A COMPARATIVE STUDY ON AFLATOXIN B₁ METABOLISM IN MICE AND RATS

M. STEYN, M. J. PITOUT AND I. F. H. PURCHASE

From the Division of Toxicology, National Institute for Nutritional Diseases, South African Medical Research Council, Private Bag 380, Pretoria, South Africa

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SUMMARY.—In vivo metabolic studies on rats and mice revealed a marked difference in the fluorescent compounds produced after ingestion of aflatoxin B₁. The mouse converted aflatoxin B₁ to three unknown fluorescent compounds, designated \( x_1 \), \( x_2 \) and \( x_3 \) and the known aflatoxin M₁, while the rat was only capable of producing aflatoxin M₁. The results suggested that metabolites \( x_1 \), \( x_2 \), \( x_3 \) and aflatoxin M₁ were not part of a major metabolic pathway, but produced independently. These unknown yellowish-green fluorescent compounds did not seem to be conjugated with sulphate or glucuronic acid.

In vitro incubations of various mouse liver cell fractions with aflatoxin B₁ showed that metabolites \( x_1 \), \( x_2 \), \( x_3 \) and aflatoxin M₁, could only be produced by the microsomal fraction and that NADPH was needed as a co-factor. The differences in aflatoxin metabolism by mice and rats are discussed in relation to the apparent resistance of the mouse to the carcinogenic effects of this toxin.

AFLATOXIN B₁ is acutely toxic to a number of animal species, including albino mice, which have approximately the same susceptibility to the acute oral effects of aflatoxin B₁ (Butler, 1969) as rats (Butler, 1964). In contrast to the rat, the mouse is resistant to the carcinogenic action of aflatoxin B₁ (Platonow, 1964; Newberne, 1965; Wogan, 1966).

It is well known that aflatoxin B₁ is converted by rats to its hydroxylated derivative, aflatoxin M₁, in vivo (Patterson and Allcroft, 1970), as well as in vitro (Schabort and Steyn, 1969). Portman et al. (1968) reported the conversion of aflatoxin B₁ to M₁ by washed microsomes prepared from mouse liver, but Patterson and Allcroft (1970) and Bassir and Emafo (1970) could not confirm this observation.

This study was undertaken to investigate and establish the difference between mice and rats in their ability to convert aflatoxin B₁ into fluorescent metabolites.

MATERIALS AND METHODS

All chemicals used were of analytical reagent grade. Nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH) was obtained from Boehringer, West Germany. Protein concentrations were determined as described by Lowry et al. (1951). \( \beta \)-glucoronidase (bovine-liver) and aryl sulphatase (Helix pomatia) were obtained from Calbiochem., Switzerland. \(^{14}\)C-Carboxyl-labelled acetate (specific activity 25 Ci/mole) was obtained from The Radiochemical Centre, Amersham, and used to synthesize \(^{14}\)C-uniformly labelled aflatoxin B₁ according to the method of Adye and Mateles (1964) and purified as described by Steyn (1970).
Albino mice (± 23 g.) obtained from Onderstepoort Veterinary Research Institute and our own Wistar-derived albino rats (weight ± 200 g.) were used in these experiments.

In vivo studies

Aflatoxin B<sub>1</sub> metabolism in mice and rats.—Two male rats (average weight 220 g.), and eight adult male mice (mean weight 23 ± 2 g.) were dosed per os with aflatoxin B<sub>1</sub> (10 mg./kg.) in dimethylsulphoxide (DMSO) (0-1 ml.) and killed with ether 2 hours later. Their livers, kidneys, stomachs plus intestines and bladders plus urine were excised, weighed and extracted according to Purchase and Steyn (1969), utilising an azotrope consisting of acetone : chloroform : water (58 : 38 : 4). The concentration of aflatoxin B<sub>1</sub> was assayed according to Pons, Robertson and Goldblatt (1966).

Absorption of aflatoxin B<sub>1</sub> from the stomachs of mice.—Twelve male mice (average weight 23 g.) were each dosed per os with aflatoxin B<sub>1</sub> (10 mg./kg.) in DMSO (0-1 ml.). Two mice were killed with ether at 1/4, 1, 2, 4, 6 and 7 1/2 hours after dosing and their stomachs and intestines removed. These organs were assayed for aflatoxin B<sub>1</sub> as described above.

Rate of aflatoxin B<sub>1</sub> metabolite formation in mice.—Twelve male and 12 female mice (average weight 23 ± 2 g.) each received an oral dose of 3·0 mg. aflatoxin B<sub>1</sub> in 0-1 ml. DMSO. Two males and two females were killed as before at 20, 40, 70, 100, 150 and 180 minutes after dosing. Their livers, stomachs plus intestines, kidneys and bladders plus urine were removed and treated as above. The relative amounts of the unknown metabolites are expressed in μg. aflatoxin B<sub>1</sub> equivalents as no quantitative standards of the unknown were available.

Two male mice (± 23 g.) each received an oral dose of 3 mg. 14C aflatoxin B<sub>1</sub> (30 × 10<sup>3</sup> d.p.m./mg. aflatoxin) in 0-1 ml. DMSO and were killed after 100 minutes as described before. Urine was collected from the bladders by means of a 1 ml. syringe (total volume of urine, 0·7 ml.) and chromatographed on 1 mm. thin-layer chromatography (t.l.c.) plates. The fluorescent bands, containing metabolites x<sub>1</sub>, x<sub>2</sub> and aflatoxin M<sub>1</sub>, were separately collected and the radioactivity measured in a Beckman liquid scintillation system.

In vitro studies

Preparation of liver cell fractions.—Two adult male mice were decapitated, their livers (total weight 6 g.) removed and homogenized in 18 ml. 0·32m sucrose-3mM MgCl<sub>2</sub>-0·02M TRIS buffer, pH 7·6, in a Dounce homogenizer. The suspension was centrifuged in an MSE-mistral 2 L centrifuge at 1800 g for 20 minutes to remove the nuclei, which were discarded. The supernatant was then centrifuged at 9000 g for 20 minutes in a Spinco L-4 ultracentrifuge, to yield the crude mitochondrial fraction, which was suspended in 10 ml. 0·02M TRIS buffer, pH 7·4, and kept. The microsomes were obtained by centrifugation of the resultant supernate at 105,000 g for 60 minutes, washed twice with 10 ml. of the above mentioned buffer and diluted with the same buffer to a protein concentration of 10–15 mg. per ml. The supernatant, which consisted of the soluble cell fraction, was used undiluted.

Incubation of aflatoxin B<sub>1</sub> with different cell fractions.—The incubation mixtures, with a final volume of 5 ml., were made up as follows:
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1. Aflatoxin B₁ in methanol or 1,2-propylene glycol (7 mg./ml., 0.4 ml.);
2. 0.02M TRIS buffer, pH 7.4 (3.4 ml.);
3. MgCl₂ solution (14.2 g./L, 0.2 ml.);
4. NADPH (74.5 mg./1 ml. TRIS buffer solution): 0.5 ml.;
5. Cell fraction: 0.5 ml.

All incubations were carried out in a shaking water bath at 37°C. in cotton wool plugged test tubes for 30 to 60 minutes. The reactions were terminated by the addition of 10 ml. acetone. Reaction mixtures were transferred quantitatively to suitable flasks by washing with two more 10 ml. portions of acetone.

Conjugates.—Enzyme incubations were performed as described by Fishman (1946) and Whitehead et al. (1952), after addition of chromatographically pure $x_1$, $x_2$ and $x_3$. These metabolites were also boiled in 6N HCl for 5 minutes (Öser, 1965).

Chromatographic methods

All extracts were evaporated to dryness in a rotary evaporator (under reduced pressure) at 45°C., and dissolved in 2 ml. benzene : acetonitrile (95 : 5). T.l.c. plates were prepared from Camag D-5 silica gel (Camag, Switzerland), wet layer thickness 0.5 mm., heated at 100°C. for 2 hours and cooled at room temperature immediately before use. After application of suitable quantities of extracts, as well as a quantitative aflatoxin B₁ standard, the chromatograms were developed in an unlined tank containing 100 ml. chloroform : acetone (80 : 20) as the mobile phase.

The following procedures were used to identify aflatoxin M₁ in several mouse organs:

1. T.l.c. employing four different mobile phases, namely, chloroform : acetone (80 : 20); chloroform : methanol (95 : 5); trichlorethylene : acetone (10 : 90), and benzene : ethanol : water (46 : 45 : 19).
2. Paper chromatography, as described by Holzapfel et al. (1966).
3. Ultra-violet spectrophotometry, employing a Beckman model DK-2A spectrophotometer.

RESULTS

In vivo experiments

Metabolism of aflatoxin B₁ by the mouse.—Fig. 1 illustrates the typical metabolic conversion pattern of aflatoxin B₁ by various organs of the mouse and Fig. 2 demonstrates the difference in metabolism of aflatoxin B₁ between rat and mouse livers.

From Fig. 1 and 2 it is clear that only the mouse is able to metabolize aflatoxin B₁ to three unknown fluorescent metabolites, two major and one minor, designated $x_1$, $x_2$ and $x_3$, respectively. These compounds all fluoresce green-yellowish. The 4th blue fluorescent metabolite with an Rf value between that of $x_1$ and $x_2$ was shown to be chromatographically identical to aflatoxin M₁ and to have a similar ultra-violet spectrum. The rate of production of $x_1$, $x_2$ and aflatoxin M₁ from combined organ extracts, expressed in μg. aflatoxin B₁ equivalents, is shown in Fig. 3.

From Table I it is evident that metabolites $x_1$, $x_2$ and aflatoxin M₁ recovered from the urine of mice dosed with ¹⁴C-aflatoxin B₁ are radioactive. Metabolite
Fig. 1.—Chromatogram of metabolites extracted from various mouse organs as viewed under ultra-violet light—1: liver; 2: stomach; 3: kidneys; 4: intestines; 5: urine plus bladder.

Fig. 2.—Chromatogram of metabolites extracted from rat and mouse liver 2 hours after administration of aflatoxin B₁. 1: rat; 2: qualitative standard containing aflatoxins B₁, M₁ and B₂a; 3: mouse.

Fig. 3.—Rate of fluorescent metabolite formation in mice. ● : x₁; ○ : M₁; ○ : x₂.
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Table I.—Distribution of Radioactivity in Metabolites $x_1$, $x_2$ and Aflatoxin $M_1$ in the Urine of Mice Following an Oral Dose of $^{14}$C-aflatoxin $B_1$

| Metabolite | d.p.m./ml urine |
|------------|-----------------|
| $x_1$      | 700             |
| $M_1$      | 1410            |
| $x_2$      | 3810            |

$x_3$ was obtained in too low a yield to confirm its relationship to aflatoxin $B_1$ in this way.

No fluorescent substances resembling $B_1$ or its metabolites were observed in extracts from control animals.

The ability of the mouse to absorb consistently more aflatoxin $B_1$ from its stomach than the rat, is illustrated in Fig. 4.

![Graph showing amounts of aflatoxin B1 in rats and mice](image)

Fig. 4.—The amounts of aflatoxin $B_1$ detected in the stomachs of rats and mice up to 8 hours after dosing. ○: rat; ●: mouse.

In vitro experiments

Conversion of aflatoxin $B_1$ by mouse liver cell fractions.—The ability of the various fractions to metabolize aflatoxin $B_1$ is illustrated in Fig. 5, which distinctly indicates that only the microsomal fraction is capable of producing a variety of fluorescent metabolites. Furthermore, it is also clear that the conversion mechanism, present in the microsomal fraction, needs NADPH (Fig. 5).

Assay for conjugates.—Treatment of chromatographically pure $x_1$, $x_2$ and $x_3$ with $\beta$-glucuronidase (bovine liver) aryl sulphatase (Helix pomatia) or 6N HCl showed no effect.
DISCUSSION

When a comparison is made between the amount of aflatoxin B₁ in rat and mouse stomachs after a single dose, it is clear that the mouse absorbs the toxin more quickly than the rat. As the acute toxicity in the mouse and rat are approximately the same, the mouse must either be more resistant to the effects of the toxin *per se* or it must be able to metabolize (detoxify) the toxin at a greater rate than the rat. The ability of the mouse to produce numerous fluorescent metabolites, and the absence of large quantities of aflatoxin B₁ in liver and kidneys indicate that the mouse is capable of metabolizing aflatoxin efficiently.

The absence of carcinogenicity of aflatoxin in mice may also be related to the rapid metabolism. Alternatively, if aflatoxin B₁ is converted into an "ultimate" carcinogen by metabolism in the rat, the lack of carcinogenicity could be due to the different metabolic conversion products produced by the mouse. Further studies on the identity and biological activity of aflatoxin metabolites may indicate which of these two suggestions is correct.

The *in vitro* experiments clearly indicate that only the microsomal fraction of mouse liver contains the enzyme(s) necessary for the conversion of aflatoxin B₁ to M₁ and the other three fluorescent components.

In addition, it is also possible that the cytochrome P-450 component, which is the rate-limiting step in drug conversion of mouse liver microsomes, has a higher affinity for aflatoxin B₁ than that of the rat. Since we accept the observation that the mouse is resistant to aflatoxin B₁-induced carcinogenicity, the conversion of aflatoxin B₁ to the three fluorescent compounds could be used as a monitoring system to study the susceptibility of various animal species, including man, to aflatoxin B₁-inducible carcinogenesis. Obviously, much more work has still to be done to validate such a statement.

Although highly unlikely, the possibility existed that the metabolites x₁, x₂ and aflatoxin M₁ were formed in mice through the synergistic action of aflatoxin
B₁ and DMSO from some other compound normally present in the animals. This possibility was excluded by using ¹⁴C-aflatoxin B₁ (Table I).

Whether aflatoxin M₁, metabolites x₁, x₂ and x₃ are minor metabolites or intermediates in a major metabolic pathway in the mouse cannot be deduced from our results. However, the results in Fig. 3 suggest that these metabolites are synthesized independently, since their excreted concentrations are similar at any particular time. Both mouse and rat livers could also metabolize aflatoxin B₁ to non-fluorescent compounds which are not perceptible, or to fluorescent metabolites which are not extracted with the acetone: chloroform: water azeotrope. It also seems that x₁, x₂ and x₃ are not glucuronide or sulphate conjugates.

The results of this study clearly demonstrate that the mouse can hydroxylate aflatoxin B₁ to aflatoxin M₁. This is in agreement with the results from Portman et al. (1968) who suggested that mouse liver microsomes hydroxylate aflatoxin B₁ (forming M₁) faster than those of rat liver. However, these authors did not mention the presence of other fluorescent components. Although Bassir and Emafo (1970) failed to demonstrate M₁ production by mouse liver, they did describe the presence of a green-yellow fluorescent substance. Whether their unknown fluorescent compound corresponds with our x₁, x₂ or x₃ is at present unknown. These differences could be due to different strains of mice or to different extraction techniques used by the various authors.

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REFERENCES

Adye, J. and Mateles, R. I.—(1964) Biochem. biophys. Acta, 86, 418.
Bassir, O. and Emafo, P. O.—(1970) Biochem. Pharmac., 19, 1681.
Butler, W. H.—(1964) Br. J. Cancer, 18, 756.—(1969) In ‘Aflatoxin, Scientific Background, Control and Implications’, by L. A. Goldblatt. New York and London (Academic Press), p. 234.
Fishman, W.—(1946) J. biol. Chem., 166, 757.
Holzapfel, C. W., Steyn, P. S. and Purchase, I. F. H.—(1966) Tetrahedron Lett., 25, 2799.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.—(1951) J. biol. Chem., 193, 265.
Newberne, P. M.—(1965) In: ‘Mycotoxins in Foodstuffs’ edited by G. N. Wogan. Cambridge, Mass. (M.I.T. Press), p. 187.
Oser, B. L., editor (1965) ‘Hawk’s physiological chemistry’. New York (McGraw Hill), p. 1181.
Patterson, D. S. P. and Allcroft, R.—(1970) Fd Cosmet. Toxic., 8, 43.
Platonow, N.—(1964) Vet. Rec., 76, 589.
Pons, W. A. Jr., Robertson, J. A. and Goldblatt, L. A.—(1966) J. Am. Oil Chem. Soc., 43, 665.
Portman, R. S., Plowman, K. M. and Campbell, T. C.—(1968) Biochem. biophys. Res. Commun., 33, 711.
Purchase, I. F. H. and Steyn, M.—(1969) Br. J. Cancer, 23, 800.
Schabert, J. C. and Steyn, M.—(1969) Biochem. Pharmac., 18, 2241.
Steyn, M.—(1970) Ass. Off. Anal. Chem., 53, 619.
Whitehead, J. E. M., Morrison, A. R. and Young, L.—(1952) Biochem. J., 51, 585.
Wogan, G. N.—(1966) Bact. Rev., 30, 460.