SPECIES DIFFERENCES IN THE *IN VITRO* METABOLISM OF AFLATOXIN B_1

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1. Introduction

It is well established that the duration of some foreign compounds in the animal body depends on enzyme metabolism [1, 2]. The enzymes concerned are located primarily in the liver microsomes. Liver slices and microsome-plus-soluble fractions of liver have been employed for the study of the metabolic fate of aflatoxin B_1 [3–6]. Our experiments showed that aflatoxin B_1 is metabolised in the rat, golden-hamster, mouse, sheep and goat by demethylation and hydroxylation. The possibility of employing *in vitro* techniques for the determination of metabolites of aflatoxin B_1 in the toad, lizard, duck and cockerel is the subject of this report.

2. Materials and methods.

White Rock cockerel (2.0–2.2 kg), duck (1.8–2.2 kg) of a local strain, lizard, *Agama agama* (70–80 g) and toad, *Bufo regularis* (50–60 g) were used. Male animals were used throughout. Microsome-and-soluble fractions of the livers of the animals were prepared as described by Bassir and Emafo [6]. The incubation of the liver microsome-plus-soluble fraction and substrate, and the estimation of the metabolites of aflatoxin B_1 were performed as described earlier [6]. The flasks were incubated in air for 1 hr in a Gallenkamp reaction shaking incubator at 37.0 ± 0.5° for the microsomes of the cockerel and duck, but for the toad and lizard the incubation was at room temperature, 25–26°. Controls consisting of a flask in which the microsome-plus-soluble fraction was replaced by inactivated (boiled) microsome-plus-soluble fraction and a flask in which aflatoxin B_1 was omitted were included. The protein in the incubation medium was precipitated with 2 ml of 20% zinc sulphate solution and 2 ml of saturated barium hydroxide solution. The medium was centrifuged at 5,000 *g* and the supernatant concentrated in a rotary evaporator. The concentrate was applied to thin-layer chromatoplates of silica-gel G and developed in 10% acetone in chloroform v/v.

The metabolite with zero R_f value was scraped off and eluted with a mixture of methanol–chloroform–water (5:2:2, by vol). The eluate was concentrated to a small volume by bubbling nitrogen gas through it at 37°. The concentrate was applied in one experiment to thin-layer chromatoplates (TLC) of silica-gel H (E. Merck, Darmstadt, Germany) and in another experiment to Whatman no. 1 chromatography paper, and developed in *n*-butanol–glacial acetic acid–water (10:1:1, v/v). Because of the high polarity of the fluorescent metabolite at the origin of the silica-gel G TLC developed in 10% acetone in chloroform v/v, it was suspected of being a metabolic conjugate of either aflatoxin B_1 or aflatoxin M_1. The suspected conjugate was tested for its stability in boiling 0.3 N hydrochloric or 0.3 N sulphuric acid. It was further tested for mercapturic acid conjugation by the method of Knight and Young [8] for amino acid conjugation according to Williams and Kirby [9], for sulphate conjugation by the method of Burma [10] as modified by Schneider and Lewbart [11] and for glucuronide conjugation by the method of Bridges, Kibby, and Williams [12].

3. Results and discussion

In order to study the nature of the metabolites of aflatoxin B_1, the thin-layer chromatogram of the concentrated incubate was examined under intense ultra-violet light in the dark. Unaltered aflatoxin B_1 and one or two metabolites which were more polar than aflatoxin B_1 were found to be present in the concentrated incubates. The lizard and the toad metabolized aflatoxin B_1 into a blue-violet fluorescing substance (R_f 0.20 in 10% acetone in chloroform v/v solvent system). This metabolite has the same R_f value as standard aflatoxin M_1. This standard was obtained by extracting with chloroform the urine of a sheep injected intraperitoneally with aflatoxin B_1, and subsequently isolating and purifying the extracted aflatoxin M_1 by thin-layer chromatography. Apart from the R_f value, the blue-fluorescing metabolite (R_f 0.20) showed an ultra-violet spectrum with maxima peaks at 210, 226, 265 and 357 nm. On the basis of the ultra-violet absorption peaks [7] and R_f value, this metabolite was identified as aflatoxin M_1. The isolation of aflatoxin M_1 as an *in vitro* metabolite of aflatoxin B_1 by the lizard and the toad is an indication of the presence of liver-microsomal hydroxylating enzymes in these species. The duck and the White Rock cockerel did not metabolize aflatoxin into aflatoxin M_1 (table 1). This observation is an indication of species differences in the *in vitro* metabolism of aflatoxin B_1 by hydroxylation to form aflatoxin M_1. These species differences in the hydroxylation of aflatoxin B_1 call to mind previous reports in which species differences were observed in the hydroxylation of coumarin and biphenyl [13, 14]. Also, there was no statistically significant difference in the amount of aflatoxin M_1 produced by the lizard and the toad (table 1).

Another metabolite with zero R_f value in the same solvent system was a common metabolite to the animal species studied. On the basis of the R_f values of this metabolite on silica-gel H chromatoplates and on paper chromatogram, only one type of conjugate was identifiable. This conjugate has an R_f value of 0.50 on silica-gel H chromatoplates developed in *n*-butanol–glacial acetic acid–water solvent system. The conjugate was hydrolysed by 0.3 N hydrochloric acid or 0.3 N sulphuric acid treatment. This hydrolysis of the metabolite proved conclusively that it existed in a conjugated form. The conjugate showed negative reaction to the ninhydrin test, mercapturic acid test, mercapturic acid test and glucuronide test and was, therefore, thought to be a sulphate conjugate because of its ready hydrolysis with 0.3 N mineral acid. However, the inability of the conjugate to produce a yellow colour with the potassium rhodizonate spray after exposure of the silica-gel H chromatoplates and paper chromatogram, respectively, to concentrated hydrochloric acid fumes [11] seems to indicate

Table 1
Influence of species differences on the metabolism of aflatoxin B_1 by liver microsomes.

Animal species	Aflatoxin B_1 in the incubation medium (nmoles)	% Aflatoxin B_1 metabolized during the 1 hr. incubation period	% Aflatoxin B_1 converted to free aflatoxin M_1 during 1 hr incubation period	Formaldehyde (nmoles) produced in 1 hr by the equivalent of 1 g liver	No of animals
White rock Cockerel	160	99.4 ± 0.4	0	49.8 ± 8.9	3
Duck	150	98.6 ± 4.5	0	0	3
Lizard *Agama agama*	160	99.6 ± 0.4	0.63 ± 0.20	0	30
Toad *Bufo regularis*	160	98.6 ± 0.8	0.56 ± 0.14	0	24

The results from a given incubation were well within ± 10% of the mean.

that the conjugate may after all, not be a sulphate conjugation product. Further work is in progress in our laboratory with a view to identifying this conjugate which is a common metabolite to the species used in this study. Since the duck and White Rock cockerel have been shown to metabolize aflatoxin B_1 by conjugation, it is possible, therefore, that aflatoxin B_1 is first hydroxylated before conjugation takes place. The inability to detect the hydroxylated toxin may be a result of rapid conjugation which occurs soon after hydroxylation. The implications of the yet unidentified conjugation product in determining aflatoxin B_1 toxicity are yet not known.

Most of the aflatoxin B_1 (98.6–99.6%) in the incubation medium was metabolized *in vitro* during an hour's incubation (table 1). With the duck and White Rock cockerel, differences in the metabolic rate of aflatoxin B_1 are not very marked, and so factors other than metabolism may account for differences in susceptibility in these species. The result of the *in vitro* metabolic studies seems to support the view that aflatoxin B_1 is rapidly metabolized into non-fluorescent metabolites other than the conjugated metabolite in the duck and White Rock cockerel. This rapid metabolism of aflatoxin B_1 in the cockerel might account for the absence of aflatoxin B_1 and its fluorescent metabolite(s) in the liver, meat or blood of chickens fed on toxic groundnut meal [15, 16]. It is important to point out that the choice of the solvent system used for extracting the chicken might have prevented Platonow [15] from isolating the yet unidentified conjugate.

In the demethylation studies of aflatoxin B_1 (table 1) the results showed that only the White Rock cockerel demethylated aflatoxin B_1.

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