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DIFFERENCES IN THE EFFECT OF PHENOBARBITAL TREATMENT ON THE *IN VITRO* METABOLISM OF AFLATOXIN AND ANILINE BY DUCK AND RAT LIVERS

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Abstract—Groups of ducks and rats were treated with phenobarbital sodium for 14 days (1 mg/ml in the drinking water) and the effects of this treatment on *in vitro* rates of microsomal aniline and aflatoxin metabolism were observed.

Aniline hydroxylase activity was enhanced in microsomes of both species. Normal levels of activity were comparable in duck and rat livers.

Aflatoxin metabolism was stimulated in crude microsomal preparations (9000 *g* supernatant fraction) from rat but not from duck liver. Normal mean rates of total aflatoxin metabolism were 0·31 nmole/g/min in rat liver and 47·0 nmoles/g/min in duck liver.

Phenobarbital treatment had the effect of stimulating total aflatoxin metabolism, hydroxylation and demethylation in isolated rat liver microsomes.

The failure of this treatment to stimulate aflatoxin metabolism in the duck liver suggested that microsomal metabolism was not rate limiting. Aflatoxin was actually metabolised 90 times more rapidly by soluble enzymes (105,000 *g* supernatant) from duck liver than from rat liver.

THE METABOLIC fate of aflatoxin in the liver is only partially understood. 4-Hydroxylation to form the milk toxin or aflatoxin M_1 was the first biotransformation to be described in rats, cows and sheep.[1–7] Two other routes of metabolism have been less intensively investigated: demethylation[8–10] and hydration of the vinyl ether double bond to form aflatoxin hemiacetal.[11–13] Conversion of aflatoxin by crude liver fractions from chicks, ducks, guinea-pigs and mice to an unknown metabolite designated BX (but thought to be aflatoxin hemiacetal, B_{2a}, or a closely related compound) has also been described.[13] Five other species, including the rat, were normally almost incapable of this transformation.[12] Structural formulae of aflatoxin B_1 and its metabolites are shown in Fig. 1.

In all these investigations it has been generally accepted that microsomal metabolism was paramountly important. Indeed, stimulation of microsomal aflatoxin processing enzymes was produced by phenobarbital treatment or aflatoxin feeding in the rat:[11] the *in vitro* conversion of aflatoxin B_1 to M_1 was enhanced and the evidence also suggested that some B_{2a} was formed.

As there appeared to be substantial differences between aflatoxin metabolism in the rat and duck,[12] this report describes a comparative study of microsomal metabolism as stimulated by phenobarbital treatment. The results that duck microsomal aniline but not aflatoxin metabolism was stimulated led to the conclusion that cytoplasmic enzymes were quantitatively important in duck liver for the detoxication of aflatoxin.

MATERIALS AND METHODS

Materials. Substrates for enzyme assays were either redistilled aniline (Hopkin & Williams, Ltd.) or crystalline aflatoxin B_1 (98 per cent pure; Calbiochem Ltd., London). NADP, glucose-6-phosphate and its dehydrogenase were obtained from Boehringer Corporation (London) Ltd. For thin layer chromatography (TLC), prepared Kieselgel F silica plates (Merck F254; Anderman & Co. Ltd.) were used or, particularly for semi-quantitative procedure, 250 μm layers of silica gel G (Merck) were spread on glass and activated for 60 min before use.

Animals. Male rats (Sprague–Dawley: 8 weeks old) and male ducks (Khaki–Campbell: 6 weeks old) were used throughout these experiments. Groups of six rats or ducks were treated with phenobarbital for a period of 14 days by including 1 mg sodium salt/ml in the drinking water.

Liver homogenates were prepared in 0·15 M KCl as described previously.[14] A supernatant fraction obtained after centrifuging at 9000 *g* or a suspension of three times washed microsomes (sedimented at 105,000 *g*) were used in the enzyme assays.

Aniline hydroxylase activity was assayed by measuring colorimetrically the production of *p*-aminophenol for 30 min at 37°[15] by 1–2 ml microsomal suspension (equivalent to 0·2–0·4 g liver tissue).

Aflatoxin metabolism. Overall metabolism was assayed by incubating limiting amounts of liver enzyme in 5 ml incubation mixture containing NADP (1 μmole), glucose-6-phosphate (40 μmoles), $MgCl_2$ (25 μmoles), nicotinamide (50 μmoles), sodium phosphate buffer (pH 7·4; 140 μmoles) and aflatoxin B_1 (approximately 0·13 μmole in 50 μl ethanol). Before and after incubating at 37° in a shaking water bath (gasphase, air) an aliquot (2 ml) was deproteinized with an equal volume of methanol saturated with sodium sulphate. An incubation blank was set up without aflatoxin. Instead of using a semi-quantitative TLC procedure as originally described,[12] residual aflatoxin was calculated from decreases in absorbance at 363 nm of the aqueous methanolic extracts obtained after incubation (Pye Unicam SP 800 recording spectrophotometer). This was a valid procedure because of the broad agreement with less reproducible TLC assays and because excellent recoveries of aflatoxin from non-incubated reaction mixtures indicated that there was little if any loss of substrate by adsorption on to protein precipitated by methanol.

To obtain measurable rates of metabolism, 0·1–0·5 ml of duck liver 9000 *g* supernatant (≡ 20–100 mg liver) were incubated for 15–30 min at 37° but in the case of rat liver 3 ml of the same fraction was incubated for 60 min. Where the rate of microsomal metabolism was being measured in rat liver, the sedimented microsomes were suspended in small volumes of KCl and aliquots equivalent to 0·3–1·2 g tissue were incubated for 60 min. A supplement of 2 μl Boehringer glucose-6-phosphate dehydrogenase suspension was added to each incubation of microsomes.

Rates of total aflatoxin metabolism were assayed in the 105,000 *g* supernatant fraction under the same assay conditions. The amount of aflatoxin metabolised was approximately proportional to the volume of liver enzyme added to the reaction mixture when 3 ml rat liver supernatant or 0·2 ml duck liver supernatant was used. In the latter case, the incubation was supplemented with glucose-6-phosphate dehydrogenase.

The rates of formation of aflatoxin M_1 or a metabolite fluorescing yellow in ultraviolet light (probably a demethylation product: see Fig. 1) were measured by semi-

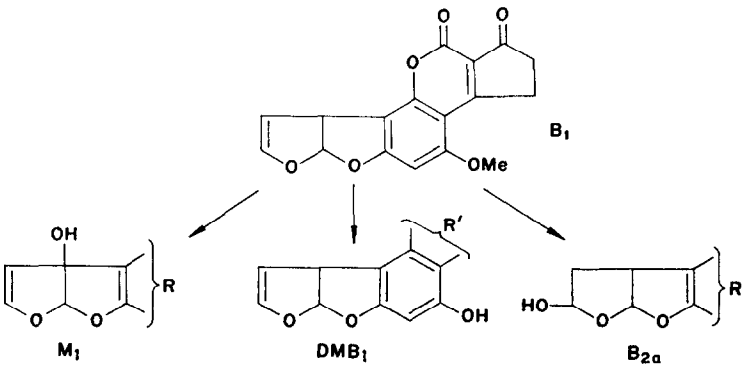

FIG. 1. Structural formula of aflatoxin B_1 with partial structures of the metabolites M_1 and B_{2a}, (aflatoxin hemiacetal). The presumed partial structure of the demethylated toxin (DMB_1) is also shown. This may be identical with metabolite Y formed by rat liver microsomes. R and R^1 in this figure denote unchanged portions of the aflatoxin molecule.

quantitative TLC.[12] Fluorescence of aflatoxin M_1 was simply matched with that of a pure standard but in the case of the yellow metabolite (Y) only a very approximate assessment was possible by comparing dilutions at the limit of visibility with an aflatoxin B_1 standard.

Protein in microsomal suspension was estimated by the colorimetric method of Lowry *et al.*[16]

RESULTS

Aniline metabolism. As shown in Table 1, phenobarbital treatment had the effect of stimulating microsomal aniline hydroxylation by a factor of more than 5 in both species. Rates of unstimulated aniline metabolism were quite comparable.

Aflatoxin metabolism in crude microsomes (9000 *g supernatant fraction*). While phenobarbital treatment enhanced aflatoxin metabolism in rat liver preparations (a 3-fold increase), there was no demonstrable effect in duck liver fractions (Table 2). The mean control or unstimulated rate of aflatoxin metabolism was 150 times greater in the duck than in the rat.

TABLE 1. THE EFFECT OF PHENOBARBITAL TREATMENT ON ANILINE METABOLISM BY DUCK AND RAT LIVER MICROSOMAL SUSPENSIONS

	Controls [μmoles *p*-nitrophenol formed/g protein/min at 37° (mean ± S.E.M.)]	Stimulated	Statistical significance of difference (P value)
Ducks (6 in each group)	0·158 ± 0·007	1·10 ± 0·025	< 0·001
Rats (6 in each group)	0·200 ± 0·018	1·013 ± 0·032	< 0·001

TABLE 2. DIFFERENT EFFECTS OF PHENOBARBITAL TREATMENT ON AFLATOXIN METABOLISM BY 9000 *g* SUPERNATANT LIVER FRACTION FROM DUCKS AND RATS

	Controls [nmoles aflatoxin metabolized/g fresh weight/min at 37° (mean ± S.E.M.)]	Stimulated	Statistical significance of difference (P value)
Ducks (6 in each group)	47·0 ± 1·83	49·5 ± 2·00	Not significant
Rats (6 in each group)	0·31 ± 0·067	0·91 ± 0·056	<0·001 compared with either groups of stimulated rats
	—	0·95 ± 0·101	

In previous studies, the formation of metabolite BX had been detected when excess duck liver enzyme was used to completely metabolise 0·13 μmole aflatoxin by observing maximum light absorption at 400 nm in aqueous methanolic extracts of liver.[12,13] As rate limiting amounts of enzyme were being used here, only a small decrease in absorbance at 363 nm (total aflatoxin metabolism) was measurable. The formation of BX could not, therefore, be confirmed. No other metabolites were detected by TLC.

Aflatoxin metabolism in isolated rat microsomes. The rate of total aflatoxin metabolism was increased approximately ten times in isolated rat microsomes as a result of phenobarbital treatment (Table 3). Not only the rate of metabolism per gram fresh tissue but also the specific activity (rate per g microsomal protein) was increased indicating a genuine stimulation of microsomal enzyme activity. As expected this was apparently a non-specific effect on drug processing enzymes as aniline hydroxylase had also been stimulated.

TLC of chloroform extracts obtained from aqueous methanol "protein-free filtrates" usually revealed two metabolites of aflatoxin. The 4-hydroxylated product aflatoxin M_1 was seen as a bright blue fluorescent spot in ultraviolet light. (R_f 0·24 in chloroform containing, 3% v/v methanol) but in addition there was a yellow fluorescent spot (metabolite "Y": R_f 0·38) sometimes accompanied by one or two other very weakly fluorescent yellow spots. The major yellow fluorescent metabolite was probably a demethylation product[10] and its rate of formation was very approximately assessed by assuming that the minimum amount visible was 2 ng as in the case of aflatoxin B_1. When sufficient metabolite Y had been collected it was purified by small scale preparative TLC to give a yellow methanolic solution absorbing light maximally at 306 nm.

Microsomes from stimulated rats produced aflatoxin M_1 and metabolite Y at much enhanced rates. Specific activities were increased by about 4·5 and 14 times the control values respectively (Table 3). While the assessment of aflatoxin M_1 and particularly metabolite Y formation by TLC was very crude (although reasonably reproducible), the sum of these two separate activities appear to form a substantial part of the measured rate of total aflatoxin metabolism.

An attempt was made to measure more directly the rate of aflatoxin demethylation by estimating the formation of formaldehyde during incubation. This failed for several reasons. Firstly, the maximum quantity of formaldehyde released assuming complete

TABLE 3. PHENOBARBITAL STIMULATED METABOLISM BY ISOLATED RAT LIVER MICROSOMES

Group (No. animals)	Rate/g fresh weight (nmoles aflatoxin metabolized or converted into stated metabolites/g fresh weight or protein/min at 37°) (mean ± S.E.M.)			Rate/g microsomal protein		
	Total metabolism	M_1	Y	Total metabolism	M_1	Y
Controls (6)	0·35 ± 0·087	0·034 ± 0·096	0·075 ± 0·017	13·75 ± 3·98	1·35 ± 0·449	2·93 ± 0·841
Stimulated (6)	3·36 ± 0·169	0·222 ± 0·018	1·18 ± 0·050	115·9 ± 4·28	6·10 ± 1·02	42·1 ± 1·85
Statistical significance of difference (P value)	<0·001	<0·001	<0·001	<0·001	<0·01	<0·001

Note: For completeness, TLC assessment of M_1 and Y formation has been given the same statistical treatment as the more reproducible spectrophotometric assay of total metabolism. Values for the formation of Y are very approximate (see text).

demethylation of the substrate, under the present assay conditions, would be 0·13 μmole and close to the limit of detection by colorimetry. Using rate limiting amounts of liver enzyme the quantities of formaldehyde would be much less. The concentrations of aflatoxin could not be raised to overcome this difficulty without adding relatively large volumes of solvents (e.g. ethanol and DMF) which can interfere with aflatoxin metabolism (unpublished observations). Secondly, when the colorimetric method of Nash[17] was employed to estimate formaldehyde it was found that residual aflatoxin interfered seriously with spectrophotometric measurements at 415 nm. This appears not to have been considered by Bassir and Emafo[10] who used this procedure with tungstic acid filtrates of liver incubations in their assay of aflatoxin demethylation. Finally, it was found that unlike deproteinisation with methanol/Na_2SO_4, tungstic acid precipitates adsorbed a large proportion of the substrate: about 66 per cent in a typical incubation with 3 ml of 9000 *g* liver supernatant. As erroneously high rates of aflatoxin metabolism would have been measured in this way, the procedure of Bassir and Emafo[10] was abandoned. Alternative use of a chromotropic acid procedure for the estimation of metabolically formed formaldehyde was not successful because of very high blank values.

TABLE 4. AFLATOXIN METABOLISM IN 105,000 *g* SUPERNATANT FRACTION OF DUCK AND RAT LIVER

	nmoles aflatoxin metabolized/g liver/min at 37°
Duck (5)	28·22 ± 6·21
Rat (6)	0·32 ± 0·092

Aflatoxin metabolism in 105,000 *g supernatant fraction of liver.* Rates of total aflatoxin metabolism were measured in five randomly selected male ducks (4–8 weeks old) and adult rats. As shown in Table 4, the cytoplasmic fraction of duck liver had more than 90 times the activity of rat liver.

DISCUSSION

Results of these experiments show that, as expected, phenobarbital treatment resulted in the stimulation of microsomal enzymes. Aniline hydroxylase activity was enhanced in both duck and rat. However, using a 9000 *g* supernatant liver fraction (crude microsomes) aflatoxin metabolism was stimulated in the rat but not in the duck. Closer examination of aflatoxin metabolism by isolated rat microsomes showed that both 4-hydroxylation to aflatoxin M_1 and the production of a yellow fluorescent metabolite (Y) were stimulated by phenobarbital treatment. The latter transformation almost certainly involved demethylation since not only does the evidence of Bassir and Emafo[10] point to this but the earlier *in vivo* studies of Shank and Wogan[8] using [^{14}C]methoxy-labelled aflatoxin provided incontrovertable evidence of demethylation in the rat. The two activities formed a large part of the total metabolism of

aflatoxin in our experiments and when more accurate methods become available for the assay of aflatoxin demethylation, it may be found that, hydroxylation and demethylation completely account for aflatoxin metabolism in the rat. One of the minor yellow fluorescent spots seen on TLC may have been a demethylated and hydroxylated metabolite of aflatoxin.

It appears that normally about twice as much aflatoxin is demethylated as is hydroxylated by rat liver and phenobarbital treatment has the effect of stimulating demethylation more than hydroxylation. Although it is not yet known whether the demethylated produce is toxic, aflatoxin M_1 is as acutely toxic as B_1.[18] Since aflatoxin M_1 and possibly metabolite Y are excreted in the bile in a conjugated form,[19] phenobarbital treatment might be considered to provide a means of clearing aflatoxin more rapidly from rat liver rather than enhancing detoxification in the classical sense.

The lack of stimulation by phenobarbital treatment of aflatoxin metabolism in duck liver 9000 *g* supernatant fractions suggested that microsomal metabolism was not rate limiting. These results do not conflict with our earlier observation[12] that both microsomes and supernatant fractions were required for the complete metabolism of aflatoxin by duck liver. They merely emphasize the quantitative importance of metabolism by the cytoplasmic fraction. In fact, examination of the metabolizing activity of 105,000 *g* supernatant fractions showed that duck liver was very active in this respect. The nature and relative importance of cytoplasmic aflatoxin metabolism in different animal species will form the subject of a separate paper.

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