BIRDS AND RODENT LIVER ENZYMES RESPONSIBLE FOR AFLATOXINS B1 AND B2 DETOXIFICATIONS

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Abstract

In the birds and some mammals, about two-thirds of the hepatic activity responsible for detoxifications is located in a soluble fraction obtained after centrifuging liver samples at high rpm. values. In the rodent liver samples detoxifications activity is little or there is no activity.

In this sense that aflatoxins B1 and B2 are reduced in vitro by NADP-dependent enzymes of birds and some mammals liver to form the corresponding cyclopentenols, aflatoxicol and dihydro-aflatoxicol. This way was not previously recognized because thin layer chromatograms of those products are easily confused with other similar compounds.

Key words: aflatoxins B1, aflatoxins B2, birds liver, rodent liver

INTRODUCTION

In previous attempts to identify the major pathways of *in vitro* aflatoxin metabolism, it was found that certain animal species (chicken, duck, guinea-pig and mouse) metabolized the toxin much more rapidly than others (calf, goat, pig, rat and sheep). Although there was spectrophotometric evidence to suggest that, under standardized incubation conditions, aflatoxin hemiacetal or a closely related compound was formed by homogenates of chicken or duck liver in about 50% yield no other metabolite was identified. Subsequently, an attempt was made to stimulate the activity of this metabolic pathway in the duck by phenobarbitone treatment but this failed, although other microsomal drug-detoxifying enzymes were induced. This resul suggested that microsomal metabolism of aflatoxin was not rate-limiting in the duck.

The present paper describes and assesses the quantitative importance of a cytoplasmic pathway for aflatoxin metabolism in chicken and duck liver and presents experimental evidence suggesting the formation of a reduction product of aflatoxin B1 identical with a fungal and a protozoan metabolite. This compound was originally termed Ro, but a more meaningful name, aflatixicol, was proposed. The present results also show that an analogue of aflatoxicol is produced from aflatoxin B2 by chicken and duck-liver preparations.

MATERIALS AND METHOD

Materials. Crystalline aflatoxin B_1 and B_2 (about 98% pure). Nicotinamide adenine nucleotides, glucose 6-phosphate and a suspension of glucose 6-phosphate dehydrogenase.

Animals. Chicks, ducklings, guinea-pigs, mice, rabbits, rats and turkey were used to provide liver tissues for these experiments. We take samples from mammals and aviar fauna.

Aflatoxin metabolism in liver samples. Liver tissues were first homogenized with four volumes of 0·15 M-KCl (or in some experiments with 0·15 M-sucrose) and then filtered. The first enzyme preparation was obtained as a supernatant fraction by centrifuging the homogenate at 14000 g for 15 min. the second remained as the supernatant after sedimenting particulate matter from the first preparation for 60 min at 105,000 g. Differential centrifugation of liver homogenates was also carried out at a series of g values between 5000 and 145,000. Assay conditions were those described previously, the disappearance of aflatoxin from the incubation mixture being measured as a diminution in absorbance at 363 nm of a 50% aqueous methanolic extract. The substrate concentration (0·026 mM) was limited because of the material's low solubility in whater and because ethanol (the solvent for aflatoxin) inhibited metabolism when used at concentrations above 10 µl/ml. When aliquots of up to 0·5 ml of the 9000 g fraction or up to 0·2 ml of the 105,000 g fraction were incubated for 15 or 30 min at 37ºC, the amount of aflatoxin metabolized was linearly related to enzyme concentration.

Thin-layer chromatography (TLC). Layers of silica gel G (250 µ thick) were spread o glass and activated at 100ºC for 60 min before use. Sometimes, prepared kieselgel F silica plates were used. Chromatograms were developed using the following solvent systems: (1) chloroform-methanol (97:3, v/v), (2) chloroform-acetone-water (88:12:1-5, by vol.), (3) chloroform-acetone-propan-2-ol (33:6:1, by vol.) or (4) diethyl ether.

Prepared cellulose plates were flooded with formamide-acetone (1:9, v/v) and soaked for 20 sec and the surplus solvent was discarded. After air drying, chromatograms were prepared using benzene saturated with formamide as the developing solvent.

RESULTS AND DISCUTIONS

Aflatoxin-metabolizing activity was studies in liver supernatants

Fractions prepared from liver homogenates of the samples were investgated for aflatoxin-metabolizing activity (Table 1). The data showed considerable animal-to-animal variation but avian and rodent livers were clearly the most active. Where aflatoxins B_1 and B_2 were compared as substrates, the former was always metabolized the more readily. When livers from ducks and chickens of different ages were compared, there was a tendency for tissues from the older birds to have grater enzyme activity.

TABLE 1

In vitro *aflatoxin metabolism by two fractions prepared from the livers of seven animal species*

Species	Age (weeks, days)	Metabolism of aflatoxins (n-moles/g/min*)					
		By 9000 g liver supernatant			By 105,000 g liver supernatant		
		No. of assays	Aflatoxin B1	B2	No. of assays	Aflatoxin B1	B2
Duck	8	4	65·7 (50·2-81·2)	39·3 (23·4-56·3)	8&2	43·6† (29·9-61·3)	18·2† (14·3-22·0)
Turkey	26	3	58·3 (56·7-60·3)	-	3	37·9† (31·4-41·9)	31·0† (14·9-40·1)
Duck	6	6	47·0 (41·9-53·2)	-	-	-	-
Chicken	8	4	37·3 (23·0-49·1)	31·3 (18·1-43·5)	2	26·2† (23·2-29·1)	16·6† (15·7-17·5)
Rabbit	Mature	2	31·3 (13·4-49·1)	18·7 (10·9-26·5)	2	17·0† (15·2-18·7)	19·3† (18·1-20·4)
Chicken	13 days	4	29·4 (26·7-31·0)	27·1 (16·5-37·2)	2	18·7† (17·9-19·5)	9·9† (8·8-10·9)
Duck	10 days	4	23·3 (19·2-29·4)	15·4 (11·9-19·4)	2	14·1† (12·6-15·5)	4·1† (1·6-6·6)
Guinea-pig	Mature	3	10·0 (4·9-15·6)	8·1 (1·9-12·5)	3	0	0.3 (0-0·6)
Mouse	Mature	3	5·1 (0·9-9·2)	9·6 (4·7-12·3)	2	0·35 (0-0·7)	0·40 (0·1-0·7)
Rat	8	6	0·31 (0·15-0·57)	-	6	0·32 (0-0·58)	-

* Values given are the mean values with the range in parentheses.

† Metabolites F1 and F2 were identified by TLC.

Aflatoxin-metabolizing activity by soluble liver enzymes

Results given in Table 1 also show that the activity of the cytoplasmic fraction was some two-thirds of that of the 9000 g supernatant. This proportion was not evaluated more accurately as the two fractions were not always assayed at the same time. Again, avian and rabbit livers were most avtive but the difference from guinea-pig, rat and mouse was even greater than in the case of the 9000 g supernatant. A marked effect of age

was again demonstated in chicken and duck, and aflatoxin B_1 was metabolized more readily than aflatoxin B_2.

Distribution of aflatoxins metabolism in the avian liver

Differential centrifugation of liver homogenates showed that aflatoxin hemiacetal was formed from aflatoxin B_1 in particles sedimented at g values greater than 40,000. This was confirmed by the appearance of an absorbance maximum at 400 nm in aqueous methanolic extracts of incubated supernatant fractions (5000-40,000 g). Metabolite F_1 (see below) was detected by TLC following the incubation of supernatant fractions obtained by centrifuging at 40,000 g or more, but it was produced maximally (as measured by absorbance at 333-335 nm) when particulate matter had been sedimented at 80,000 or 145,000 g. Table 2 shows the results of an experiment using the liver from 8-wk-old chicks.

TABLE 2

Metabolites of aflatoxin B1 formed in vitro by fractions of chicken-liver homogenate in 0·15 M-KCl

Supernatant fraction* (g value)	Absorbance maxima (nm) in aqueous methanolic extract			Hemiacetal, B2a			
				Absorbance at 400 nm after adding ammonia	TLC evidence	Metabolite F1	
	1	2	3			Absorbance at 333 nm	TLC 'fast spot'
5000	-	348	396	0·106	+	-	0
10,000	-	348	396	0·108	+	-	0
20,000	-	340	396	0·110	+	-	0
40,000	335	340	394	0·180	0	0·128	+
80,000	335	-	-	-	0	0·240	+
145,000	333	-	-	-	0	0·232	+
Whole homogenate	-	358	396	0·086	0	-	0

* Fractions (3 ml in each case) were incubated under the conditions specified under 'Experimental' for 60 min at 37ºC.

Broadly similar results were obtained in experiments with duck liver when the metabolic activities of 9000 g supernatants were compared with the corresponding 105,000 g fractions and when 0·15 M-KCl was replaced by 0·25 M-sucrose as the homogenizing medium.

Cofactor requirements for the formation of metabolites F_1 and F_2 in cytoplasmic liver fractions

As shown in Table 3, metabolism of aflatoxin B_1 and B_2 by 0·2 ml of the soluble enzymes of duck or chicken livers proceeded more rapidly in the presence than in the absence of a $NADPH_2$- generating system (NAPD with glucose 6-phosphate plus its dehydrogenase).

TABLE 3

Dependence of cytoplasmic aflatoxin metabolism on NADPH2 generating system

Species	Age	Metabolism of aflatoxin (n-moles/g/min)			
		With NADPH2		Without NADPH2	
		B1	B2	B1	B2
Duck	10 days	15·5	6·6	6·4	4·0
		12·6	4·5	1·6	1·6
	8 wk	39·8	22·0	Nil	Nil
		29·9	14·3	0·5	Nil
Chicken	13 days	19·5	8·8	0·8	1·6
		17·9	10·9	4·0	4·8
	8 wk	29·1	17·5	Nil	Nil
		23·2	15·7	Nil	Nil
Presence of 'fast spots' on TLC…		+	+	0	0

In the former case, new metabolites could be distinguished in chloroform extracts of the incubation mixtures. These were termed "fast spots", F_1 and F_2 respectively, owing to their behaviour when subjected to TLC on silica gel using solvent systems 3 and 4 and on cellulose using formamide-saturated benzene. Metabolites F_1 and F_2 ran at slightly lower R_F values than the parent aflatoxins on silica gel in solvent 2, and with solvent 1 were distinguishable from aflatoxins B_1 and B_2 only after acetylation, when they again ran as a 'fast spot'.

Further observations on duck liver showed that $NADH_2$ was a poor substitute for $NADPH_2$ when incorporated in the assay system at a final concentration of 0·2 mM. Thus, aflatoxin B_1 was metabolized at a rate of 31·3 n-moles/g/min compared with 105·3 n-moles/g/min. In the same experiment, the $NADPH_2$-generating system was shown to be superior to either reduced nucleotide when used alone, supporting a rate of 124·7 n-moles/g/min.

The high rate of activity observed in the latter experiment using a 105,000 g liver-supernatant fraction was achieved by omitting nicotinamide from the incubation medium and this result has since been confirmed. Metabolism of aflatoxin by the 9000 g supernatant is, by contrast, relatively insensitive to the presence of 10 mM-nicotinamide.

Metabolite F_1 or F_2 was always detected by TLC when aflatoxin B_1 or B_2 was incubated with duck-, chicken-, turkey- or rabbit-liver preparations, but particularly when excess enzyme was used, i.e. 1 ml or more of the 105,000 g fraction. But, while a single metabolite (F_1 or F_2) was apparently formed by avian liver tissues, rabbit liver produced several

additional metabolites. Therefore, what follows in this and the following section relates solely to avian liver.

Probable identities of metabolites F_1 and F_2

Accumulated evidence elating to metabolite F_1 is summarized in Table 4 and similar evidence is available for the reduction of aflatoxin B_2 to F_2. The inference is that the cyclopentenone ring of aflatoxin B_1 is reduced by a NADA-linked oxidoreductase to form a secondary alcohol, identical with aflatoxicol.

The much-reduced light absorbance by methanolic extracts of the incubation mixture following the metabolism of aflatoxin by 105,000 g liver supernatants is also very suggestive of aflatoxicol formation. Thus, the ratio of absorbance at λ max for the product to that for the substrate was, under optimal conditions, about 0·6, which agrees fairly well with the ratio of molecular absorbances in methanol for aflatoxicol (14,100 at 325 nm) and aflatoxin (21,800 at 362 nm).

TABLE 4

Evidence for the bioreduction of aflatoxin B1 to its cyclopentenol

Experimental evidence	Inference
Enzyme present in 105,000 g supernatant liver fraction; requires NADPH2 (or NADH2) as cofactor	Post-microsomal nucleotide-linked okidoreductase
Both aflatoxin B1 and aflatoxin B2 are substrates	Vinyl ether double bond not involved in this transformation
Product fluoresces blue in ultraviolet light and absorbs light maximally in the region of 333nm	Loss of cyclopentenone conjugation. Four possibilities: (i) aflatoxicol (ii) aflatoxin B3 (parasiticol) (iii) tetrahydrodesoxo aflatoxin (iv) hydrolysis product produced with fission of δ-lactone ring
Further absorbance peak at 264 nm; shoulder at 254 nm	Possibility (iv) excluded: Coumarin nucleus intact
TLC on silica (solvent 2) RF=0·58; RF of B1= 0·66	Consistent with (i) but excludes (ii), RF=0·44 in this solvent system and (iv), RF= 0·18, 0·25
TLC on silica (solvent 4) RF=0·37; RF of B1=0·05	Comparable with the behaviour of (i) on column chromatography
Readily forms an acetate with acetic anhydride/pyridine (RF= o·65 in solvent 1; RF of B1= 0·51)	Excludes (iii). Consistent with (i), (ii) and (iv), but (ii) and (iv) excluded above
Products of B1 metabolism have low toxicity	Consistent with properties of (i)

An analogous product, F_2, was formed from aflatoxin B_2 under identical incubation conditions by duck- and chicken-liver preparations. It has a slightly different absorbance maximum (330nm compared with 327 nm for F_1 in methanol) and on TLC its R_F values were consistently lower than those for F_1. This metabolite can be conveniently referred to as dihydroaflatoxicol (Fig. 1)

Fluorescence of metabolically formed aflatoxicol

Solutions of metabolite F_1 prepared on a micro-scale by TLC were standardized spectrophotometrically assuming that the metabolite was

indeed identical with aflatoxicol. Fluorescences of this metabolite and of pure aflatoxin B_1 were then compared following TLC on silica plates using developing solvent system 2. Observing the blue fluorescence under a "Phillips" ultra-violet lamp (HPW, 125 w), aflatoxicol was found to be approximately one-third as fluorescent as the parent aflatoxin. Diminished fluorescence had long been noticed following the incubation of aflatoxin B_1, with liver enzymes, but hitherto this could not be accounted for since the previously recognized metabolites were either more fluorescent (aflatoxin M_1) or about as fluorescent (aflatoxin hemiacetal) as aflatoxin B_1 itself.

CONCLUSIONS

In agreement with the conclusions drawn from an experiment in which phenobarbitone treatment failed to stimulate the *in vitro* metabolism of aflatoxin in the duck, it appears that, in chicken, duck and turkey, post-microsomal enzyme activity accounts for about two thirds of the total metabolism of aflatoxin assayed in 9000 g liver supernatants. The metabolites of aflatoxins B_2 and B_2 formed by soluble enzymes of the 105,000 g liver supernatant could easily be confused with the parent aflatoxins using what was hitherto our standard TLC procedure (silica gel, solvent 1). Under assay conditions where limiting amounts of enzyme are used, these products of metabolism could be mistaken for unmetabolized B_1 or B_2, particularly in any semi-quantitative TLC procedure, and it is not improbable that our tentative report of aflatoxin B_1 reduction by excess 9000 g supernatant to form B_2 was at least partly in error because aflatoxicol cannot readily be distinguished from aflatoxin B_2 under the TLC conditions used (silica gel, solvents 1 and 3).

The *in vitro* reduction of aflatoxin B_1 to aflatoxicol and of aflatoxin B_2 to dihydroaflatoxicol by avian liver enzymes, as described above, appears to be the first report of its kind. Hitherto studies of *in vitro* aflatoxin metabolism in animal tissues have generally been confined to microsomal oxidative metabolism.

It is even arguable whether this pathway truly constitutes a detoxication. These properties may favour increased clearance from the liver and the present results show that reductive activity increases with age, a finding which parallels the known increased resistance to aflatoxin poisoning in older birds. It was shown that aflatoxicol in non-toxic in day-old ducklings, but this result needs to be interpreted with caution, as the transportation into the liver cell is more important than the metabolic fate of aflatoxin. Thus, aflatoxicol might be relatively non-toxic because it fails to enter the liver cell as rapidly as aflatoxin. In this connexion, the low toxicities of other compounds closely related to aflatoxin, like the

hemiacetal, B_{2a} and parasiticol, B_3, possibly suggest relatively rigid structural requirements for transport.

The effect of intracellularly formed aflatoxicol may be quite another matter. As the cytoplasmic fraction of the avian liver contains enzymes that reduce aflatoxin so readily, it seems likely that aflatoxicol and not the parent toxin is primarily responsible for the toxic effects in chicks and ducklings. However, this hypothesis awaits experimental verification.

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