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Species Differences in the Anticoagulant Activities of Aflatoxin B_1 and 4-Hydroxycoumarin

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Summary. A study of the effects of aflatoxin B_1 (dose 58 μg/kg) and 4-hydroxycoumarin (dose 50 mg/kg) on blood clotting in eleven animal species has been made by following the thrombo-test times.

The results of the experiments indicated that there is a species variation in the anticoagulant activities of aflatoxin B_1 and 4-hydroxycoumarin.

A relationship between the anticoagulant activities of these coumarin compounds and the nutritional status of each animal species is discussed.

Résumé. On a étudié les effets de l'aflatoxine B1 (a 58 μg/kg) et de l'hydroxycoumarine 4 (à 50 mg/kg) sur la coagulation du sang chez onze espèces animales, en suivant la méthode des thrombotests.

Les résultats des expériences révèlent des variations entre les espèces dans l'activité anticoagulante de l'aflatoxine B1 et de l'hydroxycoumarine 4.

Une relation entre les activités anticoagulantes de ces composés de la coumarine et le régime alimentaire de chaque espèce est évoquée.

Several coumarin derivatives have been studied from the point of view of their anticoagulant action (Arora & Mathur, 1963). Bababunmi & Bassir (1969) reported 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.

Schoental (1967) had observed that amongst the animal species which are very susceptible to the toxicity of aflatoxin (1 mg/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 mice and sheep are quite resistant and will tolerate large doses of aflatoxin without ill-effects.

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 & Williams, 1969). The lengthening of thrombo-test time in rat after 3 hr by a sublethal dose (58·0 μg/kg

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body weight) of aflatoxin B_1 may not apply to other species. Therefore, we have examined the extent to which aflatoxin B_1 (dose 58 μg/kg) and 4-hydroxycoumarin (dose 50 mg/kg) affect thrombo-test times in eleven animal species, namely, cat, dog, monkey, goat, chicken, duck, guinea-pig, mouse, hamster, rabbit and rat. Both the young and adult animals have been compared because the activity of some drug-metabolizing enzymes varies with age in animals of many species (Kato *et al.*, 1964). Male animals have been used because, in general, they metabolize drugs and foreign compounds more rapidly than females (Kato & Gillette, 1965; Williams, 1969). In the present paper it is shown that there is a species variation of the anticoagulant activities of aflatoxin and 4-hydroxycoumarin. This variation seems to fit into a dietary classification of the mammalian species.

MATERIALS AND METHODS

Materials

Aflatoxin B_1 was prepared as described by Bababunmi & Bassir (1969) and crystals were obtained using the procedure of Robertson, Pons & Goldblatt (1967). The crystals were considered pure when the materials exhibited a single spot when analysed by thin-layer chromatography. 4-Hydroxycoumarin, m.p. 212–214 °C, was used as purchased from Hopkins & Williams, Essex, U.K. Thrombo-test reagent (Nyegaard & Co., Norway) was purchased from Duncan, Flockhard & Evans Ltd, London, U.K. It was supplied as a freeze-dried substance in vacuum-sealed ampoules of 2·2 ml each.

Animals

Rat and mouse were collected from the animal house of our Department. They were fed with a commercial diet purchased from Livestock Feed Ltd, Ikeja, Nigeria. The diet was composed of carbohydrates (71·5%), crude protein (21·0%), fibre (4·0%) and oil (3·5%). Rabbit, guinea-pig and hamster were also collected from the animal house. Their diet which was composed of carbohydrates (72·9%), crude protein (20·0%), fibre (3·4%) and oil (3·7%) was purchased from Livestock Feed Ltd, Ikeja, Nigeria. Duck and chicken 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%. Cat and dog were purchased from a local market in Ibadan and were maintained on meat and bones. Goat was also purchased from a local market and had access to green leaves and various bruised vegetables. Monkey was supplied by the University of Ibadan Zoological Garden. It was maintained on various kinds of foodstuff ranging from vegetables to meat.

Cannulation of animals and dosing. Table 1 shows the details of the body weights of the different animals used, the dose level of anaesthetic and the sizes of the polyethylene cannulae used in the collection of blood. Except for rabbit, all the other experimental rodents were decapitated. Young chickens and ducklings were also decapitated. Care was taken to avoid contamination of the blood with tissue fluid by allowing the blood to drip freely from the decapitation wound into the container. The operational procedure for cannulation was as follows: The animals were anaesthetized with sodium pentobarbitone (B.P. Abbott's Veterinary Nembutal, containing 60 mg/ml of Nembutal in a mixture of alcohol and propylene glycol) injected slowly intraperitoneally. The anaesthesia was given in doses of 0·6–1·0 ml/kg body weight (see Table 1). The left femoral vein was cannulated with the

appropriate size polyethylene tubing and 0·4 ml/kg body weight of heparin (4000 units/ml) was injected and washed in with saline; this level of heparin did not affect the thrombo-test time at the time of blood sampling. Blood was collected after 3 hr in the aflatoxin-treated animals (Bababunmi & Bassir, 1969) whilst it was collected after 48 hr in the 4-hydroxycoumarin-treated animals (Arora & Mathur, 1963; Bababunmi & Bassir, 1969). The maximum anticoagulant effects of these compounds were found, in our experiments, to occur at these times in all the animal species studied. In the case of the animals which were to be decapitated, two separate groups of six similar animals were injected intraperitoneally with (a) aflatoxin B_1 (58·0 μg/kg) (b) 4-hydroxycoumarin (50 mg/kg). 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 and age were kept as control and injected with 1·0 ml of pure distilled water.

TABLE 1. Species, body weights, dose of anaesthetic and size of cannulae

Species	Body weight (kg)		Dose of Nembutal (ml/kg body weight)		Diameter of cannulae (mm)			
	Young	Adult	Young	Adult	Young		Adult	
					Internal	External	Internal	External
Dog (familiaris. L)	2·4 ± 1·1	9·1 ± 0·3	0·5	0·6	0·56	0·80	1·21	1·44
Cat (domestica)	0·55 ± 0·2	2·9 ± 0·6	0·6	0·6	0·56	0·80	0·65	0·93
Mouse (albino, musculus)	0·028 ± 0·002	0·04 ± 0·003	—	—	—	—	—	—
Hamster (golden	0·32 ± 0·01	0·81 ± 0·05	—	—	—	—	—	—
Rat (albino, wistar)	0·18 ± 0·01	0·30 ± 0·013	—	—	—	—	—	—
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 ± 0·003	—	—	—	—	—	—
Monkey (Papio, W. Africa)	—	8·4 ± 0·7	—	0·8	—	—	1·21	1·44
Chicken (Whiterocks, domestica)	0·056 ± 0·002	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

Collection of blood. Blood from each animal was collected in a different plastic tube (11 cm × 1·5 cm) and stopped from clotting by mixing with 3·13% (w/v) sodium citrate solution in a 1 : 9 (v/v) ratio of sodium citrate and blood.

Preparation of thrombo-test reagent. The freeze-dried thrombo-test 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.

Clotting time determination. 0·05 ml of the citrated blood was pipetted off and blown into the thrombo-test reagent immediately (see Owren, 1959), holding the top of the 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 bath. 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°±0·1 °C.

RESULTS

Since the relationship between the log of the clotting time and percentage clotting activity is linear, and tissue thromboplastin is species specific (Owren, 1959), results for comparison have been expressed as percentage increase of normal clotting activity which is brought about by the drug, that is, percentage increase of the log of clotting times. In order to indicate absolute differences in clotting or anticoagulant action the Student's *t*-test has been used. *P* values are given for the comparison of each experimental group with normal and are considered statistically significant if $P<0{\cdot}05$; where $P>0{\cdot}05$, the values are designated as NS (not significant).

Carnivorous mammals

With aflatoxin treatment there was no significant anticoagulant action on the young cats and dogs ($P>0{\cdot}1$). The effect of 4-hydroxycoumarin is similar to that of aflatoxin. In adult cats and dogs, also, the anticoagulant effects of both 4-hydroxycoumarin and aflatoxin are not statistically significant.

Omnivorous mammals

Aflatoxin and 4-hydroxycoumarin increase markedly clotting time in rat, mouse and hamster. At the standard doses used 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 the anticoagulant activity of aflatoxin on both young and adult mice is quite marked despite the reported resistance of this species to aflatoxin poisoning (Newberne & Butler, 1969). However, when the various *t*-values were compared, 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. The results obtained from the adult monkey are somewhat similar to those of cat and dog.

Herbivorous mammals

The actions of these drugs 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 anticoagulants, except in the rabbit where the activities of the two drugs were increased in 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 the anticoagulant activity of each drug is also pronounced in both the adult and in the young. In the duck, however, the anticoagulant effects of the two drugs seem to increase with the age of the animal.

TABLE 2. Anticoagulant activities of aflatoxin B_1 and 4-hydroxycoumarin in the young of various species. Males were used. The dose of aflatoxin B_1 was 58·0 μg/body weight, injected intraperitoneally in 1·0 ml distilled water. Blood was collected at 0 hr and after a 3-hr period. The dose of 4-hydroxycoumarin was 50 mg/kg body weight, injected intraperitoneally in 1·0 ml distilled water. Blood was collected at 0 hr and after a 48-hr period. For determination of blood clotting time, thrombo-test reagent was used (see the text). Results are expressed as mean values for six animals.

Species	Normal thrombo-test time* (sec)	Anticoagulant activities (as mean % increase of the log of clotting times)	
		Aflatoxin B_1	4-hydroxycoumarin
Dog	26·0 ± 5·9	3·0 (NS)	5·5 (NS)
Cat	32·0 ± 7·1	0·0 (NS)	2·1 (NS)
Mouse	15·8 ± 0·3	34·5 ($P < 0·001$)	32·0 ($P < 0·001$)
Hamster	19·4 ± 0·1	51·0 ($P < 0·001$)	36·0 ($P < 0·001$)
Rat	32·0 ± 0·2	26·5 ($P < 0·001$)	33·3 ($P < 0·001$)
Rabbit	26·0 ± 0·3	32·0 ($P < 0·001$)	41·0 ($P < 0·001$)
Goat	25·0 ± 0·2	25·0 ($P < 0.001$)	30·2 ($P < 0·001$)
Guinea-pig	29·6 ± 0·1	32·7 ($P < 0·001$)	36·0 ($P < 0·001$)
Chicken	51·7 ± 0·2	19·4 ($P < 0·001$)	22·5 ($P < 0·001$)
Duck	101·5 ± 1·4	28·0 ($P < 0·001$)	23·0 ($P < 0·001$)

* Mean ± SEM.

DISCUSSION

The action of foreign compounds can vary with the nutritional status of the animal (Williams, 1938; Dingell, Joiner & Hurwitz, 1966). And in 1968, Osiyemi reported that rats maintained on a diet deficient in protein showed a diminished rate of aflatoxin metabolism.

Our results obtained on the species variation of the anticoagulant activities 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 (see Tables 2 and 3), except for adult mouse and adult duck 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 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 habit is of importance.

Foreign compounds undergo metabolism in the gastro-intestinal tract by the action of the gut microflora (Dacre & Williams, 1968; Renwick & Williams, 1969). The microflora of the gut also give rise to the formation of toxic compounds such as amines and aromatic hydrocarbons (see Parke & Williams, 1969). Many toxic chemicals, including aflatoxin B_1, are excreted in the bile (Bassir & Osiyemi, 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

TABLE 3. Anticoagulant activities of aflatoxin B_1 and 4-hydroxycoumarin in adult animals of various species. Males were used. The dose of aflatoxin B_1 was 58·0 μg/kg body weight, injected intraperitoneally in 1·0 ml distilled water. Blood was collected at 0 hr and after a 3-hr period. The dose of 4-hydroxycoumarin was 50 mg/kg body weight, injected intraperitoneally in 1·0 ml distilled water. Blood was collected at 0 hr and after a 48-hr period. For determination of blood clotting time, thrombo-test reagent was used (see the text). Results are expressed as mean values for six animals, except for monkeys where two animals were used.

Species	Normal thrombo-test time* (sec)	Anticoagulant activities (as mean % increase of the log of clotting times)	
		Aflatoxin B_1	4-hydroxycoumarin
Dog	15·5 ± 3·4	3·4 (NS)	6·0 (NS)
Cat	26·5 ± 5·0	5·9 (NS)	4·0 (NS)
Mouse	18·2 ± 0·2	50·1 ($P < 0·001$)	51·0 ($P < 0·001$)
Hamster	19·3 ± 0·2	57·0 ($P < 0·001$)	49·4 ($P < 0·001$)
Rat	28·5 ± 0·3	41·2 ($P < 0.001$)	48·0 ($P < 0·001$)
Rabbit	28·1 ± 0·2	39·0 ($P < 0·001$)	46·0 ($P < 0·001$)
Goat	29·0 ± 0·3	30·0 ($P < 0·001$)	27·5 ($P < 0·001$)
Guinea-pig	33·5 ± 0·2	41·0 ($P < 0·001$)	36·4 ($P < 0·001$)
Monkey	44·0 ± 2·1	0·2 (NS)	2·6 (NS)
Chicken	66·8 ± 0·1	26·0 ($P < 0·001$)	23·0 ($P < 0·001$)
Duck	118·5 ± 1·1	35·3 ($P < 0·001$)	35·8 ($P < 0·001$)

* Mean ± SEM.

molecular weight greater than 400 (Millburn, Smith & Williams, 1967). Parke & Williams (1969) reported that conjugates excreted in the bile may be hydrolyzed by the action of intestinal micro-organisms to give the original toxins or may be decomposed to give new toxic substances which are subsequently re-absorbed from the gut (Williams, Millburn & Smith, 1965; Smith, 1966). In the present paper the drugs were administered intraperitoneally and not orally. Therefore, the species differences in 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. Species differences in the metabolism of foreign compounds occur frequently and some of their enzymic bases have been discussed by Williams (1967). Bassir & Bababunmi (1969) observed that a sublethal dose of aflatoxin B_1 inhibits blood clotting

factors 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. The ultrastructure of the rat liver cell has been shown to be intact during the anticoagulant action of the dose of aflatoxin B_1 used in these experiments (Bassir & Bababunmi, 1970). Therefore, the differences in the response of various animal species to the anticoagulant activities of aflatoxin and 4-hydroxycoumarin could be due to the extent of competition between these drugs and vitamin K in the liver parenchymal cell of each animal.

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