THIS DISSERTATION SUBMITTED BY

ENITAN ABISOGUN BABARUNMI

WAS ACCEPTED IN PART FULFILMENT

OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR IN THE FACULTY

OF SCIENCE

OF THE UNIVERSITY OF IBADAN

THE DEGREE WAS AWARDED ON

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A STUDY OF THE EFFECT OF THE AFLATOXINS ON BLOOD CLOTTING.

A THESIS

PRESENTED BY

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ABSTRACT

The structural configurations of the synthetic coumarins have been shown to be of major importance in their action on blood clotting. In view of the similarities in the structures of the synthetic coumarins and the aflatoxins, attempts have been made to compare the blood clotting activities exhibited by 4-hydroxycoumarin with similar effects obtained with the aflatoxins.

The effect of a balanced diet, which had been infested by a toxic strain of Aspergillus flavus, was fed to a set of rats. The blood clotting times of these animals and their controls were determined. A "Thrombotest" reagent, which contains simultaneously quantities of blood clotting factors II, VII, IX and X present in a known volume of blood, was used. The clotting time of blood withdrawn from the poisoned animal was prolonged by approximately 65% of the normal time. There was a suggestive evidence that the increase in blood clotting time was due to the action of aflatoxins which are metabolites of Aspergillus flavus. The mouldy diet was therefore extracted with methanol and the mixture of aflatoxins purified using chloroform in thin layer chromatography. The different effects on blood clotting time of (a) the infested diet, (b) the aflatoxin mixture, and (c) pure aflatoxin B_1, were compared. The average percentage increase in clotting times in the three cases were the same.

ABSTRACT

The structural configurations of the synthetic coumarins have been shown to be of major importance in their action on blood clotting. In view of the similarities in the structures of the synthetic coumarins and the aflatoxins, attempts have been made to compare the blood clotting activities exhibited by 4-hydroxycoumarin with similar effects obtained with the aflatoxins.

The effect of a balanced diet, which had been infested by a toxic strain of Aspergillus flavus, was fed to a set of rats. The blood clotting times of these animals and their controls were determined. A "Thrombotest" reagent, which estimates simultaneously quantities of blood clotting factors II, VII, IX and X present in a known volume of blood, was used. The clotting time of blood withdrawn from the poisoned animal was prolonged by approximately 50% of the normal time. There was a suggestive evidence that the increase in blood clotting time was due to the action of aflatoxins which are metabolites of Aspergillus flavus. The mouldy diet was therefore extracted with methanol and the mixture of aflatoxins purified using chloroform in thin layer chromatography. The different effects on blood clotting time of (a) the infested diet, (b) the aflatoxin mixture, and (c) pure aflatoxin B_1, were compared. The average percentage increase in clotting times in the three cases were the same.

The antagonism of therapeutic amounts of 4-hydroxycoumarin by vitamin K preparations was studied in rats, and comparison was made with the effect of these vitamin K preparations on the blood clotting of aflatoxin B_1 - treated animals. Vitamin K_1 and, to a lesser degree, 2 - methyl-1:4 - naphthoquinone (Menadione) were effective in decreasing the prolonged clotting times which were induced by 4 - hydroxycoumarin and aflatoxin B_1.

By using the "thromboplastin" reagent, factors II and VII were found to be deficient in the plasma obtained from the blood of the aflatoxin - poisoned animal. A study of the "In vitro" synthesis of these two factors by rat liver slices was attempted. Inhibition of the syntheses of these factors was caused by the presence of aflatoxin and also by the administration of 4-hydroxycoumarin. In each case, reversal of inhibition with vitamin K_1 has also been demonstrated.

It was necessary to investigate whether the deficiency of one or both of factors II and VII was responsible for this prolongation of blood clotting. For this purpose, thromboplastin was replaced with viper venom in the clotting time determinations of plasma. Only the depression of prothrombin content of the plasma was measured.

Some liver function tests were performed on the experimental rats when the effects of aflatoxin B_1 and 4-hydroxycoumarin on blood clotting were maximal, side by side with their controls, in order to determine whether prothrombin was being destroyed in the parenchymal cells of the liver or whether the aflatoxins were acting like the coumarins by competing with vitamin K, the latter being an essential cofactor in the production of prothrombin in the liver cells.

Two liver function tests were performed on the experimental rats when the effects of aflatoxin B_1 and 4-hydroxycoumarin on blood clotting were noted, side by side with their controls, in order to determine whether prothrombin was being destroyed in the parenchymal cells of the liver or whether the aflatoxins were acting like the coumarins by competing with vitamin K, the latter being an essential cofactor in the production of prothrombin in the liver cells.

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INTRODUCTION

In man, the complex mechanism of blood coagulation breaks down only in conditions such as thrombosis, when excessive clotting may block essential blood vessels, or in haemophilia, when the blood fails to clot.

Early in this century, haemorrhagic septicaemia, a disease associated with excessive bleeding and a long whole blood clotting time was recognised in Canadian Cattle. This 'sweet clover disease' was traced to ingestion of improperly cured hay made from common types of sweet clover (Schofield, 1924). It was found that the disease disappeared when the cattle ceased to be fed on the spoiled hay. Roderick (1931) was unable to prepare prothrombin (blood clotting factor II) from the blood of affected animals, and he also found that when prothrombin was added to the plasma of diseased animals, the clotting time was reduced. Later, Quick's one-stage prothrombin technique was found to give abnormal results with the plasma of the diseased animals (Quick, 1936).

Link (1941) developed a bio-assay for the haemorrhagic agent in the spoiled sweet clover using rabbits. Quick's one-stage technique was used. The plasma was diluted to give a considerable "spread" to the clotting time figures as compared with the test on the concentrated plasma. These studies culminated in the isolation and synthesis of dicoumarol, and in the establishment

of its chemical formula —3,3' - ethylene-bis-4-hydroxycoumarin. Campbell and Link (1942) suggested that the biological synthesis of this substance from coumarin during spoilage of the hay might be due to oxidation of coumarin to 4-hydroxycoumarin, which couples with formaldehyde to give dicoumarol.

Arora and Mathur (1963) postulated that in the anticoagulant activity associated with the coumarin molecule, the anticoagulant competes with Vitamin K in its blood clotting role of producing prothrombin in the liver.

Olson et al (1966) obtained evidence for the synthesis of prothrombin and proconvertin (factor VII) by the liver. They also demonstrated inhibition of the syntheses of these factors by 3(α-acetonyl benzyl)-4-hydroxycoumarin and a reversal of the inhibition by vitamin K.

The discovery of aflatoxins as contaminants in animal feeds (De Lough, 1962: Hampton, 1962) has aroused the interest of research workers to determine the effects of these toxins on various biological systems. Aflatoxins are produced by strains of Aspergillus flavus, a common fungus. Aspergillus flavus, can be grown on both natural and synthetic media. Sargeant et al (1961) first reported that the toxic properties of certain samples of peanuts were due to metabolic products of Aspergillus flavus. Nesbitt et al (1962) and Ambrecht et al (1963) used a Cowpak-Dox

medium to which zinc sulphate was added to increase the yield of aflatoxin in cultures of A. flavus. Bassir (1964) demonstrated that the production of aflatoxin, and hence the incidence of "aflatoxicosis", varied appreciably when spores of A. flavus were grown on sterilised crushed wheat, rice, beans, guri, and soya, or mixtures of these materials. Calyeud et al (1967) indicated that if an equimolecular mixture of fructose and glucose is used as the carbon source in the Czapek-Dox medium in place of sucrose, the production of the toxic metabolite of A. flavus is greatly enhanced.

Asao et al (1963) and Hartley et al (1963) have indicated that there are at least four aflatoxins. They have also determined the structures of B_1, B_2, G_1 and G_2. These aflatoxins have a furocoumarin configuration.

The study of the biological effects of the aflatoxins has become a major project in several laboratories, especially in those countries where groundnut constitute a major feeding-stuff.

Thorpe (1965) examined the histochemistry and electron microscopy of acute liver lesions induced by aflatoxin B_1 in ducklings, and he suggested that this toxic principle was transported by the red blood cells. He showed that at least one of its cytotoxic effects was due to a direct action on the liver cell embranes.

Butler (1964) showed that a single dose of aflatoxin B_1 depressed protein synthesis for over 48 hours. He suggested that in these circumstances, the inhibition of protein synthesis could play an important part in the development of periportal necrosis.

Basadir (1964) showed that rats fed on balanced mixed diets on which toxic strains of Aspergillus flavus had been growing for 6 days, 9 days, and 12 days respectively, lost weight rapidly. The reduction of growth rate on the less contaminated diets was in proportion to the concentration of the aflatoxin produced in the diets. The liver cells of the animals were carcinomatous, with rapid deterioration of the bile ducts.

The classical theory of blood clot formation which was put forward by Howell's (1905) suggested that thromboplastin (factor III) released from damaged tissue or blood cells activated prothrombin, to produce thrombin, in the presence of calcium (factor IV). The thrombin in turn activated fibrinogen to produce fibrin. However, prothrombin activation is now thought to be due to a whole series of factors taking effect in the presence of phospholipids and calcium. The phospholipid is derived either from damaged tissue cells in the extrinsic system or from blood platelets. At least 13 blood clotting factors have been described, so far. These factors appear to be protein which are

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present only in trace amounts in normal plasma. Unlike fibrinogen (factor I), they cannot be readily detected by electrophoretic or other physico-chemical techniques.

Their presence or absence in a given plasma must be inferred from the results of addition experiments in suitably designed "in vitro" clotting systems.

Owren (1947) indicated that the prolongation of the clotting time of blood induced by dicoumarol was not necessarily due to a deficiency of prothrombin, but could be caused by a deficiency of some other coagulation component. In view of the similarities in the structures of the cyclotic coumarins and the aflatoxins, the experiments described in this thesis were undertaken to determine the effect of aflatoxin on blood clotting.

METHODS

1. **The Preparation of a Balanced Diet for the Experimental Rats.**

The method used for the preparation of a balanced diet was that described by O. Bassir (1964). The diet was composed of Garri flour (1000 grams), Soya bean flour (1000 grams), Salt Mixture (100.5 grams), Methionine (40 grams) and Lysine (2.0 grams).

360.0 grams of this diet was made into a dough with 370.0 ml. of boiling water in a bowl and then 50.0 gram-portions of the dough were plated out in petri dishes. The dishes with the food were then sterilized for twenty minutes at 15 lbs. pressure.

2(1) **The Preparation of "Chromatoplate" for Thin-Layer Chromatography.**

The adsorbent used was Silica Gel G. It was spread on glass plates using Stanton's thin-layer chromatographic equipment according to the procedure of Stahl (1962).

30 gm. of the adsorbent was used to coat five, (20 cm. x 20 cm.) "Chromatoplates" with a layer 250 u. thick. Activation of "chromatoplates" was carried out in a drying oven at 110° C. for 90 minutes. The prepared plates were then stored in a cabinet.

2(ii) Extraction and Estimation of Daily Yields of the Aflatoxins from Dist :

a) Inoculation of dist :

A pinpoint of the sporulum of a toxic strain of Aspergillus flavus, which had been sub-cultured in a Czapek medium containing sodium Nitrate (3.0 grams), Magnesium Sulphate crystals (15.0 grams), Potassium Chloride (15.0 grams), Ferrous sulphate crystals (0.01 grams) Sucrose (150.0 grm.), Agar (30.0. grams), and water (1000 ml.) was transferred aseptically into the centre of the dist in each petri dish. It was covered and allowed to incubate for five days at room temperature (28° C.) (O. Bankir, 1983).

b) Extraction of the Aflatoxins:

This contaminated dist was extracted continuously in a clean soxhlet extractor for six hours, using methanol as solvent (de Iongh et al, 1964). The methanolic extract was then diluted with water to a methanolic concentration of 60% and extracted with chloroform (de Iongh et al, 1964).

c) Thin-layer Chromatography of the Toxins.

The chloroform extract was concentrated to 25 ml. under vacuum (Adye and Mateles, 1964) and aliquots of the extract was spotted on activated thin-layer plates made of Merck A.G. Silica Gel G (de Iongh et al, 1964).

The plates were immediately developed in a solution of 3 per cent. Methanol in pure chloroform. The plates were then examined for fluorescence under ultraviolet light at 365 mu.

a) Assessment of levels of Aflatoxin.

The mull-fluorescence dilution technique (Tropical Products Institute Report No. 6, 1965) was used to estimate the amount of aflatoxins in the daily intake of the contaminated diet.

0.2 ml. of the concentrated extract (described above) was made up to 20.0 ml. with chloroform. This gave a 1.100 dilution. Serial dilutions of this solution were then examined for the characteristic blue or green fluorescence of the aflatoxins under ultraviolet light at 365 mu, after spotting on the chromatoplate and running on 3 per cent. Methanol in chloroform.

The amount of Aflatoxin B in the spot with the least fluorescence is given as 4×10^{-4} ugm; whilst that of Aflatoxin G is 3×10^{-4} ugm. (Tropical Products Institute Report, 1965).

3. Determination of Blood clotting time:

A commercial "thrombotest" reagent was used in the determination of blood clotting time. This method was developed by Owren (1939). In this thrombotest system, all clotting factors were held constant except factors II, VII, IX, and X.

The "thrombotest" reagent is specifically deficient in these four factors, and the clotting time is therefore dependent exclusively upon the concentration of these four factors in the blood sample to be tested. The reagent was supplied as a freeze-dried substance in a vacuum - sealed ampoule graduated to measure 2.2 ml. for 8 tests (Nycomed and Co., Oslo, Norway).

Venous blood was collected in the proportion of 9 parts of blood and 1 part of 3.13 per cent sodium citrate dihydrate solution in water. 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. 0.05 ml. of the citrated blood was pipetted off and blown into the reagent immediately, holding the tip of pipette just above the surface of the reagent and against the inner wall of the test tube, and starting the stop-watch simultaneously.

The test-tube containing the citrated blood and the reagent was flicked once and the mixture was then left in the water bath 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 moment of coagulation of the mixture in the test tube was recorded.

4. Determination of The Combined Effect of Prothrombin and Proconvertin.

The method used for the determination of the combined effect of prothrombin and proconvertin was that described by Owren and Aas (1951).

Blood was collected into 2.5 per cent potassium oxalate solution in distilled water in the proportion of one part of oxalate solution to nine parts of blood. The oxalated 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. The dilution solution was made of Owren's buffer, pH 7.35 (200 ml.), 0.9 per cent saline (600 ml.) and solution A (200 ml.) Solution A was made of 3.13 per cent. (w/v) trisodium citrate dihydrate aqueous solution (240 ml.) and Distilled water (760 ml.). Owren's buffer was prepared by dissolving 5.88 grams of sodium diethylbarbiturate (Barbitone-sodium) and 7.34 grams of sodium chloride in a mixture of 785.0 ml. of distilled water

and 215.0 ml. of 0.1N. Hydrochloric acid. 0.1 ml. of the diluted plasma was transferred into the bottom of a clean dry small test tube in a water-bath at 37° C. 0.1 ml. of thromboplastin (UDCO LABORATORIES, DETROIT 1, MICHIGAN) was added and the tube was twirled to mix the contents. The tube was allowed to stand in the water bath for 30 seconds to come to the working temperature of 37° C. "Bacto-thromboplastin" was supplied in ampoules of 150 mgm. each. Before the thromboplastin was used, it was extracted and centrifuged as follows :- one ampoule was emptied into a clean, dry test tube. 4.0 ml. of saline was added to it, and the tube was twirled in order to suspend all the particles. The tube was then placed in a 45° C. water bath. At 3 minute intervals, the tube was twirled gently to resuspend the solids. After 10 minutes, the tube was removed from the water bath and centrifuged for 3 minutes at 1,500 r.p.m. in order to sediment the particles. The supernatant was transferred to a clean tube and stored in a freezer.

0.1 ml. of 0.02 M. calcium chloride was blown forcibly and directly into the plasma-thromboplastin mixture and the stop-watch 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 horizontal position and was observed for a formation of clot, which was

the end-point. At this point the stop-watch was stopped and the time was recorded to the tenth of a second. Each plasma sample was run in duplicate.

The clotting time in this system depended on the combined effect of prothrombin and proconvertin in the tested plasma. The clotting time was transferred to per cent, of normal activity by using a correlation graph (Owren, 1948). The dilution curve of normal plasma, as used in the prothrombin and proconvertin method of Owren and Aas (1951) and Denny (1958), was obtained by plotting the concentration of normal plasma (taking the 1 in 10 dilution as 100 per cent) against the clotting time of the plasma (in seconds) on a double-logarithmic paper.

5. "In vitro" Synthesis of Coagulation Factors by Rat Liver Slices :-

a) Incubation.

A male albino rat which weighed 300 grams approximately was anaesthetized with 0.25 ml. Nembutal, by intraperitoneal injection. Nembutal had been found (Fouts and Robinson, 1959) to be the most efficient anaesthetic agent for use in liver perfusion experiments in rats. The abdomen of the rat was then opened and a perfusion needle was tied into the portal vein; the inferior vena cava was cut, and 15.0 ml. of normal saline followed by 15.0 ml. of cold bicarbonate-buffered

balanced salt solution (Peters and Anfinsen, 1950) were perfused through the liver under a pressure of 70 cm. of water. Rate of perfusion was 0.5 ml. per minute. The liver was then excised, frozen and sliced into 1.0 mm. slices. These slices were prepared rapidly and washed in a beaker of the fresh, cold salt solution; they were then placed in 50.0 ml. of the salt solution in a stoppered 150 ml. Erlenmeyer flask and washed for five minutes in a shaker at room temperature. The slices were then drained on a filter paper, and one gram was weighed and placed in a 150 ml. Erlenmeyer flask containing 5.0 ml. of the buffered solution. The flask was equilibrated with a 95% oxygen - 5% carbon dioxide mixture, stoppered and placed in a 37° C. water bath for incubation. Four 1 - gm. quantities of liver slices were prepared in this way in four flasks and incubated for 0, 2, 4, 6 hours, respectively.

b) Sampling.

1.0 ml. of 3.8 per cent solution of sodium citrate dihydrate in distilled water was added to the medium in each of the four flasks which were incubating. 0.2 ml. of the citrated incubation medium was then withdrawn and immediately mixed with 0.2 ml. of Indiacola buffer (pH 7.3) and assayed for clotting factors (Barts and Owen, 1940). The Indiacola

buffer was prepared by dissolving 1.72 grams of Imidazole (Glycerine) in 90.0 ml. of 0.1 N. Hydrochloric acid and diluting this mixture with distilled water to make a 100 ml. solution in a graduated volumetric flask. The liver slices and the remaining incubation media were poured into a homogeniser and thoroughly macerated. 0.2 ml. of the homogenate was also withdrawn and immediately mixed with 0.2 ml. of the Imidazole buffer and assayed for clotting factors (Pool and Robinson, 1939).

6. Estimation of Prothrombin using Russell Viper Venom.

The test for determining the combined effect of prothrombin and proconvertin employed thromboplastin prepared from tissue extract (Quick, 1938).

This technique of estimating prothrombin with Russell Viper Venom was exactly the same as that of Fullerton, 1940 and that of Russell and Page, 1940 in which (a) 0.2 ml. each of plasma and 0.02 M. calcium chloride solution was used, instead of 0.1 ml. as in the Quick's (1938) method.

b) Russell Viper Venom (0.2 ml.) instead of thromboplastin was used as the thrombokinase. 0.1 mgm. of the Venom was dissolved in 1.0 ml. of distilled water immediately before use.

Russell Viper Venom possesses the following advantages :

(1) It is constant in potency.

(2) It is in a clear solution.

(3) Recognition of the earliest formation of a fibrin web is probably a more accurate end-point than the development of a fibrin and adherent clot.

7. Tests of Liver Function.

Alkaline Phosphatase in Serum

Bodansky (1947) and Hanson (1948) distinguished obstructive from parenchymatous jaundice by estimating the quantity of alkaline phosphatase in serum.

The buffer which was used in this method was made by dissolving 6.3 gr. of anhydrous sodium carbonate and 3.36 gr. of sodium bicarbonate in distilled water and made up to 1 litre. The substrate was made by dissolving 2.18 gr. of disodium phenyl phosphate in exactly 1 litre of distilled water (King, 1956). For the experiment, 1.0 ml. of the buffer described above, and 1.0 ml. of the substrate were mixed in a test tube to give a solution of pH 10. The tube was allowed to remain in a water bath at 37° C. for 3 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 0.5 N. of sodium hydroxide solution in distilled water. The control was prepared by mixing 1.0 ml. of buffer, 1.0 ml. of substrate and 0.8 ml. of 0.5 N. sodium hydroxide solution, followed by 0.1 ml. of normal serum in

another test tube. The standard was prepared by mixing 1.1 ml. of buffer with 1.0 ml. of a phenol standard solution (containing 1 mg. of phenol in 100 ml. of distilled water) and 0.8 ml. of 0.5 N. of sodium hydroxide solution in a different tube. To each of the test, control and standard solutions 1.2 ml. of 0.5 N. sodium bicarbonate aqueous solution followed by 1.0 ml. of amino antipyrine solution (containing 6.0 gm. of 4 - amino antipyrine in 1 litre of distilled water) and 1.0 ml. of potassium ferricyanide solution (containing 24.0 gm. of potassium ferricyanide in 1 litre of distilled water) were added. Each tube was mixed well after each addition.

The standard, control and test solutions were then read in the spectrophotometer (E.F. 601) against a blank (1.1 ml. of buffer, 1.0 ml. of distilled water, 0.8 ml. of 0.5 N. sodium hydroxide aqueous solution, 1.2 ml. of 0.5 N. sodium bicarbonate aqueous solution, and 1.0 ml. of the potassium ferricyanide aqueous solution) at 510 mu.

b) Bilirubin in Serum.

Values of bilirubin, a yellow pigment in normal blood, fall within the range 0.1 to 0.8 mgm. per 100 ml. of serum. But the majority of the values are within the limits 0.3 to

0.5 (Vaughan and Blackwood, 1958; Boston, Maclaurin - Smith and King, 1950).

The indirect reaction of van den Bergh was used. In this, a red colour is given by bilirubin with diazotised sulphanilic acid in the presence of ammonium sulphate (to precipitate protein), and alcohol (to liberate and dissolve the uro-bilirubin).

A Diazo-reagent was made by mixing 10.0 ml. of solution A and 0.3 ml. of solution B. Solution A was prepared by dissolving 1.0 gram. Sulphanilic acid in 15 ml. of concentrated hydrochloric acid and diluting with distilled water to 1 litre. Solution B was prepared by dissolving 0.5 grams of sodium nitrite and making up to 100 ml. with distilled water.

0.05 ml. of the diazo-reagent was added to 1.0 ml. of serum in a 10 ml. centrifuge tube and, after 3 minutes, 3.0 ml. of 95% alcohol and 0.5 ml. of saturated ammonium sulphate were added in that order. After mixing, and allowing the tube to stand at room temperature for a further 15 minutes, the mixture was centrifuged and the colour of the clear supernatant fluid was read in the spectrophotometer (S.P. 600) at 540 m, after the instrument had been set to zero with a blank.

The blank was prepared by diluting 1.5 ml. of concentrated hydrochloric acid with 98.5 ml. of distilled water. The standard solution was prepared by mixing 1.0 ml. of the diazo-reagent (described above) with 4.0 ml. of 95% alcohol in a tube. 30 minutes were allowed for colour development, when the colour was equivalent to that produced by serum containing 4.0 mgm. bilirubin per 100 ml.

c) Urobilinogen in Urine.

Maier (1951) concluded that the test for urinary urobilinogen is one of the reliable aids of differential diagnosis of liver function.

2.5 ml. of centrifuged test urine and 2.5 ml. of Ehrlich's reagent were mixed in a test tube and 5.0 ml. of saturated sodium acetate solution was added to the mixture. Ehrlich's reagent was made by dissolving 0.7 gm. of p-dimethyl-amnobenzaldehyde in 150 ml. of concentrated hydrochloric acid and adding 100 ml. of distilled water. A blank was prepared by mixing well, 2.5 ml. of normal urine and 5.0 ml. of saturated sodium acetate and adding 2.5 ml. of Ehrlich's reagent to the mixture. After all effervescence has ceased, the optical densities of both the standard solution and the test urine were read at 540 mu. For the standard, the instrument was

put at zero with water; and for the test urine sample, it was set at zero with the blank described above.

The standard solution for Urobilinogen (Watson, 1925) was prepared by dissolving 0.5 gm. of phenolphthalein in 95% (v/v) alcohol and made up to 1 litre in the alcohol. 1.0 ml. of this alcoholic solution was transferred to a 100 ml. volumetric flask, and 5.0 ml. of saturated sodium carbonate solution was added, and enough distilled water was added to make 100 ml. This phenolphthalein standard has a similar colour to that given by 0.537 mg. Urobilinogen in 100 ml. of Urine (Watson, 1957).

4) Thymol turbidity test.

Thymol turbidity test was successfully used by Mulligan (1944) as an additional means of distinguishing hepatitis from obstructive jaundice.

A thymol reagent, (King, 1951) which is a buffered solution of thymol, was prepared by heating, just to boiling point, 1.58 gm. of barbitone, 1.03 gm. of sodium-barbitone, 5.0 gm. of thymol and 500 ml. of water. The turbid mixture was then cooled, shaken, and allowed to stand overnight at room temperature. Before use, it was shaken, and filtered from the excess of thymol which was precipitated. 0.05 ml. of serum was added to 5.0 ml. of the thymol reagent. It was mixed and

allowed to stand for 60 minutes. The turbidity was read against albumin standards. These standards consisted of a permanent suspension of formalin in gelatin. The formalin was prepared as follows : 10.0 grams of hexamine (hexamethylene tetramine) was dissolved in 100 ml. distilled water. 25.0 ml. of this aqueous solution of hexamine were added to 25.0 ml. of a solution of hydrazine sulphate (1 gm. of hydrazine sulphate was dissolved in 100 ml. of distilled water). The mixture was stoppered, shaken, and left at room temperature for 15 hours. The resulting precipitate of formalin was carefully mixed by gently shaking it until it was evenly dispersed throughout the liquid.

14.5 ml. of this formalin suspension were added to 100 ml. gelatin solution (60.0 gm. of pure gelatin was dissolved at about 50° C. in 900 ml. of distilled water) together with 0.3 ml. of 40 per cent formaldehyde to ensure permanent "setting". Such a gelatin suspension of formalin has been found to be equivalent to a concentration of serum albumin of 100 mgm. per 100 ml. The gelatin suspension was then diluted with clarified gelatin (containing 0.3 ml. of 40 per cent formaldehyde per 100 ml. of the gelatin solution) to give standards corresponding to other albumin concentrations. Clarified gelatin was prepared as follows: the "white" of an egg, in approximately twice its volume of distilled water, was vigorously stirred into solution.

The mixture was heated, with continual stirring, on a boiling water bath for 1 hour. It was then filtered through a large paper in a hot funnel. The clear, slightly yellow filtrate was kept liquid at about 50° C. for use in the preparation of the standards described above. The following mixtures of gelatin and formalin-gelatin suspension were made in small tubes :-

Tube	ml. of gelatin solution.	ml. of gelatin-formalin suspension.	value in mgm. of albumin per 100 ml.
1	3.6	0.4	10
2	3.2	0.8	20
3	2.8	1.2	30
4	2.4	1.6	40
5	2.0	2.0	50
6	1.6	2.4	60
7	1.2	2.8	70
8	0.8	3.2	80
9	0.4	3.6	90
10	0.0	4.0	100

The mixture was heated, with continual stirring, on a boiling water bath for 1 hour. It was then filtered through a large paper in a hot funnel. The clear, slightly yellow filtrate was kept liquid at about 50° C. for use in the preparation of the standards described above. The following mixtures of gelatin and formalin-gelatin suspension were made in small tubes :-

Tube	ml. of gelatin solution.	ml. of gelatin-formalin suspension.	value in mgm. of albumin per 100 ml.
1	3.6	0.4	10
2	3.2	0.8	20
3	2.8	1.2	30
4	2.4	1.6	40
5	2.0	2.0	50
6	1.6	2.4	60
7	1.2	2.8	70
8	0.8	3.2	80
9	0.4	3.6	90
10	0.0	4.0	100

EXPERIMENTS AND RESULTS

1. THE EFFECT ON BLOOD CLOTTING OF RAISING THE RATE OF MOULTING.

Apparatus:

1. Small glass test tubes 7.5cm. x 1cm).
2. Graduate pipettes (2.2 ml., 0.25 ml., 0.05 ml.,)
3. Plastic tubes (10cm x1.5 cm), for collection and storage of citrated blood.
4. A water bath (at 37°C).
5. A large pair of scissors, for decapitation.
6. A stop watch.

Reagents:

1. Thrombotest (Hyegaard & Co A/S - OSLO)
2. 3.2 M. aqueous solution of calcium chloride (solvent for thrombotest reagent).
3. 3.13 per cent (w/v) sodium citrate dihydrate solution in distilled water.

Procedure:

A set of twelve male, albino rats weighing approximately 300 grms each, were obtained from the Department of Pharmacology. Six of them were fed on the balanced diet (described on page .10..) for a period of four days and the remaining six were fed on the contaminated diet (described on page .11..) continuously for a period of four days.

Collection of blood

At the end of the feeding period, each rat was decapitated, using a pair of large, sharp scissors. Blood, from each rat, was collected in a different plastic tube (containing 0.5 ml. of 3.1% (w/v) aqueous solution of sodium citrate) to a mark of 5.0 ml., that is, 9 parts of blood have been mixed with 1 part of sodium citrate solution, to stop the blood from clotting.

Clotting time determination.

The method which was used to determine the clotting times of the various samples of blood is described on pages ..13... to ..14....

Results

The average clotting time of the blood obtained from the control rats (those which fed on the balanced diet) and that obtained from the poisoned rats (those which fed on the contaminated diet) were 26.05 ± 0.05, 48.05 ± 0.05 seconds, respectively. The results are shown in Table and Figure 1 .

TABLE 1

EFFECT ON BLOOD CLOTTING OF FEEDING RATS ON MOULDY DIET.

Coagulation Time (seconds)	
Balanced diet	Mouldy diet.
26.0	48.0
26.5	48.0
26.0	47.5
26.0	48.0
26.0	47.5
25.5	48.0
26.0	48.5
26.5	48.0
Mean = 26.05 ± 0.05	Mean = 48.05 ± 0.05

FIGURE 1. CHANGES IN BLOOD COAGULATION TIME OF RATS FED ON NORMAL AND MOULDY DIETS.

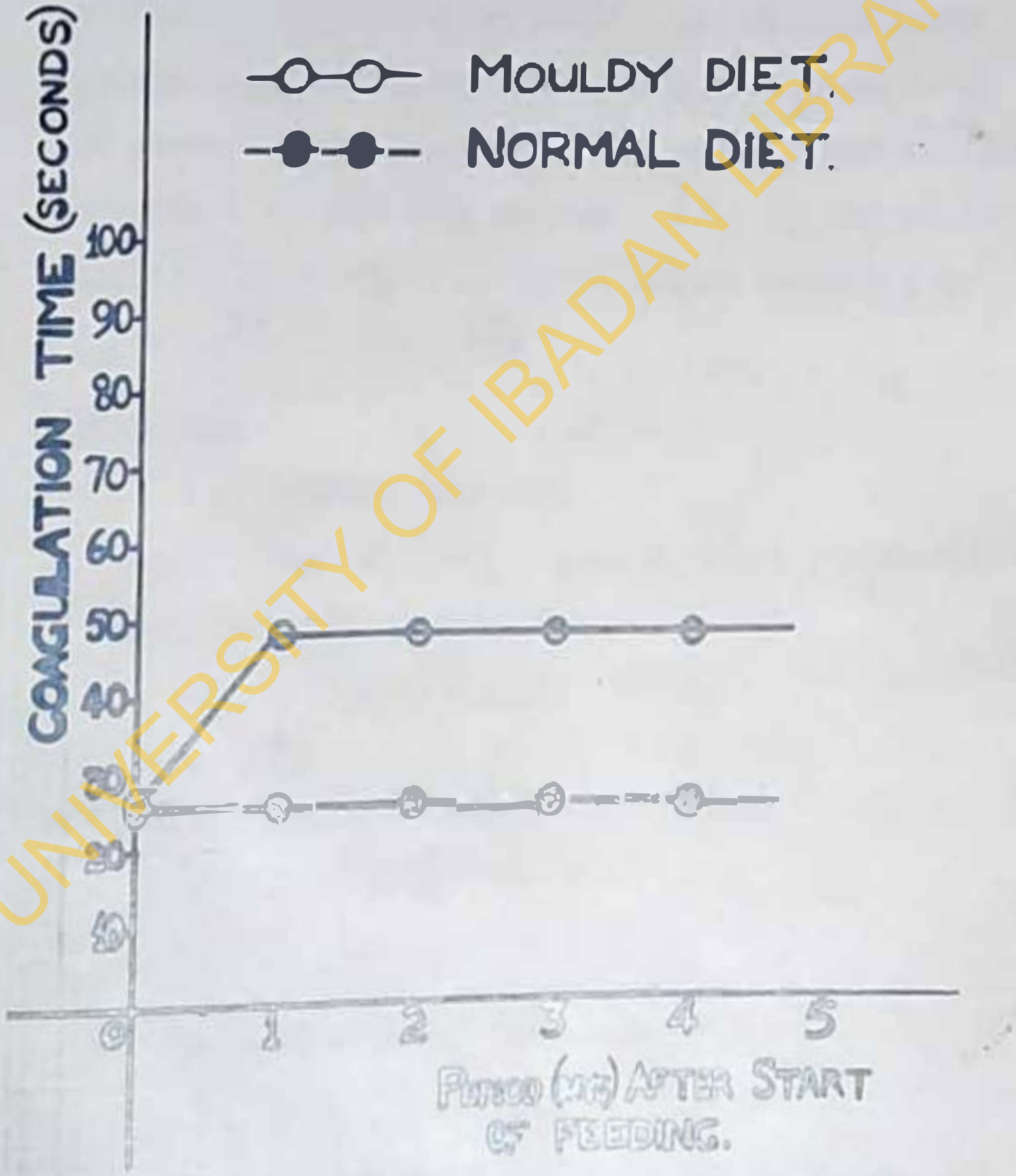
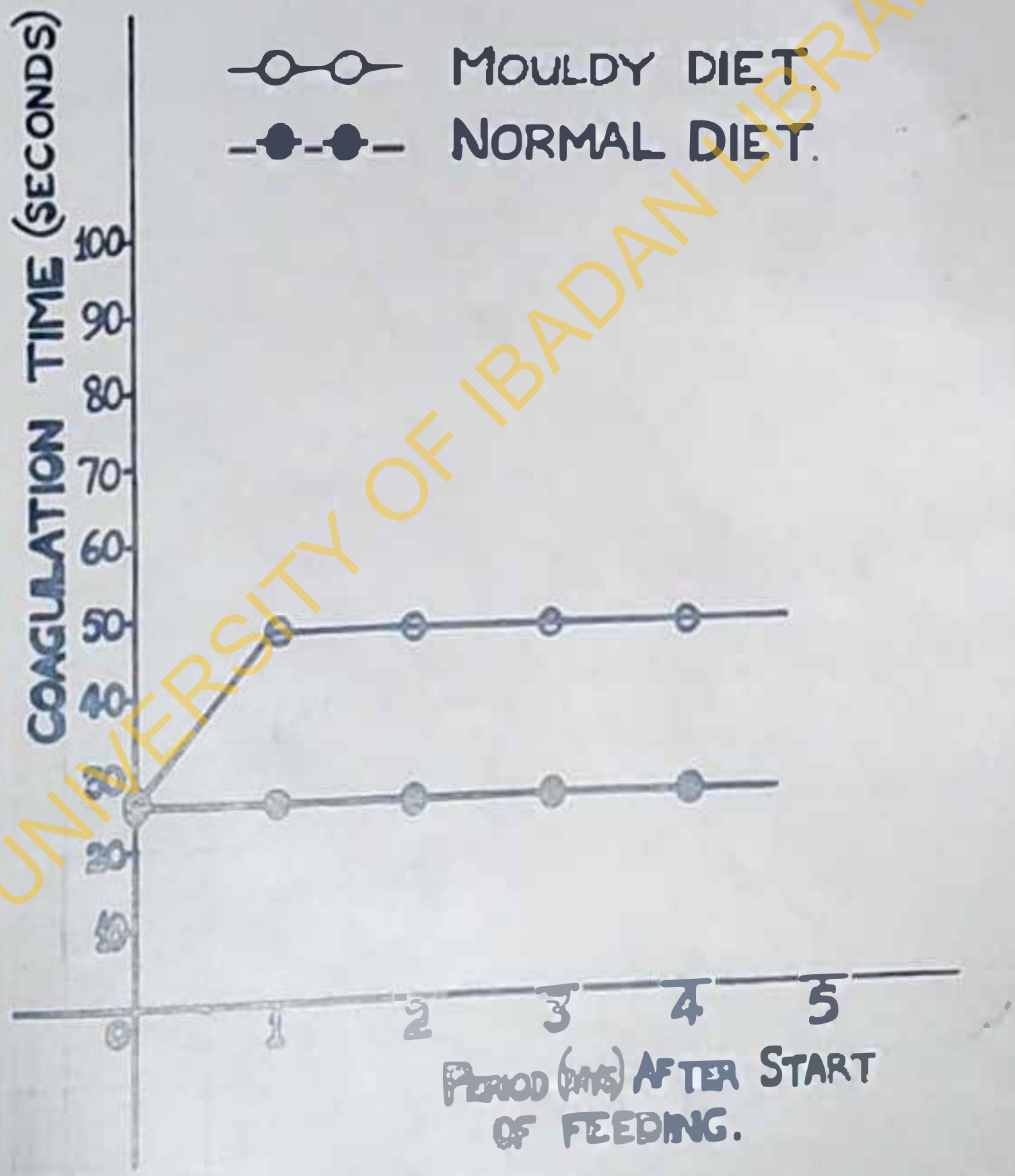


FIGURE 1. CHANGES IN BLOOD COAGULATION TIME OF RATS FED ON NORMAL AND MOULDY DIETS.



2. EXTRACTION AND PURIFICATION OF THE AFLATOXINS FROM THE MOULDY DIET

The amount of food consumed by each rat was measured daily throughout the feeding period by finding the difference between the amount of food given to the rat at the start of feeding and the amount left uneaten at the end of 24 hours. The average daily intake, measured using this method, was found to be 12 grams. This quantity of the mouldy diet was then extracted as described on page ...11... and the mixed extract was purified using the method described on pages ...11... to ...12...

Materials:

1. Soxhlet Extractor
2. 250 ml. Round-bottomed flasks (Quick-Fit)
3. Glass plates (20 cm. x 20 cm)
4. Ultraviolet light (365 mμ).

Adsorbent for thin-layer chromatography:

Silica Gel G (Merck A.G.)

Solvent:

3% (v/v) Methanol in chloroform.

Results:

Aflatoxins B and G were identified, under ultraviolet light at 365 mμ, in the purified extract of the mouldy diet.

5. **Estimation of Aflatoxins in the Dietary Intake:**

In the assessment of the levels of Aflatoxins in the daily dietary intake, the Full-fluorescence technique was used, as described on page ...12.....

Apparatus:

1. Graduated pipettes (1 ml., 10 mls.)
2. Test tubes (12 cm. x 1.5 cm).
3. Glass plates (20 cm. x 20 cm).
4. Ultraviolet light (365 mp).

Adsorbent for thin-layer chromatography.

Silica Gel G (Merck A.G.) was used.

"Chromatoplates" were prepared as described on page..10..

Solvent:

3% (v/v) methanol in chloroform.

Procedure:

Eight dilutions of the concentrated chloroform extract of aflatoxins obtained as described on page..11.. were prepared, using the method described on page...12... as follows:-

$\frac{1}{100}$, $\frac{1}{200}$, $\frac{1}{400}$, $\frac{1}{800}$, $\frac{1}{1600}$, $\frac{1}{3200}$, $\frac{1}{6400}$, $\frac{1}{12800}$

Results:

The dilution with the least fluorescence in the Aflatoxin B extract was $\frac{1}{200}$, and that of the Aflatoxin G extract was $\frac{1}{200}$ also.

These dilutions were equivalent to 4×10^{-4} ppm. and 3×10^{-4} ppm. for Aflatoxins B and G, respectively (Coomes et al, 1965).

Since 0.2 ml. of the concentrated chloroform extract (25 ml) was used for the dilutions, the amount of Aflatoxin B present in the daily intake of the contaminated diet will be equal to $\frac{1}{0.2} \times \frac{200}{1} \times \frac{25}{1}$ $\times 4 \times 10^{-4}$ ppm.

$= 10$ ppm.

and the amount of Aflatoxin G present in the daily intake of the contaminated diet will be equal to

$\frac{1}{0.2} \times \frac{200}{1} \times \frac{25}{1} \times 3 \times 10^{-4}$ ppm.

$= 7.5$ ppm.

Therefore, the total amount of mixed aflatoxins in 32.0 grms of the mouldy diet was 17.5 micrograms.

4. INFLUENCE OF (i) THE EXTRACTED MIXED AFLATOXINS (ii) PURE AFLATOXIN B_1 AND (iii) 4-HYDROXYCOUMARIN ON BLOOD CLOTTING.

The effect of pure aflatoxin B_1 on blood clotting time of rats was compared with that of the mixed aflatoxins extracted from the contaminated diet, using the "thrombotest" method which is described on pages..13. to.. 14...A similar experiment was performed using 4-hydroxycoumarin in place of aflatoxin B_1.

Apparatus:

Same as those described on page ..26....

Reagents:

1. Saline (0.9% (w/v) aqueous solution of sodium chloride (A.R.).

2. Mixed aflatoxins, extracted from the moldy diet as described on page ....11....

3. Pure aflatoxin B_1 (Arthur D. Little Inc., Massachusetts,) U.S.A)

4. 4-hydroxycoumarin A.R. (Hopkins and Williams Ltd., Essex).

Procedure

A set of twenty-four male, albino rats, of the same strain, weighing approximately 300 grams each, were obtained. Six rats were each injected intraperitoneally with 17.5 µgm. of mixed aflatoxins in 1.0 ml. distilled water. Six rats received 17.5 µgm. of pure aflatoxin B_1 suspended in 1.0 ml. of distilled water each. The third group of rats received 15.0 mgm. of 4 hydroxycoumarin suspension in 1.0 ml. distilled water each. The remaining six rats were each injected with 1.0 ml. of distilled water to serve as control.

One animal from each set of rats, except the 4-hydroxycoumarin-treated ones was decapitated, every hour after injection. The 4-hydroxycoumarin-treated rats were decapitated daily, since the anticoagulant activity of 4-hydroxycoumarin is maximal only after 48 hours (Arora.1963).

Blood from each animal was obtained, after decapitation, and clotting time was determined for each sample.

Result:

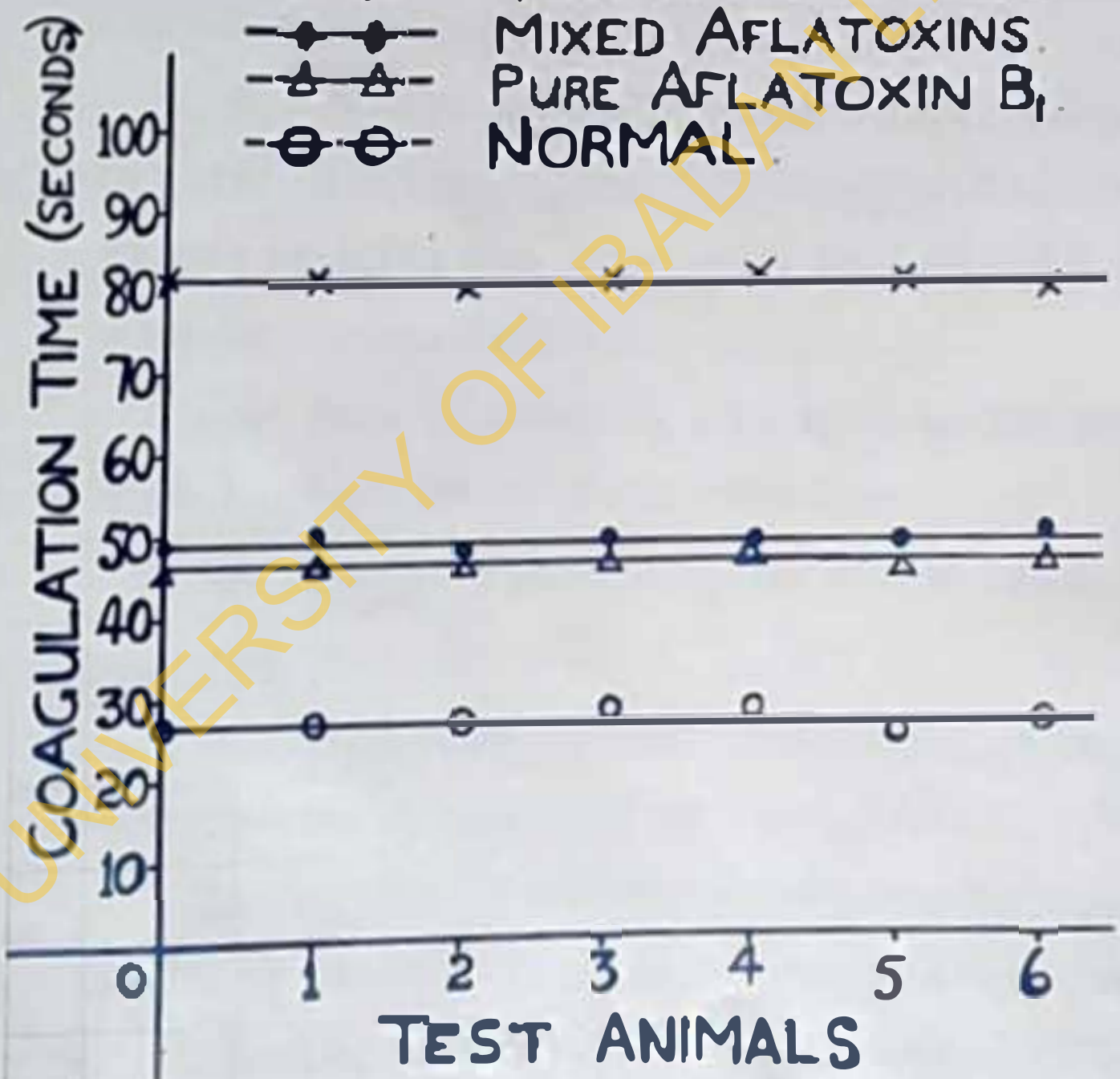
The results are shown in Table 2 and Figure 2. After a period of 3 hours, both the mixed aflatoxins and pure aflatoxin B_1 have prolonged the normal clotting time maximally. The peak action of 4-hydroxycoumarin was reached after 48 hours.

TABLE 2.

THE EFFECT OF (i) THE EXTRACTED MIXED AFLATOXINS (ii) PURE AFLATOXIN B_1, AND (iii) 4-HYDROXYCOUMARIN ON BLOOD CLOTTING TIME.

Completion times (seconds)			
Control (Normal)	Extracted Mixed Aflatoxins	Pure Aflatoxin B_1	4hydroxy-coumarin
27.0	49.0	48.5	80.5
27.5	49.5	48.5	81.0
27.5	49.0	49.0	81.5
27.0	49.0	48.5	80.5
26.5	49.5	48.0	80.5
27.0	49.0	48.5	80.0
27.5	49.0	48.0	80.0
27.5	49.0	48.0	80.5
Mean=27.2 ± 0.06	Mean= 49.12±0.05	Mean= 48.37±0.05	Mean = 80.56 ± 0.05

FIGURE 2. THE INFLUENCE OF (i) MIXED AFLATOXINS (ii) PURE AFLATOXIN B_1 AND (iii) 4-HYDROXY-COUMARIN ON BLOOD COAGULATION TIME OF RATS.



5. THE EFFECT OF (a) 2-METHYL-1:4-NAPHTHOQUINONE (b) VITAMIN K_1 ON THE ANTICOAGULANT PROPERTIES OF AFLATOXIN B_1 AND 4-HYDROXYCOUMARIN.

Apparatus:

Same as those described on page..26...

Reagents:

1. 2-methyl-1:4-naphthoquinone(Antigen Ltd., Ireland), supplied in ampoules, each containing 5.21 mg. in 1 ml. aqueous solution.

2. Vitamin K_1 (Roche Products Ltd., England), supplied in ampoules, each containing 10.0 mg. in 1 ml. aqueous solution with a few drops of 0.5% (w/v) of phenol added as a bacteriostatic.

3. Pure aflatoxin B_1 (Arthur D. Little Inc., Mass. U.S.A.) suspension in distilled water.

4. 4-hydroxycoumarin (A.R.) suspension in distilled water.

Procedure:

The method used in the determination of blood clotting time is described on page ..13... to ..14..

The effects of 2-methyl-1:4-naphthoquinone and vitamin K_1 on the anticoagulant properties of aflatoxin B_1 and 4-hydroxycoumarin were determined in two different ways:-

A set of ten rats were each injected intraperitoneally with 17.5 ugm. of aflatoxin B_1 in 1.0 ml. aqueous suspension. When the effect on blood clotting of the aflatoxin was maximal, 1.0 ml. of an aqueous solution containing 5.21 mg. of 2-methyl-1:4-naphthoquinone was injected intraperitoneally to each of these rats. A similar experiment was performed on another set of ten rats, administering 5.0 mgm. of vitamin K_1 in 1.0 ml. aqueous solution in place of 2-methyl-1:4-naphthoquinone. Their controls were ten rats into each of which was injected 1.0 ml. of an aqueous suspension containing 17.5 ugm. of aflatoxin B_1 and 1.0 ml. of distilled water. The blood coagulation times of ten normal, comparable rats were also determined.

A different set of ten rats were each injected intraperitoneally with 15.0 mgm. of 4-hydroxycoumarin (Arora and Mathur, 1963) in 1.0. ml. aqueous suspension. When the effect on blood clotting of the 4-hydroxycoumarin was maximal, 1.0 ml. of an aqueous solution containing 5.21 mgm. of 2-Methyl-1:4-naphthoquinone was injected intraperitoneally to each of these rats. A similar experiment was performed on another set of ten rats, administering 5.0 mgm. of Vitamin K_1 in 1.0 ml. aqueous solution in place of 2-Methyl-1:4-naphthoquinone. Their controls were ten rats into each of which was injected 1.0 ml. of an aqueous suspension containing 15.0 mgm. of 4-hydroxycoumarin and 1.0 ml of distilled water.

The blood coagulation times of ten normal, comparable rats, were also determined.

Blood was obtained from each rat by decapitation and clotting times of the various blood samples were determined hour after hour .

Results.

When vitamin K_1 was administered, there was a reversal of the anticoagulant effect of both 4-hydroxycoumarin and aflatoxin B_1. The reversing effect of 2-methyl-1,4-naphthoquinone was of a lesser degree. The results are shown in Tables 3 and 4.

TABLE 3

THE EFFECT OF 2-METHYL-1:4-NAPHTHOQUINONE WHEN THE ANTICOAGULANT ACTION OF (i) AFLATOXIN B1 AND (ii) 4-HYDROXYCOUMARIN WAS MAXIMAL.

ne(hours)after jection of -methyl-1:4 phthoquinone.	Coagulation time(seconds)				Coagulation time(seconds) of normal rats.
	Aflatoxin B1-treated rats		4-hydroxycoumarin treated rats.		
	Control	Test	Control	Test	
1	47.5	47.5	81.0	80.0	28.0
2	47.5	47.0	81.0	80.0	27.5
3	47.0	40.5	80.5	69.0	27.5
4	47.5	40.0	80.5	69.0	27.5
5	47.5	40.5	80.5	68.5	28.0
6	47.5	41.0	81.0	69.0	27.0
7	48.0	40.0	80.5	68.5	27.5
8	47.0	40.5	80.0	68.0	27.0
9	48.0	40.0	80.5	68.5	27.0
10	47.5	40.0	80.5	68.5	27.5

TABLE 4.

THE EFFECT OF VITAMIN K_1 ON THE ANTICOAGULANT ACTION OF (I) AFLATOXIN B_1 AND (II) 4-HYDROXYCOUMARIN IN RATS

Time(hours)after injection of Vitamin K_1	Coagulation time (seconds)				Coagulation time(seconds) of normal rats.
	Aflatoxin B_1-treated rats		4-hydroxycoumarin TR treated rats		
	Control	Test	Control	Test	
1	48.0	48.0	80.5	80.5	27.0
2	48.0	47.5	80.5	80.5	28.0
3	48.0	42.5	81.0	47.5	27.0
4	47.5	34.3	80.5	34.0	27.5
5	48.0	30.0	80.5	30.7	27.0
6	47.0	29.0	80.5	28.5	27.0
7	47.5	27.5	80.0	27.0	27.5
8	47.0	27.0	80.5	27.5	28.0
9	47.5	27.5	80.0	26.5	27.0
10	47.5	27.5	80.0	27.0	27.0

B.

0.5 ml. of an aqueous solution containing 5.0 mgm. of vitamin K_1 was injected intramuscularly to each of a set of ten rats, and 0.5 ml. of an aqueous suspension containing 17.5 µgm. of aflatoxin B_1 was injected intraperitoneally into each of the same set of rats at the same time. A similar experiment was performed, injecting 0.5 ml. of distilled water in place of vitamin K_1. A third set of ten rats were each injected with 0.5 ml. of an aqueous solution containing 5.0 mgm. of vitamin K_1 intramuscularly, but 0.5 ml. of an aqueous suspension containing 15.0 mgm. of 4-hydroxycoumarin was injected, at the same time, intraperitoneally to each of this set of rats. A similar experiment was also performed, injecting 0.5ml. of distilled water in place of Vitamin K_1. Blood was obtained by decapitation, from a rat in each of the sets, every hour. Clotting times of the various samples were determined using the method described on page ..13..to ..14.

Result.

Vitamin K_1 completely inhibited the anticoagulant activities of both 4-hydroxycoumarin and aflatoxin B_1, as shown in Tables 5 and 6.

TABLE 5.

THE EFFECT OF SIMULTANEOUS ADMINISTRATION OF VITAMIN K_1 AND 4-HYDROXYCOUMARIN ON BLOOD COAGULATION TIME.

Time(hours) after simultaneous injection of Vitamin K_1 and 4-hydroxycoumarin.	Coagulation time(seconds)		Coagulation time(seconds) of normal rats.
	Test	Control	
	4-hydroxycoumarin with Vitamin K_1.	4-hydroxycoumarin with distilled water.	
1	27.0	80.5	27.5
3	26.0	80.0	27.0
5	25.5	80.0	27.0
9	25.0	80.0	27.0
12	26.0	81.0	27.5
24	26.0	80.5	27.5
36	27.0	80.0	27.5
48	27.5	80.5	28.0
60	28.0	28.0	27.5
65	28.5	80.0	28.0

TABLE 6.

THE EFFECT OF SIMULTANEOUS ADMINISTRATION OF VITAMIN K_1 AND AFLATOXIN B_1 ON BLOOD COAGULATION TIME.

Time (hours) after simultaneous injection of Vitamin K_1 and Aflatoxin B_1	Coagulation time (seconds)		Coagulation time (seconds) of normal rats.
	Test: Aflatoxin B_1 with Vitamin K1.	Control: Aflatoxin B_1 with distilled water	
1	27.5	48.0	27.5
2	27.0	48.0	27.5
3	27.0	47.5	27.0
4	27.5	48.5	27.5
5	27.0	48.0	28.0
6	27.5	47.5	27.0
7	27.5	48.0	27.0
8	27.0	48.5	27.0
9	26.5	48.5	27.5
10	27.0	48.5	27.0

6.

THE EFFECT OF VITAMIN K_1 ON THE "IN VITRO" SYNTHESIS OF COAGULATION FACTORS BY THE LIVER OF RATS TREATED WITH (a) AFLATOXIN B_1 AND (b) 4HYDROXYCOUMARIN.

Apparatus.

1. Burette (50ml.) on a retort stand (85cm. tall).
2. Dissecting board (60cm. x 60 cm).
3. Stadie-Riggs slicer
4. Shaker (E. Mickle, Surrey)
5. Equilibruting mixture (95% oxygen-5% carbon dioxide).
6. Potter-Elvehjem homogenizer.

Reagents

1. Saline (0.9 gm. of sodium chloride (A.R.) dissolved in 100ml. of distilled water).
2. Bicarbonate-buffered balanced salt medium, containing 10ml. of calcium chloride (A.R), 30 ml. of sodium carbonate (A.R.), 105 ml. of sodium chloride(A.R.) and 10ml. of Potassium Bicarbonate in 1 litre of aqueous solution.
3. 3.8% (w/v) sodium citrate (A.R.) aqueous solution.
4. Imidazole Buffer, made by dissolving 1.72 gm of Imidazole (Glycine) in 90.0 ml. of 0.1N. Hydrochloric acid and diluting with water to 100ml.

Anaesthetic Agent.

Nembutal.

Procedure

A standard curve, shown in Figure 3 was prepared as described on pages ...14.... to ...16..

Liver slices were prepared, as described on page ..17.. from two groups of rats which had been treated with aflatoxin B_1 and 4-hydroxycoumarin respectively, as described on page..33... One gram of the liver slices from each group of experimental animals were incubated as described on page ..17.. for 0 hour, 2 hours 4 hours, and 6 hours respectively, to allow for syntheses of coagulation factors. Prothrombin and Prooonvertin were assayed both in the incubation medium and in the liver homogenate which was prepared using the method described on page ..18.. Thromboplastin (Difco Laboratories) was used as the thrombokinase as described on page 15 . The amounts of Prothrombin and Proconvertin present in the plasma at the end of each incubation period, were read off the standard dilution curve.

The effect of vitamin K_1 on the syntheses of these factors was determined by adding 0.2 mg. of vitamin K_1 in 0.5 ml. of diluted aqueous solution to the liver slices which had been incubating for 4 hours. Progress in the syntheses of the coagulation factors was followed for the next four hours.

FIGURE 3. STANDARD CURVE OF NORMAL PLASMA COAGULATION.

Result

Tables 7 and 8, and Figures 4 and 5 show that the liver slices prepared both from the rats treated with 4 hydroxycoumarin, and those given Aflatoxin B_1 could not synthesize measurable quantities of prothrombin and proconvertin. But the condition was reversed when vitamin K_1 was added to the incubated liver slices. The plasma obtained from the incubation medium withdrawn at every period did not clot. When the medium was assayed for coagulation factors.

TABLE 7. THE EFFECT OF VITAMIN K_1 ON THE SYNTHESIS OF PROTHROMBIN AND PROCONVERTIN BY LIVER SLICES OF RATS TREATED WITH 4HYDROXYCOUMARIN.

Time(hours) after start of incubation	Clotting Time of Homogenate (seconds)			Units of factors in Homogenate (Normal = 100)		
	Normal	4 hydroxycoumarin - treated	After the addition of Vitamin K_1	Normal	4hydroxycoumarin - treated	After the addition of Vitamin K_1.
0	70.0	274.0		2.8	< 1	
2	40.0	225.5		21.5	< 1	
4	35.0	185.0		43.9	< 1	
6	29.0	182.0	65.0	81.4	< 1	3.2
8			42.0			18.5
10			29.0			65.0

FIGURE 4. SYNTHESIS OF COAGULATION FACTORS IN LIVER SLICES.

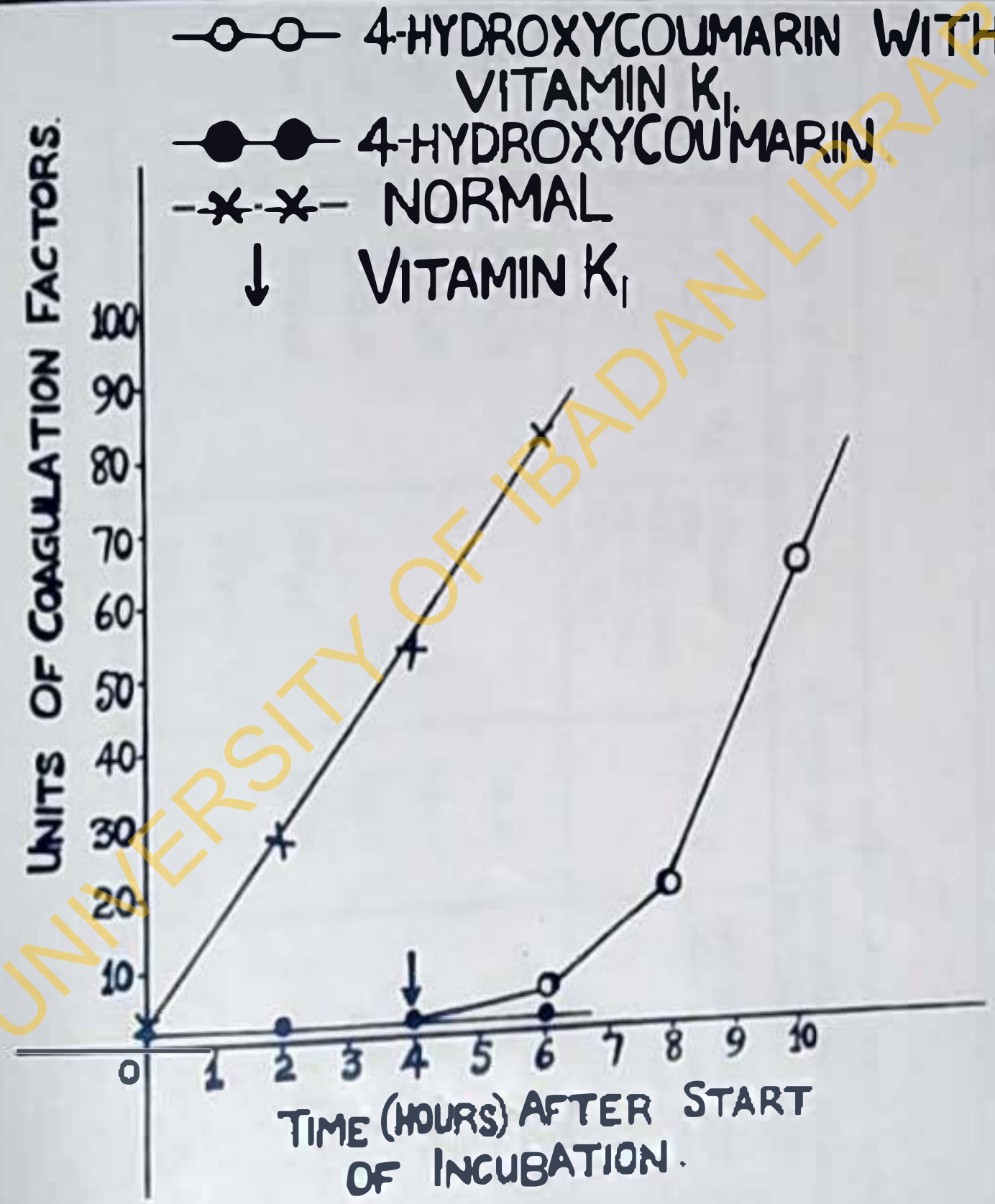
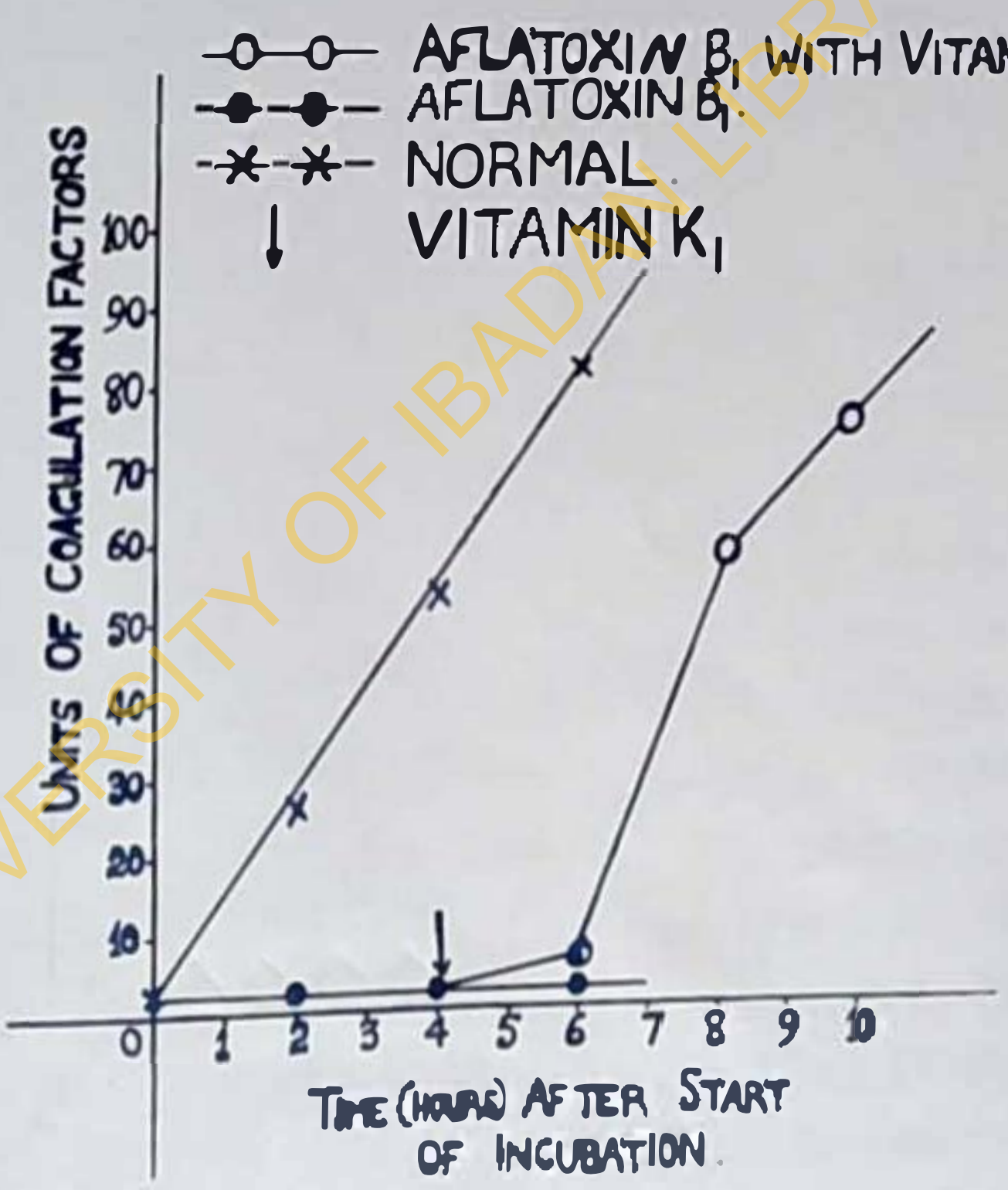


TABLE 8.

THE EFFECT OF VITAMIN K_1 ON THE SYNTHESES OF PROTHROMBIN AND PROCONVERTIN BY LIVER SLICES OF RATS TREATED WITH AFLATOXIN B_1

Time(hours) after start of incubation.	Clotting Time of Homogenate (seconds)			Units of factors in Homogenate (MAXIMUM = 100)		
	Normal	Aflatoxin B_1 -treated	After the addition of Vitamin K_1	Normal	Aflatoxin B_1 -treated	After the addition of Vitamin K_1
0	70.0	256.0		2.8	<1	
2	40.0	242.0		21.5	<1	
4	35.0	205.0		43.9	<1	
6	29.0	207.0	62.0	81.4	<1	4.36
8			33.0			59.0
10			29.5			75.0

FIGURE 5. SYNTHESIS OF COAGULATION FACTORS IN LIVER SLICES



7. PROTHROMBIN SYNTHESIS IN RATS TREATED WITH (a) AFLATOXIN B_1 AND (b) 4 HYDROXYCOUMARIN.

Apparatus:

Same as those on page 44.

Materials:

Same as those described on page 44, except that thromboplastin was replaced with Russell Viper Venom (Koch-Light Laboratories, Bucks, England).

Procedure.

A standard curve was prepared, using the method described on pages 15 to 16 but Russell Viper Venom was used in place of thromboplastin in the chromboloimes.

Liver slices were prepared from Aflatoxin B_1- and 4 hydroxycoumarin-treated rats as described on page 17. One gram quantities were incubated as described on page for 0 hour, 2 hours, 4 hours, 6 hours, respectively, to allow for synthesis of coagulation factors. Prothrombin was assayed both in the incubation medium and in the liver homogenate (obtained as described on page 18), using Russell viper Venom as described on pages 18 to 19

The effect of Vitamin K_1 on the synthesis of prothrombin was determined by following the same procedure as described on page 45.

Result

Tables 9 and 10 show that the liver slices prepared both from the rats treated with 4 hydroxycoumarin and from those given Aflatoxin B1 synthesise practically no prothrombin during the period of the experiment. But the condition was reversed when vitamin K was added to the incubated liver slices. The plasma obtained from the incubation medium, withdrawn at the various periods, did not clot when they were assayed for prothrombin.

TABLE 9.

THE EFFECT OF VITAMIN K_1 ON THE SYNTHESIS OF PROTHROMBIN BY LIVER SLICES OF RATS TREATED WITH 4HYDROXYCOUMARIN.

Time(hours) after start of incubation.	Clotting Time of Homogenate (Seconds)			Units of Prothrombin in Homogenate (MAXIMUM = 100)		
	Normal.	4 hydroxy-coumarin - treated	After the addition of Vitamin K_1.	Normal.	4-hydroxy-coumarin-treated.	After the addition of Vitamin K_1.
0	201.0	704.0		0.95	< 0.1	
2	105.0	627.0		3.5	< 0.1	
4	79.0	460.0		6.4	0.18	
6	553.0	287.0	121.0	15.0	0.48	2.2
8			60.0			10.9
10			54.5			13.7

TABLE 10.

THE EFFECT OF VITAMIN K_1 ON THE SYNTHESIS OF PROTHROMBIN BY LIVER SLICES OF RATS TREATED WITH AFLATOXIN B_1.

Time (hours) after start of incubation	Clotting Time of Homogenate (seconds).			Units of Prothrombin in Homogenate (MAXIMUM = 100).		
	Normal	Aflatoxin B_1 -treated	After the addition of Vitamin K_1	Normal	Aflatoxin B_1 -treated	After the addition of Vitamin K_1.
0	200.0	709.0		0.95	< 0.1	
2	106.0	618.0		1.5	0.1	
4	78.0	410.0		6.4	0.23	
6	52.0	282.0	159.0	15.0	0.65	1.2
8			64.0			9.2
10			54.0			14.4

8. **LIVER FUNCTION TESTS IN RATS TREATED WITH (a) AFLATOXIN B_1 AND (b) 4 HYDROXYCOUMARIN.**

Two sets of four rats each were treated by intraperitoneal injection with (a) Aflatoxin B_1 and (b) 4-hydroxycoumarin as described below. A third set which was not treated served as control. When the effects of aflatoxin B_1 and 4 hydroxycoumarin on blood clotting were maximal, four different liver function tests were performed on the test animals, side by side with their controls. The tests performed were (a) Alkaline phosphatase in serum, (b) Bilirubin in serum, (c) Urobilinogen in urine, and (d) Thymol turbidity as described on pages 19 to 25 .

17.5 micrograms of pure Aflatoxin B_1 suspended in 1.0 ml. of distilled water was injected intraperitoneally into each of the first set of rats. Urine which was passed by the rats, three hours after injection, was collected in small beakers. Three hours after the injection, the male rats (weighing approximately 300 grms each) were decapitated to obtain serum.

15.0 mgm. of 4 hydroxycoumarin suspended in 1.0 ml. distilled water was injected intraperitoneally into each of the second set of male rats. Urine which as passed by the rats, was collected thereafter, and the

rats were decapitated after 48 hours in order to collects serum from each of them.

Tests were then performed on the urine and blood samples as follows:-

a) Alkaline Phosphatase in serum.

Procedure:

The level of alkaline phosphatase in each of the sera obtained from normal, aflatoxin B_1-treated, and 4-hydroxycoumarin-treated rats, was determined using the method described on pages 19 to 20.

Results—

The amounts of the enzyme were about the same from the sera obtained from the normal, aflatoxin B_1 treated and 4 hydroxycoumarin-treated rats as shown in Table 11.

TABLE 11

AMOUNTS OF SERUM ALKALINE PHOSPHATASE IN NORMAL, AFLATOXIN B_1-TREATED, AND 4-HYDROXYCOUMARIN-TREATED RATS.

1 King-Armstrong Unit is the production of 1.0 mg. of phenol in 15 minutes under the conditions of the test.

RATS	OPTICAL DENSITY (510 mμ)	ALKALINE PHOSPHATASE (King-Armstrong Units per 100ml. serum).
Normal	0.10	5.3
Aflatoxin B_1-treated	0.10	5.3
4-Hydroxycoumarin - treated.	0.095	5.0

b) Bilirubin in Serum.

Procedure:

1.0 ml. of serum obtained from the blood of a decapitated rat was pipetted into a test tube and tested for bilirubin, using the method described on page 20 . Tests were performed on the sera of normal, aflatoxin B_1-treated, and 4 hydroxy-coumarin-treated rats. Optical densities were read on spectrophotometer (s.p. 600) at 540 nm.

Result:

The levels of serum bilirubin were about the same for three different sera as shown in Table 12.

TABLE 12.

LEVELS OF SERUM BILIRUBIN IN (a) AFLATOXIN B_1-TREATED AND (b) [illegible] RATS.

The colour of the standard solution was equivalent to that produced by serum containing 4.0 mgm. of bilirubin per 100 ml.

RATS	OPTICAL DENSITY (540 mμ)	BILIRUBIN IN 100 ml. SERUM (mgm).
NORMAL	0.032	0.17
AFLATOXIN B_1-TREATED	0.030	0.16
4 HYDROXYCOUMARIN-TREATED.	0.039	0.18

c) Urobilinogen in Urine.

Procedure

The method described on page 22 was used to determine the level of Urobilinogen in urine samples of aflatoxin B$_1$-treated and 4-hydroxycoumarin-treated sets of four rats each. A 24hour urine sample from each treated rat was obtained and measured. A 24-hour sample of normal urine was also obtained, measured and tested. Optical densities of the normal urine, test urine samples, and the standard phenolphthalein solution were read on a spectrophotometer (S.P.600) at 540 mμ.

Result

The amounts of urinary urobilinogen were about the same for the three different sets as shown in Table 13.

TABLE 13.

AMOUNTS OF URINARY UROBILINOGEN IN NORMAL, AFLATOXIN B_1 - TREATED, AND 4-CHLOROCOUMARIN-TREATED RATS.

The phenolphthalein standard has a similar colour to that given by 0.387 mg. Urobilinogen in 100 ml. of Urine.

RATS	OPTICAL DENSITY (540 mμ)	Units of Urinary Urobilinogen per 100 ml.
NORMAL	0.600	1.33
AFLATOXIN B_1 - TREATED	0.595	1.32
4 CHLORO - COUMARIN - TREATED	0.610	1.35

a) Thymol turbidity tests.

Procedure

Standards were set up as described on pages 24 to 25. 0.03 ml. of serum, pipetted from the blood of a decapitated normal rat, was added to 3.0 ml. of the thymol reagent (described on page 23) in a test tube. The contents in the tube were mixed and allowed to stand for 60 mimtes. The turbidity was read against the albumin standards. Similar experiments were performed, using sera obtained from the blood of

(i) a decapitated aflatoxin B_1- treated rat and

(ii) a decapitated 4 - hydroxycoumarin-treated rat.

Result.

The turbidity produced by each of the normal, aflatoxin B_1-treated, and 4-hydroxycoumarin-treated sera was the same as that produced by the standard which was equivalent to 20 mgm per 100ml. serum. Therefore, the thymol turbidity (arbitrary units) of each of normal, aflatoxin-treated, and 4-hydroxycoumarin-treated rats sera was 2

DISCUSSION

The relationship between the structures and hypoprothrombinaemic activities of coumarin-type molecules have been studied extensively (Arora and Mathur, 1963), following the discovery of dicoumarol as the toxic agent responsible for the serious haemorrhagic conditions in cattle(Campbell and Link, 1941).

Clotting time determinations on the blood of rats fed on a balanced diet which had been infested with a toxic strain of Aspergillus flavus showed that there was a substance in the contaminated diet which prolonged the blood clotting time of rats which fed on the diet (Figure 1). A mixture of aflatoxins were subsequently extracted from the mouldy diet. The fact that the effects of (1) the extracted mixed aflatoxins and (2) pure aflatoxin B_1 on blood clotting time (Table 2) were similar to that of the mouldy diet (Table 1) was an evidence that the anticoagulant activity of the mouldy diet was a result of the action of the aflatoxins present in the mouldy diet on the blood clotting factors.

By using the thrombotest technique for clotting time determinations, it was shown that any of the four blood clotting factors II, VII, IX and X could have been affected (Figure 2). The blood clotting time was determined primarily by the factor which was most deficient at the time of determination, because the deficient factor would be the limiting one for the coagulation reaction.

When specific thromboplastins were employed, it appeared as if factors II and VII (prothrombin and proconvertin, respectively) were deficient in the plasma of the rat which was injected with pure aflatoxin B_1 (Table 6.). But this does not preclude the possibility that either factor IX or factor X, or both, may have been deficient in the plasma of the treated rats.

Aflatoxin B_1, a coumarin-type compound (Asao et al., 1963) has been shown to be much more effective than 4-hydroxycoumarin in prolonging the blood clotting time of rats. In this connection, the comparative effective doses were 56×10^{-6} M. aflatoxin B_1 to 93×10^{-3} M. 4-hydroxycoumarin, using a rat which weighed about 300 grams

In an attempt to determine the mode of action of aflatoxin B_1 on blood clotting, its effect on the synthesis of coagulation factors by liver slices was investigated and was compared with that of 4-hydroxycoumarin.

Aflatoxin B_1 had been shown to have a carcinogenic activity in rats (Barnes and Butler, 1964) and Clifford and Rees (1966) had indicated that aflatoxin B_1 has a site of action in the rat liver cell. Smith and Mckernan (1962) were among the earliest to report some hepatotoxic action of chromatographically separated fractions of Aspergillus flavus extracts.

The results of the four liver function tests (Table 11, 12 and 13) performed on our experimental animals were an evidence that the parenchymal cells of the livers of these animals were intact when the effects of both aflatoxin B_1 and 4-hydroxycoumarin on blood clotting time were maximal. Therefore, it seems reasonable to conclude that the synthesis of at least one of the blood clotting factors (which are proteins made in the liver parenchymal cells-Barnhart, 1960) must have been inhibited. Such inhibition would have been responsible for the deficiency of the factor(s) in the plasma of the experimental animal.

Pool and Robinson (1959) found that Vitamin K_1 reversed the inhibitory effect of 3(α-acetonylbenzyl)-4-hydroxycoumarin on the synthesis of coagulation factors by rat liver slices.

Experiments described in this thesis showed that the prolongation of the blood clotting time and the inhibition of the synthesis of blood clotting factors II and VII in rat liver slices (Tables 7,8,9, and 10) by vitamin K_1, and by 4-hydroxycoumarin, were reversed when Vitamin K_1 was administered.

By using Russell viper venom as the thrombokinase for clotting the blood plasma of aflatoxin-treated rats, the synthesis of prothrombin in the parenchymal cells of the liver has been shown to be that which is predominantly inhibited (Table 10).

In view of these evidences, the action of aflatoxin B_1 on blood clotting could be considered to involve a similar mechanism to that of dicoumarol on blood clotting (Chmielewska and Olcalak, 1936) where, by virtue of structural resemblance, the coumarin compound competes with Vitamin K for the apoenzyme in the production of prothrombin in the liver.

CONTRIBUTION TO KNOWLEDGE

The thesis has made the following additions to the knowledge of the biological properties of the aflatoxins.

1. Aflatoxin B_1 prolongs the blood clotting time of rats.

2. Aflatoxin B_1 inhibits the synthesis of blood clotting factors - particularly prothrombin-in the liver cell of the rat.

3. Aflatoxin B_1 inhibits blood clotting reactions in which vitamin K is a cofactor.

The following approach is suggested for future work.

a) Experiments should be performed to determine if either factor IX (Plasma thromboplastin component) or factor X (Stuart-Prower factor) or both factors were deficient in the plasma of rats treated with Aflatoxin B_1.

b) The effects of Aflatoxin G on the clotting time should be investigated and compared with that of Aflatoxin B.

c) Attempts should be made to find out if any of the blood clotting factors binds to the aflatoxin.

d) An attempt could also be made to determine at what stage of the synthesis of a deficient factor the aflatoxin acts.

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