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METABOLIC BASIS OF THE SPECIES DIFFERENCE TO AFLATOXIN B_1 INDUCED HEPATOTOXICITY

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Primary metabolism of aflatoxin B_1 by the liver microsomal enzymes from a range of animal species showed both quantitative and qualitative differences. Quail was shown to have the most rapid metabolism of aflatoxin B_1. The major product of metabolism in this case was found to be aflatoxin B_1-8,9-dihydrodiol suggesting that the quail microsomes produced high levels of the proposed reactive intermediate aflatoxin B_1-8,9-epoxide. Using this system to generate the epoxide, the ability of the cytosol prepared from each species to conjugate epoxide with reduced glutathione was investigated. Large differences in ability to conjugate were observed ranging from 0 to 72% for quail and mouse respectively. Differences in both primary and secondary metabolism of AFB_1 were noted between male and female Fischer 344 rats.

Animal species differ markedly in their susceptibility to both the acute and chronic toxicity of aflatoxin B_1 (1,2). Activation to AFB_1-8,9-epoxide is considered to be essential for the biological effect of this potent hepatotoxin and hepatocarcinogen (3,4,5,6,7). The *in vitro* production of epoxide as measured by the detection of the hydrolysis product AFB_1-8,9-dihydrodiol, as the Tris-diol complex (Fig. i) has been shown to parallel the *in vivo* susceptibility to acute aflatoxin B_1 poisoning in various species (8). The effect of glutathione on AFB_1 toxicity has been reported (9,10,11,12) and the formation of a glutathione conjugate has been described (13,14). Degen and Neumann (16) reported that differences in

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Abbreviations

AFB_1	aflatoxin B_1
Tris	Tris(hydroxymethyl)methylamine
AFB_1-GSH	8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-aflatoxin B_1
h.p.l.c.	high performance liquid chromatography
AFQ_1	aflatoxin Q_1
AFM_1	aflatoxin M_1
AFP_1	aflatoxin P_1
Tris-diol	Reaction product of AFB_1-8,9-dihydrodiol with Tris(hydroxymethyl)-methylamine.

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the formation of this material by rat and mouse post mitochondrial supernatant may explain different susceptibilities to AFB_1 toxicity between species and between sexes.

We have recently described an *in vitro* system of preparing 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-aflatoxin B_1 and provided ^1H n.m.r. and mass spectral evidence for the structure (15). In this paper we describe a further study into the primary metabolism of AFB_1 in a range of species combined with an investigation of the formation of AFB_1-GSH by the post-microsomal supernatant of each species using quail microsomes as a standard method of formation of AFB_1-8,9-epoxide. By this approach we hope to compare

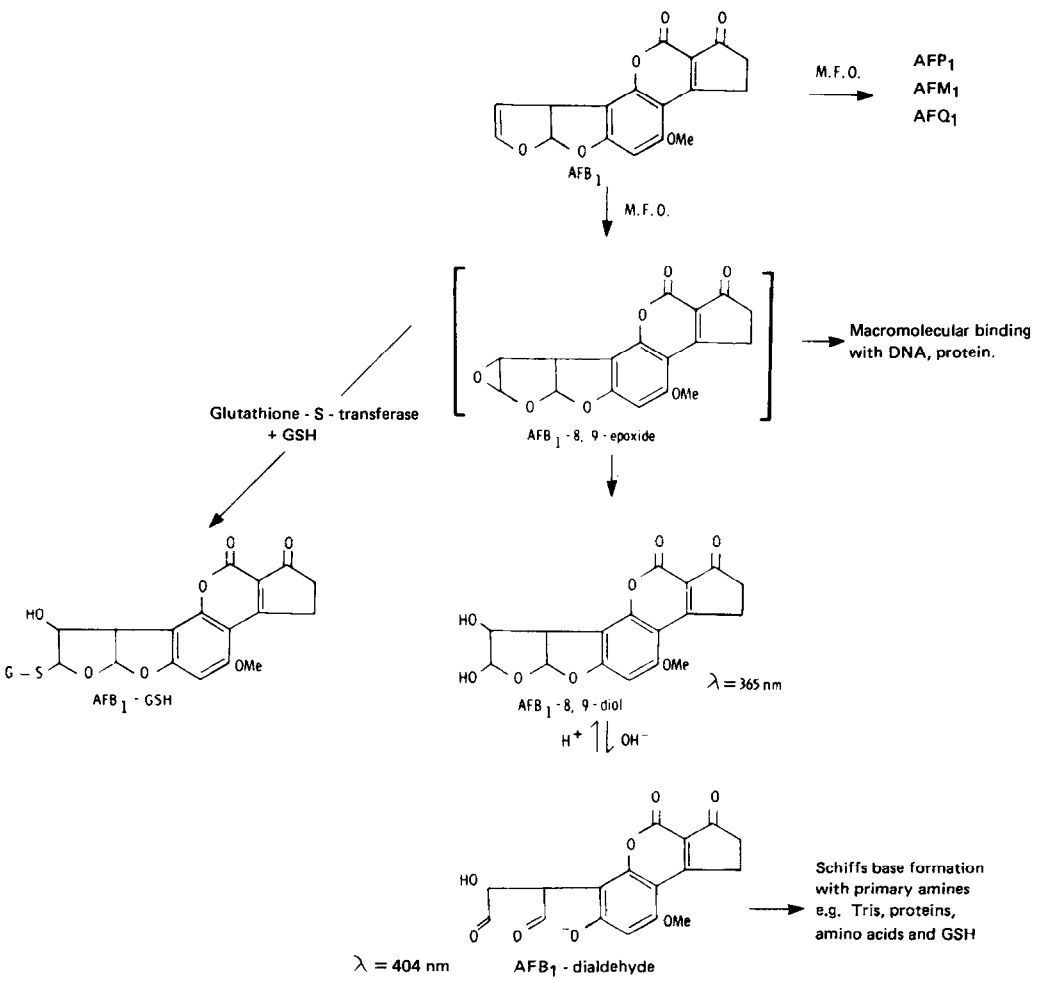

Fig. i. Metabolic pathway of AFB_1.

quantitatively the ability of each species to activate AFB_1 and the deactivation activity as measured by AFB_1-GSH production. Three mammalian and one avian species were investigated. Published LD_{50} values suggested a range of susceptibility to the acute toxicity of AFB_1 for these animals. Toxic response was investigated by *in vivo* studies to allow for strain differences from the published LD_{50} value.

The implications of the results to the importance of glutathione conjugation in AFB_1-toxicity are discussed and the results obtained from a single sample of human liver are compared to those from the animal studies.

MATERIALS AND METHODS

Materials

Aflatoxin B_1 was obtained from Makor Chemical Company, Jerusalem, Israel. Adult male and female rats (Fischer 344, body weight, 200-250 g) were supplied by MRC, Carshalton Beeches. Adult male mice (C57/BL 10 body wt. 25 g) and adult male albino guinea pigs (600 g) were supplied by Olac 1976 Ltd, Shaws Farm, Blackthorn, Bicester, Oxon. Adult male Japanese quails (200-250 g) were obtained from Lincolnshire Pheasantries, Tunby, Boston, Lincolnshire.

Tissue Preparation

Rats and quails were killed by decapitation; the mice and guinea pigs were killed by cervical fracture. Microsomes and post microsomal supernatant were prepared as described previously (17).

Metabolic Assays

Microsomal incubations using a microsomal suspension (400 µl) equivalent to 0.25 g wet weight of liver tissue, were carried out in triplicate as previously described (17) in the presence or absence of NADPH. Microsomal and supernatant metabolic incubations were essentially as for microsomal metabolism except that the mixtures were supplemented with 5 mM reduced glutathione. Post microsomal supernatant (400 µl) equivalent to 0.13 g wet weight of liver tissue was added prior to the addition of microsomes. Incubations were carried out in triplicate.

H.p.l.c anaysis

Chromatographic analysis was achieved by the method described previously (15).

In Vivo Studies

Animals were dosed intraperitoneally with aflatoxin B_1 dissolved in DMSO (6 mg cm^{-3}). One animal of each species received a high dose equivalent to twice the published LD_{50}, a low dose equivalent to the estimated LD_{50} and a control injection of DMSO. Quails received doses of aflatoxin B_1 equivalent to 1 and 2 mg kg^{-1} body weight. Mice were given the equivalent of 7.5 mg and 15 mg kg^{-1} body weight. Guinea pigs were given 2 and 4 mg kg^{-1}. Male and female rats were both given 1 and 2 mg kg^{-1} body weight. After 48 hours all surviving animals were sacrificed and the livers were removed and fixed in formal saline for 1 week. Samples were prepared for sectioning and stained with Harris' haemotoxylin and eosin.

Human Metabolism of AFB_1

A sample of normal male human liver was obtained from King's College Hospital Liver Unit. Tissue was stored in liquid nitrogen for 4 weeks prior to the experiment. Microsomes and post-microsomal supernatant were prepared as described in tissue preparation. All experiments involving microsomes and post-microsomal supernatant were carried out immediately after sample preparation.

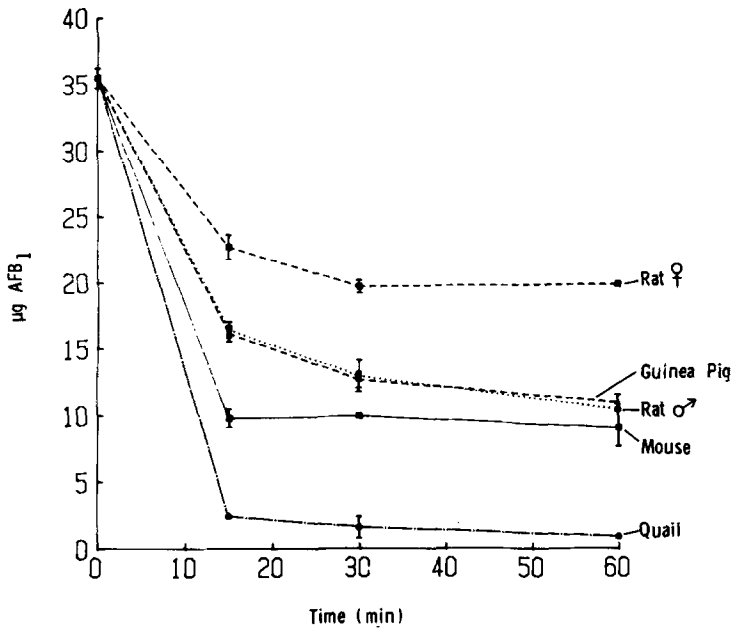

Fig. ii. Time course of microsomal metabolism of AFB_1. For each time point n=6 and the bars indicate the standard error of the mean.

RESULTS

The microsomal metabolism of AFB_1 was examined using h.p.l.c. The time course for the disappearance of AFB_1 (Fig. ii) revealed qualitative differences in the rate of AFB_1 metabolism between species (Table 1). The quail microsomes had the most rapid metabolism of AFB_1 (1.57 nmol/mg microsomal protein/min) and those of the female rat had the slowest (0.54 nmol/mg microsomal protein/min). H.p.l.c. analysis also revealed qualitative differences in the range of AFB_1 metabolites produced after a 30 minute incubation. The recoveries of soluble aflatoxins are recorded in Table 1. The production of AFB_1-8,9-dihydrodiol was measured by the detection of the Tris-diol complex by h.p.l.c. In the quail microsomal assay the recovery of soluble aflatoxins from the incubation mixtures after 30 minutes accounted for approximately 46% of the original AFB_1. 87% of the recovered aflatoxin was detected as AFB_1-8,9-dihydrodiol.

The recovered aflatoxins from mouse microsomal assays totalled 73% of the original substrate concentration. Nearly 30% of the total recovered was unmetabolized AFB_1, 30% was AFB_1-8,9-dihydrodiol and 40% was

Table 1

Species	LD_{50}* (mg/kg body wt.	Rate of AFB_1 metabolism (nmol/mg protein/min)	Recovery of soluble aflatoxins after 30 min (% of initial AFB_1 concentration)	Production of AFB_1-8,9-dihydrodiol (% of total metabolites recovered)	Production of AFB_1-GSH by quail microsomes + postmicrosomal supernatant from each species + GSH (% of total metabolite)
Quail	0.3#	1.57	46	87	0
Guinea pig	1.4-2	0.82	68	31	6.8
Rat (Male)	7.2x	0.69	63	12	6
Rat (Female)		0.54	71	5	16
Mouse (C57)	>9	0.73	73	30	72

* From Newberne and Butler (1969)

\# Estimated from duckling and in vivo studies (Table 2)

x See Table 2 and discussion.

AFP_1, AFM_1 and AFQ_1 combined. The recovery from guinea pig microsomal incubations was 68% and the detectable AFB_1-8,9-dihydrodiol was 31% of this. Unmetabolized AFB_1 (58%), and AFM_1, AFQ_1 and AFP_1 (11%) accounted for the rest of the recovered material. The recovered soluble aflatoxins from male and female rat were 63% and 71% respectively. In both cases a large percentage of this was unmetabolized AFB_1. AFB_1-8,9-dihydrodiol accounted for 12% of the soluble aflatoxins from the male rat microsomal assay and 5% from that of the female.

Using quail microsomes as an efficient system to produce AFB_1-8,9-epoxide, the ability of post microsomal supernatant from each species to convert the AFB_1-8,9-epoxide to AFB_1-GSH was investigated. The combination of quail microsomes with quail supernatant resulted in the production of AFB_1-8,9-dihydrodiol. The conjugating ability of the quail supernatant to convert AFB_1-8,9-epoxide to the glutathione conjugate is very low or absent. In contrast the combination of quail microsomes with mouse supernatant produced almost exclusively AFB_1-GSH demonstrating high conjugating ability in the mouse supernatant (Fig. iii). The relative production of AFB_1-GSH is recorded in Table 1.

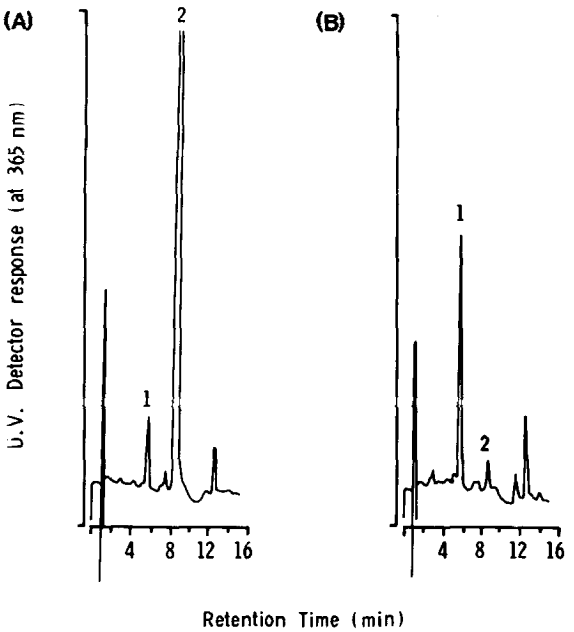

Fig. iii. H.p.l.c. analysis of AFB_1 metabolites. (A) product of incubation of AFB_1 with quail microsomes and mouse supernatant. (B) incubation of AFB_1 with quail microsomes and quail supernatant. All incubations in the presence of Tris-buffer. Peak 1 is Tris-diol. Peak 2 is AFB_1-GSH.

The effect of intraperitoneal dosing of AFB_1 to each species is recorded in Table 2.

Human Metabolism

Human microsomes metabolized AFB_1 rapidly (2.2 nmol/mg microsomal protein/min). After 30 minute incubation, 45% of the AFB_1 was recovered as soluble metabolites. Of these, AFQ_1 was the major product (63%) and AFB_1-8,9-dihydrodiol accounted for 11%. Incubations of AFB_1 with quail microsomes and the post microsomal supernatant from human liver resulted in the production of AFB_1-8,9-dihydrodiol exclusively.

DISCUSSION

From the in vivo estimation of toxicity, quail was the most sensitive species studied. Of the 4 animal species investigated, quail microsomes showed the most rapid metabolism of AFB_1, and AFB_1-8,9-dihydrodiol was the only metabolite detected in the soluble fraction from the incubation.

Table 2
Estimation of toxicity of AFB_1

Species	Dose ($mgkg^{-1}$ i.p.)	Observed toxicity after 48 hours
Quail	2	Death. Autopsy revealed haemorrhagic necrosis.
	1	Periportal necrosis and bile duct proliferation.
Guinea Pig	4	Centrilobular necrosis and bile duct proliferation.
	2	Centrilobular necrosis.
Rat (Male) (Fischer 344)	2	Severe haemorrhagic necrosis.
	1	Periportal necrosis.
Rat (Female) (Fischer 344)	2	Periportal necrosis.
	1	Mild periportal necrosis.
Mouse (C57)	15	No pathological changes observed.
	7.5	No pathological changes observed.

The low recovery of aflatoxin B_1 and metabolites in the soluble fraction was probably due to high levels of macromolecular binding.

The addition of quail post-microsomal supernatant to the quail microsomal incubation did not produce any detectable AFB_1-GSH. It would appear that the susceptible quail can rapidly activate AFB_1 to AFB_1-8,9-epoxide but lacks the ability to deactivate this reactive intermediate by conjugation with glutathione.

The published LD_{50} value for mouse is relatively high (2) and the results of the in vivo estimations of toxicity (Table 2) suggest that the LD_{50} value for the C57 strain is greater than 15 mg kg^{-1}. This contrasts with the estimated LD_{50} value of between 1 and 2 mg kg^{-1} for guinea pig and rat. The mouse and guinea pig show similar rates of AFB_1 metabolism and production of AFB_1-8,9-dihydrodiol. The resistance of mouse to AFB_1 toxicity is therefore not adequately explained by a consideration of primary metabolism alone. However, a comparison of the conjugating activity of the post-microsomal supernatant from each species, when combined with quail microsomes, shows that mouse produces over ten times the amount of AFB_1-GSH compared with guinea pig and twelve times that produced by male rat. This

efficient deactivation by mouse liver cytosol must be an important mechanism in the observed resistance to AFB_1 hepatotoxicity *in vivo*.

The Fischer 344 rats used in this study are a relatively susceptible strain. The hepatic damage observed in the male after i.p. doses of 1 and 2 mg kg^{-1} would be consistent with LD_{50} values between 1 and 2 mg kg^{-1}. It has been reported that the female rat is more resistant to the acute and chronic toxicity of AFB_1 (2). Our *in vivo* acute toxicity study (Table 2) was in agreement with this report. Microsomal metabolism of AFB_1 revealed that less AFB_1-8,9-epoxide is produced by the female and conjugating activity in the post-microsomal supernatant was higher in the female than in the male. The formation of AFB_1-GSH was considerably less by both male and female post-microsomal supernatant compared with that of mouse.

It would appear therefore that the relative species susceptibility to AFB_1-8,9-epoxide is explained by the activating and deactivating mechanisms described, and the formation of AFB_1-GSH is an important detoxification mechanism in resistant species. The h.p.l.c. analyses shown in Fig. iii demonstrate the difference in production of AFB_1-GSH by mouse post-microsomal supernatant (a) compared with that of quail (b) when both are incubated with AFB_1 and quail microsomes. The analyses also demonstrate how efficient the production of AFB_1-GSH is in competing with the alternative fates of AFB_1-8,9-epoxide e.g. the formation of AFB_1-8,9-dihydrodiol and macromolecular binding (Fig. i).

The results from a single human liver (male) suggest that humans may be rapid metabolizers of AFB_1. Approximately 5% of the AFB_1 incubated with microsomes can be detected as the Tris-diol complex, but a greater proportion of AFB_1 is detected as AFQ_1 (>20%). This result agrees with that of Büchi *et al* (18) who also noted that microsomes from a female human liver produced mainly AFQ_1. The low recoveries (45%) may be due to high levels of macromolecular binding.

The secondary metabolism of AFB_1 by human post microsomal supernatant in the presence of quail microsomes, produced only the Tris-diol

complex. There was apparently little or no conversion to AFB_1-GSH. The absence of any deactivation mechanism in this male human liver was surprising and we have repeated incubations using freshly prepared post mitochondrial supernatant. Lack of conjugating activity was again confirmed by the complete absence of AFB_1-GSH.

The data obtained from human studies would suggest a relatively high susceptibility compared with other species investigated based on the high rate of AFB_1 metabolism and apparent absence of conjugating activity. This observation may be modified slightly by the relatively low levels of Tris-diol detected, suggesting epoxidation is not a major pathway. However, as the low recoveries might suggest high macromolecular binding, other factors may influence the toxicity of AFB_1 to humans. We hope to investigate this shortly.

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