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The effect of aflatoxin on blood clotting in the rat

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1. The action of aflatoxin on blood clotting of rats was investigated. Aflatoxin B_1 prolonged the blood clotting time of rats.

2. Aflatoxin B_1 was effective as an anticoagulant in much smaller doses than 4-hydroxycoumarin.

This communication is concerned with the blood anticoagulant properties of aflatoxin. Attempts have also been made to compare these properties with those exhibited by 4-hydroxycoumarin in view of the similarities in the structures of the synthetic coumarins and the aflatoxins (see Asao, Buchi, Abdel-Kader, Chang, Wick & Wogan, 1963).

Methods

A balanced diet used for the culture of *Aspergillus flavus* was prepared according to the method of Bassir (1964). The diet was composed of gari flour (1 kg), soya bean flour (1 kg), methionine (40·0 g), lysine (2·0 g) and 100·5 g of a salt mixture containing sodium chloride (22·0 g), calcium phosphate (130·0 g), potassium citrate (125·0 g), magnesium sulphate (30·0 g), ferric citrate (5·0 g), and 0·7 g of a mixture of trace elements. The composition of the mixture of trace elements was as follows: potassium iodide (12·0 g), sodium fluoride (10·0 g), manganese sulphate (2·0 g), potash alum (1·0 g) and zinc sulphate (1·0 g).

Inoculation of diet

The balanced diet was inoculated with a toxic strain of *Aspergillus flavus*, using the method described by Bassir (1964). The Petri dish was covered and allowed to incubate for 5 days at room temperature (28° C).

Extraction and assessment of aflatoxin

The amount of food consumed by each of six rats was measured daily throughout the feeding period. The mean daily intake was found to be 32 g. This quantity of the contaminated diet was extracted continuously in a Soxhlet extractor for 6 hr, using methanol as solvent.

The methanol extract was diluted with water until the concentration of methanol

was reduced to 60% and then extracted three times with chloroform as described by de Iongh, van Pelt, Ord & Barrett (1964). The chloroform extract was concentrated to 25 ml. at 28° C under reduced pressure and aliquots of the extract were spotted on activated thin-layer plates made of Merck silica gel G. The plates were immediately developed in a solution of 3% methanol in chloroform and examined for fluorescence under ultraviolet light at 365 mμ.

The null-fluorescence dilution technique of Coomes, Crowther, Francis & Stevens (1965) was used to estimate the amount of aflatoxin in the daily intake of the contaminated diet. 0·2 ml. of the concentrated extract was made up to 20 ml. with chloroform. This gave a 1 : 100 dilution. Several dilutions of this solution were then examined for characteristic blue or green fluorescence of the aflatoxins under ultraviolet light, after spotting on the chromatoplate and running in 3% methanol/chloroform. By comparing with standards, the amount of aflatoxin B in the spot with the least fluorescence is given as 4×10^{-4} μg; while that of aflatoxin G is 3×10^{-4} μg (Tropical Products Institute, 1965).

Determination of blood clotting time

A set of six male albino rats, each weighing approximately 300 g, fed for 4 days *ad libitum* on the balanced diet on which a toxic strain of *Aspergillus flavus* Link had grown for 5 days. Three separate groups of six similar male rats were injected intraperitoneally with: (a) 17·5 μg (B/G = 1·3) of the mixed aflatoxins extracted from 32 g of the contaminated diet; (b) 17·5 μg of pure aflatoxin B_1; (c) 15·0 mg of 4-hydroxycoumarin (Hopkin & Williams, Essex, England). These substances were dissolved in distilled water, and administered in volumes of not more than 1·0 ml.

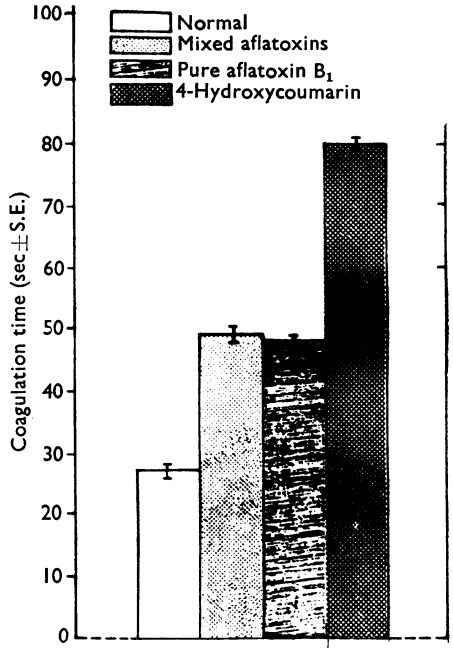

FIG. 1. Prolongation of blood coagulation time of rats treated with mixed aflatoxins, pure aflatoxin B_1 or 4-hydroxycoumarin. Each group contained six rats.

The sample of pure aflatoxin B_1 used in these experiments came from our departmental stock, and originated from mouldy local beans.

Another group of rats was kept as control on the uncontaminated diet. The blood clotting times of the test animals and their controls were determined using the thrombotest reagent (Nyegaard, Oslo, Norway) (see Owren, 1959).

Results

Production of aflatoxin in the culture of A. flavus

Aflatoxins B and G were identified under ultraviolet light at 365 mμ, in the purified extract of the mouldy diet. Using the method of Coomes *et al.* (1965) the total amount of mixed aflatoxins in 32 g of the mouldy diet was found to be 17·5 μg. The amounts of aflatoxin B and G given to each animal were assessed from thin-layer chromatograms of the extracts as described above. The ratio of the amount of aflatoxin B to that of aflatoxin G was estimated to be 1·3.

Prolongation of blood clotting time

The mean blood clotting time of the animals fed on the infested diet was $47{\cdot}9 \pm 0{\cdot}28$ sec as compared with $26{\cdot}25 \pm 0{\cdot}39$ sec for the normal rats.

After a period of 3 hr, both the mixed aflatoxins and pure aflatoxin B_1 had prolonged the normal clotting time maximally. The peak action of 4-hydroxycoumarin was reached after 48 hr.

Similar effects on blood clotting time were obtained by injecting rats fed on a normal diet with either the purified mixture of aflatoxin extracted from the contaminated diet or with 56×10^{-6}mM pure aflatoxin B_1 (Fig. 1). The prolongation of clotting time in these cases was similar to that found in rats fed on the infested diet.

In order to test whether or not the difference between the mean coagulation time of the control and that of each of the groups of experimental rats is significant, the *t* test has been used. As shown in Table 1, there was a highly significant difference between the control and the test animals in the three experiments (*a*), (*b*), and (*c*).

Discussion

In a study of the acute effects of aflatoxin B_1 in dogs, Newberne, Russo & Wogan (1966) observed occasional haemorrhage in the gastrointestinal tract, and also

TABLE 1. *Clotting times (sec) in rats following administration of 4-hydroxycoumarin (50·0 mg/kg, intraperitoneally), extracted mixture of aflatoxins (58·0 μg/kg. B/G=1·3 intraperitoneally) and pure aflatoxin B_1 (58·0 μg/kg, intraperitoneally)*

	Clotting time (sec)					
	Control	4-Hydroxy-coumarin	Control	Mixed aflatoxins	Control	Aflatoxin B_1
Mean±S.E.M.	27·3±0·2	80·7±0·2	27·3±0·2	49·4±0·2	27·3±0·2	48·1±0·1
N	6	6	6	6	6	6
t	249·0		103·2		97·2	
P	<0·001		<0·001		<0·001	

found that the blood clotting time was lengthened by at least a factor of 10. In a review by Schoental (1967), emphasis was placed on the need for studies on the anticoagulant property (amongst other pharmacological properties) of aflatoxin in doses which are not acutely toxic.

Our 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. The fact that the effect of the extracted mixed aflatoxins and that of pure aflatoxin B_1 on the blood clotting time were similar to that of the mouldy diet was evidence that the anticoagulant activity of the latter was due to the aflatoxins present in it.

The thrombo-test technique used does not enable us to distinguish between various factors involved in blood clotting; factors II (prothrombin), VII (proconvertin), IX (plasma thromboplastin component) and X (Stuart-Prower factor) could have been affected. Experiments to show which of these factors are actually involved will be published later.

Asao *et al.* (1963) presented evidence to show that aflatoxin B_1 is a coumarin-type compound, having a central 5-methoxycoumarin moiety. In our studies, aflatoxin B_1 has been shown to be much more effective than 4-hydroxycoumarin in prolonging the blood clotting time of rats.

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