enzymes in rat serum indicate that the liver was functioning normally at the end of the three-hour period of action of aflatoxin B_1. Therefore, the possibility of any abnormal physiological conditions which could have caused a leakage of these enzymes out of cells into the blood can be eliminated.

Metabolites of aflatoxin have been detected in the liver half hour after the administration of the drug (Butler and Clifford, 1965). Evidence has been presented by Oniyemi (1968) to show that liver is the major site for the metabolism

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of aflatoxin; rat on a high-protein diet excretes mainly conjugation products, but no free aflatoxin, and that the reverse is the case for the rats on low-protein diet. McLean and McLean (1965) had suggested that the activity of the drug metabolising enzymes is depressed in animals on inadequate diets. Dicoumarol, a well-known haemorrhagic agent, is not conjugated in either man or dog, and is slowly metabolised to unknown products (Parke, 1968).

Using the fluorescent antibody technique the liver parenchymal cell has been found to be the site for the synthesis and prothrombin, a blood clotting factor and also a protein (Barnhart, 1960; Anderson and Barnhart, 1964). Bassir and Bababunmi (1969) reported that prothrombin and proconvertin were deficient in the plasma of the rat which was injected with pure aflatoxin B_1 when thromboplastin was employed as the thrombokinase. Evidence is presented in this thesis to show that a sublethal dose of aflatoxin B_1 inhibits specifically prothrombin synthesis in rat in a way similar to that of 4-hydroxycoumarin where the drug competes with vitamin K for the apoenzyme in the synthesis of prothrombin by the liver. Tables 8 and 9 show the result of the effect of aflatoxin and 4-hydroxycoumarin on the <u>in vitro</u> synthesis of prothrombin by rat liver slices. In this experiment, it is

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(see Table 10) obtained from both aflatoxin and 4-hydroxy-coumarin treatments are much greater than zero, unlike that obtained from carbon tetrachloride treatment. These high "I" values are probably related to the degree of vitamin K antagonism. In the case of the carbon tetrachloride treatment, on the other hand, the resulting damaged liver would have caused a depletion of plasma constituents by the liver rather than enhance the production of a new protein inhibitor (PIVKA). These results, therefore, seem to be in agreement with those of Henker et al (1968) who demonstrated the presence of this inhibitor in patients treated with coumarin drugs.

With an increase in the dosage of aflatoxin from 58.0 µg per kg body weight to 7.0 mg per kg body weight, there was still a comparable prolongation of blood clotting time after a three-hour period of action (Figure 17). But after a duration of 48 hours, the profile obtained with the larger dose tended to be similar to that of carbon tetrachloride. "I" in this instance was nearly zero (see Table 10). This would suggest that the liver had become damaged under the conditions of (a) longer period of action, and (b) increase in aflatoxin dosage. Since the liver is the main site of protein (including prothrombin) synthesis, the level

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of plasma clotting factors will be expected to drop in hepatocellular disease. Also, the increase in the blood clotting time under any conditions of liver damage could be due to the failure of bile salt secretion into the intestine which will result in inadequate vitamin K absorption (Sherlock, Barber, Bell and Watt, 1961). The profile obtained with the low dose after three hours did not change after 48 hours. It can, therefore, be assumed that the livers of animals treated with the low dose of aflatoxin were intact after 48 hours and that at this dose level of aflatoxin, the mechanism of anticoagulant action of the compound is similar to that of 4-hydroxycoumarin. The morphology of liver cells gives a clue to the action of most carcinogens (Lee, Miles, Ayres and Sinnhuber, 1968; Schoental, 1968). The immediate effect of lethal doses of aflatoxin on the liver parenchymal cell is dilation of rough endoplasmic reticulum cisternae and dislocation of ribosomes in the periportal cells (Butler, 1966). In this study, with the dosage of aflatoxin used, no sign of pathological alteration has been observed. Butler (1964a) has also shown that toxic doses of aflatoxin B_1 induce periportal zones of necrosis. But in our paraffin-embedded tissues obtained with haematoxylin and eosin (Figures 12 and 13) this was not recognised. Also, fatty infiltration which would compare

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with the effect of a liver carcinogen such as carbon tetrachloride (Rees and Shotlander, 1963) was not observed.

It has been shown that in normal liver parenchymal cells, the mechanisms of protein synthesis are associated with the rough endoplasmic reticulum (Palade and Siekevitz, 1956; Siekevitz and Palade, 1960). Two hours after the administration of an anticoagulant dose of aflatoxin, no dilatation of the rough endoplasmic reticulum has been demonstrated. Nor was there any dislocation of ribosomes.

Changes in liver RNA content seem to reflect structural changes in the endoplasmic reticulum which possesses the drug metabolising enzyme system (Clifford and Rees, 1966a). The contents of liver RNA of the test rats in our investigations were not different from those of the control. This observation agrees very well with the conclusion of Ceuri and Vesilait (1966) that coumarin anticoagulants do not inhibit endogenous factors at the peak of their effect on prothrombin time. It appears, therefore, that the lengthening of blood clotting time by aflatoxin, arising from the reduction of prothrombin production, was independent of any alteration in microsomal RNA.

In aflatoxin poisoning, there is ample evidence indicating an inhibition of messenger-RNA production and a

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consequent inhibition of protein synthesis (Clifford and Rees, 1967); this mechanism is outlined in Figure 20. In carcinogenesis, aflatoxin binds on to DNA (Clifford and Rees, 1966, 1967). In this respect the interaction of aflatoxin B_1 to DNA is very different from that of actinomycin D (Reyreton, 1961). The binding of aflatoxin B_1 to the purine ring and purine nucleosides is relatively weaker (Clifford and Rees, 1967a). Although both aflatoxin B_1 and actinomycin D produce similar cytological changes in regenerating rat liver (Bernhard, Frayssinet, Lafarge and Le Breton, 1965; Schwartz, Sodergren, Garofalo and Sternberg, 1965) it is known that actinomycin D does not produce liver necrosis in the intact rat liver unlike aflatoxin B_1 which is a necrotic agent. Clifford and Rees (1967) have suggested that this difference in the toxities of aflatoxin B_1 and actinomycin D could be due to the relative binding strength to DNA.

Also, it has been shown by Clifford and Rees (1967) that in aflatoxin poisoning there is no inhibition of the incorporation *in vivo* of amino acids into liver proteins; this is unlike the observation of Rees and Shotlander (1963) of the inhibition of the incorporation *in vivo* of amino acids into liver protein after the action of hepatotoxic agents such

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as carbon tetrachloride and dimethylnitrosamine.

Since no hepatocellular damage has been observed at the time when a sublethal dose of aflatoxin B_1 prolongs blood clotting time maximally, there could only have been a very weak or no interaction with DNA. Also, at this dose level, the incorporation *in vivo* of amino acids into prothrombin *per se* could hardly be inhibited.

In many instances, the integrity of an enzymatic process requires the participation of one or more coenzymes. Coenzymes are organic molecules of a size intermediate between the small-molecule intermediary metabolites, which serve as the substrates of enzymic reactions, and the macromolecular proteins. A coenzyme (containing a vitamin as part of its structure) is the portion (prosthetic group) which can easily be dissociated from the protein component (apoenzyme). The combination of the apoenzyme and the coenzyme forms the complete, enzymatically active, conjugated protein (holoenzyme). Each coenzyme acts usually as acceptor or donor of some specific type of atom or group of atoms removed from or added to a small-molecule substrate in a reaction catalyzed by the holoenzyme.

Amongst quinones which play important roles in the living cell is vitamin K. Vitamin K is the cofactor of the

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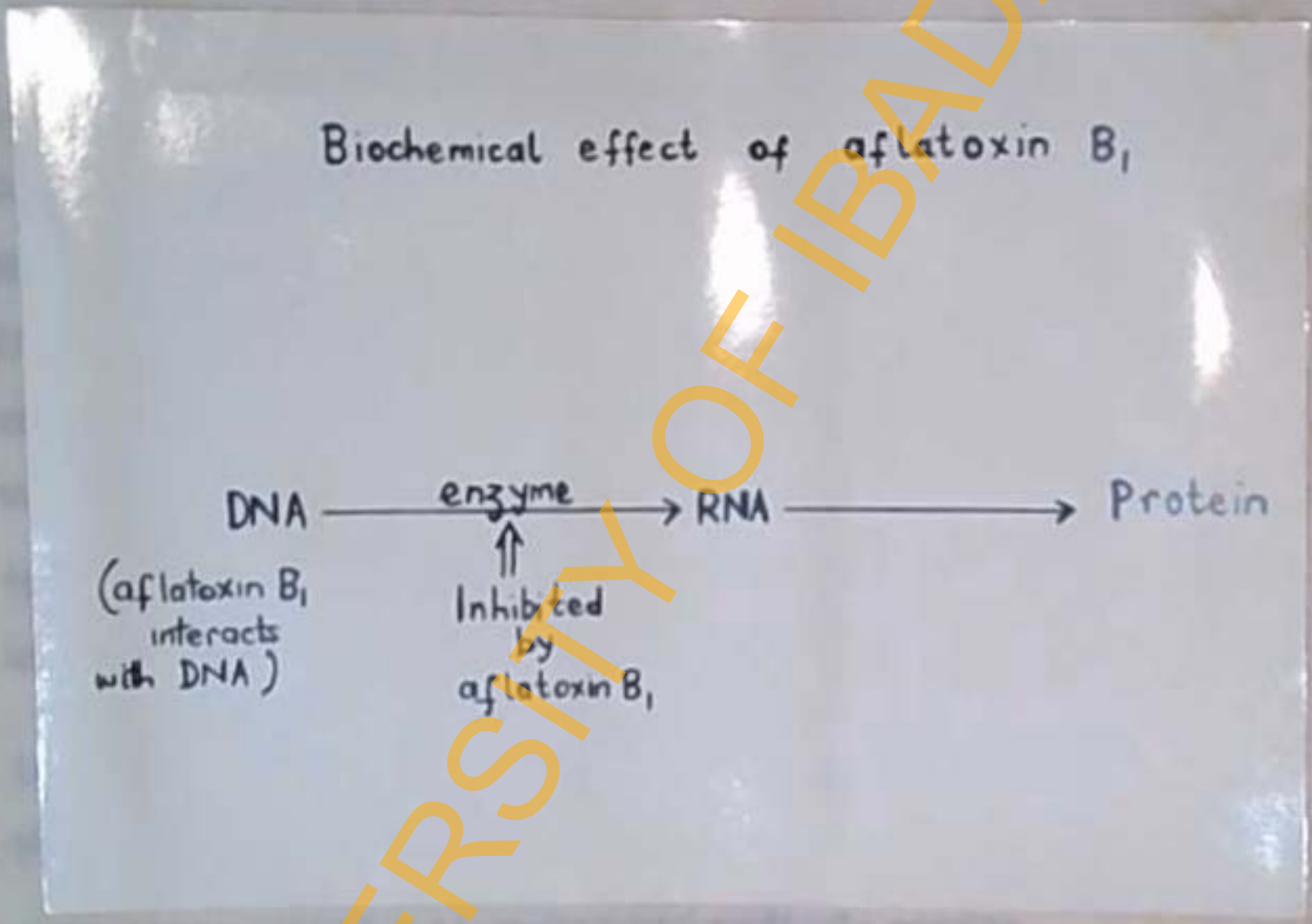

Figure 20: Biochemical effect of aflatoxin B_1.

Aflatoxin B_1 interacts with DNA under conditions in which the two compounds are brought into contact '*in vitro*' (Sporn *et al*, 1966; Clifford and Rees, 1966, 1967). Aflatoxin B_1 inhibits RNA polymerase, the enzyme responsible for DNA-directed RNA synthesis (Gelboin *et al*, 1966).

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apoenzyme which catalyses the synthesis of prothrombin, the rate-limiting step in the clotting of blood.

In this thesis, biochemical evidence has been given to demonstrate a competition between vitamin K and aflatoxin B_1 (or 4-hydroxycoumarin) for the active site of an apoenzyme. This competition might have been due to the resemblance of the aflatoxin structure to the quinone-structure of vitamin K. In view of this, kinetic and microscopic findings, the mechanism outlined in Figure 21 is proposed for the anti-coagulant action of aflatoxin:

(i) aflatoxin B_1 competes with vitamin K for the active site of the apoenzyme (AE);

(ii) as a result of this competition, the formation of prothrombin is depressed, and

(iii) this reduction in prothrombin synthesis results in the prolongation of blood clotting time.

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Antivitamin-K activity of aflatoxin B_1

$$AEK + B \rightleftharpoons AEB + K$$

K = Vitamin K

AE = Apoenzyme in liver cell

B = 4-hydroxycoumarin or aflatoxin B_1

Figure 21: The proposed mechanism of the anticoagulant action of aflatoxin B_1 - antivitamin K activity.

Vitamin K(K) is the cofactor of the apoenzyme (AE) responsible for producing prothrombin in the liver. 4-Hydroxycoumarin or aflatoxin B_1 (B) compete with vitamin K for the apoenzyme.

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SUMMARY OF RESULTS

A:

(i) There is a species variation in the anticoagulant action of aflatoxin and 4-hydroxycoumarin.

(ii) This variation seems to fit somewhat into a dietary classification of the mammalian species, that is, carnivores, omnivores and herbivores. The birds seem to form a separate class.

B:

When the removal of indocyanine green from the livers of healthy adult goat, rat and duck was investigated and compared with the removal after treatment of these animals with (a) carbon tetrachloride and (b) a sublethal dose of aflatoxin B_1, the following observations were made:

(i) there is a species variation in the plasma removal rate for indocyanine green.

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(ii) the patterns of clearance of the dye in animals treated with aflatoxin indicate that their liver parenchymal cells are intact after a three-hour ingestion of aflatoxin B_1. This period is required for aflatoxin to prolong blood clotting time maximally in these animals. The profiles of the carbon tetrachloride-treated animals were distinctly different from those of the normal.

C:

Comparative kinetic studies with clotting tests on plasmas of rats treated with 4-hydroxycoumarin, aflatoxin B_1 and carbon tetrachloride respectively, gave the following results:

(i) the minimal clotting time (t_m) - intercept of the profile with the ordinate - obtained with either aflatoxin B_1 or 4-hydroxycoumarin plasma is greater than that of the normal plasma but the t_m obtained with the carbon tetrachloride plasma is approximately equal to that of the normal plasma. t_m of a mixture of equal volumes of aflatoxin and carbon tetrachloride plasmas is only slightly lower than that of aflatoxin plasma alone.

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(ii) t_m obtained with the mixture of equal volumes of 4-hydroxycoumarin and aflatoxin plasmas is a mean of the minimal clotting times of the two plasmas at 99% level of confidence. This indicates a similarity in the mechanism of prolongation of clotting time in the aflatoxin and 4-hydroxycoumarin treatments.

(iii) t_m of aflatoxin plasma, after a three-hour period of action, was not increased by an increase in dosage. It was, however, lowered significantly by the high dose when the period of action was 48 hours. The t_m of the low dosage aflatoxin plasma, after 48 hours, was exactly the same as that after three-hour period of action.

D:

Investigations on the ultrastructure of the rat liver cell after a sublethal dose of aflatoxin B_1 has prolonged blood clotting time maximally show that:

(i) the typical hyperplastic nodules and bile duct proliferation which accompany aflatoxin carcinogenesis were not present.

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(ii) Features which characterize hepatocellular damage caused by a liver carcinogen, such as carbon tetrachloride, were also absent.

(iii) When RNA was assayed in liver homogenate extract, there was no significant difference between the RNA content of liver removed from the test rats and that from the controls.

(iv) In all the determinations of the activities of lactic dehydrogenase, glutamic-oxaloacetic transaminase and alkaline phosphatase in serum, there were no significant differences in the activities within the three-hour period of action of aflatoxin B_1.

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