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OXIDATIVE METABOLISM OF AFLATOXIN B_1 BY MAMMALIAN LIVER SLICES AND MICROSOMES

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Abstract—The metabolism of aflatoxin B_1 in mammals involves both hydroxylation and demethylation. The species differences in the metabolism of aflatoxin B_1 in demethylation and hydroxylation have been investigated. The differences in the metabolism of the toxin are due principally to differences in enzymic action of microsomal enzymes. Aflatoxin B_1 is metabolized relatively slowly in the liver tissues of dog, rat and sheep than in the mouse, goat, guinea pig, rabbit and golden hamster. Also the sheep demethylates aflatoxin B_1 poorly but the dog does not demethylate the toxin. Differences also exist in the hydroxylation of the toxin; of all the mammals studied, only the mouse does not produce aflatoxin M_1.

THE AFLATOXINS are a group of mycotoxins originally produced by some strains of *Aspergillus flavus* Link growing on groundnuts and groundnut products. The *in vivo* metabolism of aflatoxins in some animals has been reported. Cows,[1, 2] goat[3] and rats[4] fed rations containing toxic groundnut meal excreted in milk a toxic factor having a biological activity in ducklings similar to that of the aflatoxins. The toxic metabolite was later identified and designated aflatoxin M_1.[6] Aflatoxin M_1 is also present in the liver extracts, portal and systemic blood of rat,[7] and in the kidney, urine, faeces, milk and liver extracts of sheep previously given a dose of aflatoxins.[8, 9] Since the rice meal fed to cows which excreted aflatoxin M_1 also contained aflatoxin M_1, Masri *et al.*[10] suggested that the presence of aflatoxin M_1 in the urine and milk may be related to its presence in the mixed aflatoxins rather than to its exclusive biotransformation from aflatoxin B_1. Earlier in the search of aflatoxin metabolites, de Iongh *et al.*[4] had shown that aflatoxin M_1 is a *de novo* metabolite of aflatoxin B_1 in rats. More recently, however, the presence of aflatoxin M_1 in the bile of rats injected with aflatoxin B_1 has been reported.[11, 12] These reports indicate that aflatoxin M_1 is produced from aflatoxin B_1 *in vivo* in rats.

The metabolism of aflatoxin B_1 involves its hydroxylation. This may be done directly,[1–4, 6–10] or by the prior cleavage of the methoxy group [12–14]. The direct hydroxylation of aflatoxin B_1 leads to the formation of aflatoxin M_1. Metabolism of aflatoxin B_1 by demethylation appears significant and is consistent with the earlier report of Axelrod[15] that the major route of metabolism of many aromatic alkyl ethers was by cleavage of the alkyl group to yield hydroxylated aromatic compounds.

The present paper reports some results obtained on the *in vitro* metabolism of aflatoxin B_1 by some mammalian species.

MATERIALS AND METHODS

Animals

Adult male mice weighing 30–35 g; adult golden hamsters weighing 140–180 g; adult male Wistar rats weighing 140–150 g; adult male guinea pigs weighing 300–350 g; male sheep weighing 10–10·5 kg; male goats weighing 12–12·5 kg; male dogs weighing 5·4 kg; and adult male rabbit weighing 1·8–2 kg were used in all experiments.

Substrates

The aflatoxin B_1 used was pure as shown by thin-layer chromatography. The aflatoxin B_1 showed characteristic u.v. spectrum with maxima peaks at 223, 265 and 360 mμ in methanol[34].

Thin-layer chromatography. Chromatoplates of silica gel G (Chromalay, M & B) of thickness 500 μ were activated at 90° for 4 hr and cooled to room temperature before use. The plates were run in 10% acetone in chloroform (v/v) in an unlined, and unequilibrated tank.[16]

Preparation of liver slices

The animals were decapitated and bled. The livers were immediately removed and rinsed with ice-cold (0–2°) normal saline. The gall bladder was removed from the liver where necessary and slices of liver approximately 200–500 μ in thickness were prepared free hand, using a razor blade.

Preparation of liver microsomes and soluble fraction

The livers were homogenized with three volumes of 0·3 M phosphate buffer pH 7·6 in a Waring Blendor for 20 sec and centrifuged at 10,000 *g* for 20 min at 0–3° in an MSE super speed refrigerated centrifuge.

The 10,000 *g* supernatant (microsomes plus soluble fraction) was used in the incubation experiments.

Incubation mixture for isolation of metabolites of liver slices

All incubations were conducted in freshly prepared medium containing 160m μmoles aflatoxin B_1 substrate, 500 μmoles (KH_2PO_4/Na_2HPO_4) phosphate buffer pH 7·6, 120 μmoles glucose, 13·5 μmoles $MgSO_4$. $7H_2O$, 28 μmoles $CaCl_2$, 1·39 m-mole NaCl and 67 μmoles KCl in a total volume of 10 ml placed in 50-ml Ehrlenmeyer flasks. 1·0 g of liver slices (wet weight) were placed in each flask and incubated in air with continuous shaking in a GallenKamp metabolic shaking incubator at 37 $\pm$ 0·5° for 2 hr. Control with inactivated (boiled) tissue slices and control in which aflatoxin B_1 was omitted were also used.

Incubation mixture for isolation of metabolites of microsomes plus soluble fraction

The incubations were carried out in 50 ml Erlenmeyer flasks, each flask containing 100 μmoles nicotinamide, 100 μmoles of magnesium chloride, 100 μmoles of glucose-6-phosphate, 380 μmoles of potassium chloride, 1 μmole NADP, 160 mμmoles of aflatoxin B_1 and 4 ml of microsomes plus soluble fraction in a total volume of 10 ml. The flasks were incubated in air for 1 hr at 37 $\pm$ 0·5°. Controls with inactivated (boiled) microsomes plus soluble fraction and controls in which aflatoxin B_1 was omitted were also used.

Isolation of metabolites

After completion of the incubation, the content of each flask was homogenized in the case of liver slices. To each flask was added 2 ml of 20% (w/v) zinc sulphate solution and 2 ml of saturated barium hydroxide solution to precipitate the protein.[17] The mixture was then centrifuged at 5000 rpm for 20 min in an MSE refrigerated centrifuge. The supernatant was concentrated in a rotary evaporator at 27°.

Separation of metabolites and analytical techniques

Thin-layer chromatography. The concentrated supernatant was applied to chromatoplates of silica-gel G (Merck) prepared and run as described earlier.[16] Pure aflatoxin B_1 and aflatoxin M_1 were also applied to the TLC plates as reference substances. The plates were viewed under u.v. light and the metabolites located by their R_f values and fluorescence in ultra-violet light.

Aflatoxin B_1 and M_1 present in the concentrated supernatant was assayed on thin layer chromatoplates using internal standards of aflatoxins B_1 and M_1.

Ultra-violet spectra. The bands of metabolites were marked, scraped and eluted with methanol. The ultra-violet absorption spectra of the metabolites were determined with a Perkin–Elmer recording spectrophotometer.

Control elutions from similar area of chromatoplates to which was applied concentrate of control incubation medium to which aflatoxin B_1 was not added were used for the reference cell.

Incubation mixture for study of demethylation of aflatoxin B_1

The incubation mixture was as for the study of the metabolites except for the inclusion of 50 μmoles semi carbazide hydrochloride (pH 7·6) in a final volume of 10 ml.

Estimation of formaldehyde produced by demethylation of aflatoxin B_1

After incubation and cooling, the formaldehyde was assayed by the methods of Cochin and Axelrod[18] as modified by Stitzel *et al.*[19] using double strength Nash reagent.[35] The protein in the incubation medium was precipitated as stated earlier under isolation of metabolites. To 5 ml of the 5000 rpm supernatant in test tubes was added 2 ml of freshly prepared double strength Nash reagent[35] and heated at 60° for 30 min in a water bath. The tubes were immediately cooled in an ice:water mixture and assayed spectrophotometrically at 415 mμ in a Unicam SP600. A plot of known concentrations of formaldehyde treated in the same way as the 5000 rpm supernatant was used as standard. Eight flasks were incubated for each of the test and control tissues.

In a preliminary experiment the number of mμmoles of formaldehyde produced in the test incubation were 40·5, 39·0, 40·5, 41·5, 42·5, 39·0 and 40·5 respectively while 10·0 mμmoles formaldehyde were obtained in the control in which aflatoxin B_1 was omitted.

RESULTS

Qualitative studies

Species differences in the metabolism of aflatoxin B_1 is significant (Tables 1 and 2).

TABLE 1. EFFECT OF SPECIES DIFFERENCES ON THE METABOLISM OF AFLATOXIN B_1 BY LIVER SLICES

Animal species	Goat	Sheep	Rat	Mouse	Guinea pig	Rabbit	Dog	Golden hamster
Substrate concentration	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles
Formaldehyde (mμmoles) produced in 1 hour per gram liver slices	23·0 ± 1·3	6·4 ± 0·9	37·2 ± 1·9	17·8 ± 1·8	30·4 ± 1·3	36·5 ± 3·2	0	29·3 ± 2·7
% of aflatoxin B_1 metabolized during the 2-hr incubation period	91·3 ± 3·7	48·1 ± 7·4	83·8 ± 5·3	88·8 ± 5·5	83·8 ± 2·9	92·5 ± 4·8	82·8 ± 3·0	95·6 ± 2·7
% of aflatoxin M_1 produced during the 2-hr incubation period	1·8 ± 0·3	4·3 ± 0·6	1·6 ± 0·6	0	0·88 ± 0·1	1·1 ± 0·3	5·3 ± 1·4	5·4 ± 1·1
Number of animals	3	3	8	30	5	3	4	8

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

TABLE 2. EFFECT OF SPECIES DIFFERENCES ON THE METABOLISM OF AFLATOXIN B_1 BY LIVER MICROSOMES

Animal species	Goat	Sheep	Rat	Mouse	Guinea pig	Rabbit	Dog	Golden hamster
Substrate concentration	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles	160 mμmoles
Formaldehyde (mμmoles) produced in 1 hour per gram liver	61·6 ± 2·0	43·4 ± 2·5	75·1 ± 13·0	68·3 ± 7·5	0	56·3 ± 10·5	0	89·0 ± 9·0
% of aflatoxin B_1 metabolized during the 1-hr incubation period	98·0 ± 1·3	75·5 ± 1·5	88·8 ± 2·0	99·8 ± 0·4	94·4 ± 2·6	98·7 ± 0·5	85·6 ± 3·6	99·4 ± 0·6
% Concentration of aflatoxin M_1 produced during the 1-hr incubation period	1·9 ± 0·5	4·8 ± 1·0	1·7 ± 0·13	0	0·9 ± 0·07	1·0 ± 0·12	5·4 ± 1·2	1·3 ± 0·3
Numbers of animals	3	3	8	30	5	3	4	8

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

The liver slices and microsomes plus soluble fraction of all the mammals studied, except the mouse, metabolized aflatoxin B_1 into a blue–violet fluorescing substance (R_f 0·20) in ultra-violet light. In the mouse, instead of the blue–violet fluorescing metabolite, a yellowish-green fluorescing substance (R_f 0·15) in u.v. light is produced. Also the rat and the golden hamster metabolize aflatoxin B_1 into another substance (R_f 0·34) which fluoresces yellowish-green in ultra-violet light. In addition, the golden hamster metabolizes aflatoxin B_1 in trace acounts into the same metabolite (R_f 0·15) as the mouse.

The metabolites with R_f values 0·34, 0·20 and 0·15, respectively, react with acetic anhydride in the presence of pyridine. On the basis of this reaction hydroxyl group may be present in each of the three metabolites. The blue–violet fluorescing substance (R_f 0·20) exhibits maximum absorption peaks in methanol at 226, 265 and 357 mμ wavelengths and has the same R_f value as aflatoxin M_1. On the basis of the ultra-violet absorption peaks [6,10] and R_f value, this substance is identified as aflatoxin M_1.

The yellowish-green fluorescing metabolite (R_f 0·15) shows an ultra-violet spectrum with maximum peaks at 226, 257 and 360 mμ wavelengths. This metabolite has not been characterized beyond the possibility of it having a hydroxyl group. The metabolite with R_f value 0·34 was not obtained in sufficient quantity for its ultra-violet spectrum to be determined. All the species studied except the dog demethylated aflatoxin B_1.

Quantitative studies

There also exist species differences in the metabolic rate of aflatoxin B_1. The microsomes of goat, mouse, guinea pig, rabbit and golden hamster metabolize aflatoxin B_1 fairly rapidly while the rat, dog and sheep microsomes metabolize the toxin relatively slowly.

DISCUSSION

Differences in the *in vitro* metabolism of aflatoxin B_1 exist in the mammals studied. While the mouse is unable to biotransform aflatoxin B_1 into aflatoxin M_1 all the other mammals—the goat, sheep, rat, guinea pig, rabbit, dog and golden hamster biosynthesize aflatoxin M_1. Differences in the rates of biotransformation of aflatoxin B_1 are also observed; the dog and the sheep being relatively rapid producers of aflatoxin M_1 in comparison with the goat, rat, guinea pig, rabbit and golden hamster.

The rate of *o*-demethylation of aflatoxin B_1 in the various species also vary. The sheep demethylates aflatoxin B_1 poorly; the rat, mouse, goat, guinea pig and rabbit moderately; and the golden hamster rapidly. The demethylation of aflatoxin B_1 might therefore be a significant method of detoxification of aflatoxin B_1 in the golden hamster, rat, mouse, rabbit and goat. The dog does not demethylate aflatoxin B_1. The inability of the liver microsomes plus soluble fraction of the liver of guinea pig to demethylate the toxin may be due to the presence of enzyme inhibitors[15] or to the demethylating enzyme systems being unstable.[8, 20] The relatively low demethylating ability of liver microsomes of the rabbit may also be due to the instability of the liver microsomes.

Since aflatoxin B_1 is significantly metabolized by liver microsomes of the guinea pig in spite of inhibition of demethylation process, *o*-demethylation of the toxin in the guinea pig might not be a major route of aflatoxin biotransformation in this specie.

Considerable variation in susceptibility to aflatoxin toxicity has been reported for different strains and species of mammals.[2, 21-30] Aflatoxin B_1 is teratogenic to golden hamsters but not to mice or rats.[23, 31-33] This species difference in teratogenicity has been ascribed to probable differences in the metabolism and excretion of the toxin.[31] In the *in vitro* metabolic studies reported here, differences in the qualitative and quantitative metabolism of aflatoxin B_1 in the mouse, rat and golden hamster are observed. The differences in the types of metabolites of aflatoxin B_1 found in these species and the relative proportions in which these metabolites are produced might account partly or wholly for the difference in teratogenicity of aflatoxin B_1 in golden hamster, rat and mouse.

From the results given in this paper it may be concluded that the rapid metabolism of the aflatoxin B_1 as well as the inability of the mouse to biotransform the toxin into aflatoxin M_1 is directly related to the resistance of the animal to aflatoxin B_1-induced liver injury and cancer. Also the inability of the dog to detoxify aflatoxin B_1 by demethylation may be responsible for its sensitivity to aflatoxin B_1.

It is possible that the production of large amounts of aflatoxin M_1 by the dog and golden hamster is responsible for the proneness of members of these species to liver injury by aflatoxin B_1, since aflatoxin M_1 is as toxic as aflatoxin B_1. However, we are unable to correlate the resistance of the sheep to the induction of cancer and liver injury by aflatoxin with the metabolism of the toxin in this specie, since aflatoxin B_1 is metabolized slowly and aflatoxin M_1 is produced in relatively large quantities.

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