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SUBSTRATE AND PHENOBARBITAL INDUCIBLE AFLATOXIN-4-HYDROXYLATION AND AFLATOXIN METABOLISM BY RAT LIVER MICROSOMES

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Abstract—A quantitative method based on the determination of the fluorescence of B_1, B_2, M_1, M_2, G_1 and a toxin called "GM_1" (most probably a 4-hydroxylated derivative of aflatoxin G_1) on thin-layer chromatoplates was used in the assay of aflatoxin-4-hydroxylase activity. NADPH was found to be a cofactor and the pH optimum was about 7·4. Aflatoxins M_1, M_2 and "GM_1" were identified chromatographically after extraction from reaction mixtures. Aflatoxin-4-hydroxylase activity was found mainly in the microsomal fractions of male Wistar rat livers. Livers of rats dosed intraperitoneally (i.p.) for 5 days with a daily dose of phenobarbital (75 mg/kg) or with a daily dose of aflatoxin B_1 (500 μg/kg) had a microsomal hydroxylating activity 2·3 and 3·5 times higher than that of the control rats. This demonstrates the inductive effect of these compounds. The relative activity of the aflatoxin-4-hydroxylating system on aflatoxins B_1, B_2 and G_1 as substrates was investigated. Aflatoxin B_1 also significantly induced its breakdown by rat liver microsomal preparations. In this case phenobarbital had no similar effect. Results obtained during this investigation showed that aflatoxin M_1, M_2 and "GM_1" could possibly be intermediates in the breakdown of aflatoxins B_1, B_2 and G_1 in rat liver.

A TOXIC factor excreted in the milk of cows fed aflatoxin, contained in groundnut meal, and in the urine of sheep fed mixed aflatoxins, was claimed by Allcroft and Carnaghan,[1] and Allcroft *et al.*,[2] to be one single compound on the grounds of chromatographical evidence. They suggested giving the compound the trivial name aflatoxin M. Aflatoxin M_1 and M_2 were isolated from the urine of sheep given intraperitoneal doses (1 mg/kg) of mixed aflatoxins (B_1, 73%; B_2, 24%; G_1, 2% and G_2, 1%) and their structures determined.[3] Aflatoxin M_1 and M_2 were also isolated from extracts of *Aspergillus flavus* grown on peanuts. The milk toxin (aflatoxin M) was found to be present in the livers of rats given aflatoxin B_1 (see ref. 4). Purchase and Theron[5] found a green fluorescent component in tissue extracts of rats dosed with aflatoxin G_1 and assumed that it might be the 4-hydroxylated derivative of aflatoxin G_1 (called aflatoxin "GM_1" in this paper).

The structures of aflatoxins B_1, B_2, G_1 and G_2 as well as certain aspects of their toxicity, carcinogenicity and biochemical effects have been summarized by Wogan.[6] The structures of aflatoxin B_1 and M_1 are given in Fig. 1.

Studies on the metabolism of the aflatoxins became more important because of the possibility that one or more unknown metabolites of aflatoxin B_1 are more toxic than the parent compound as reported recently by Portman, Campbell and Plowman.[7] The fact that the milk toxin (aflatoxin M) was found in the livers of rats[4] stressed the

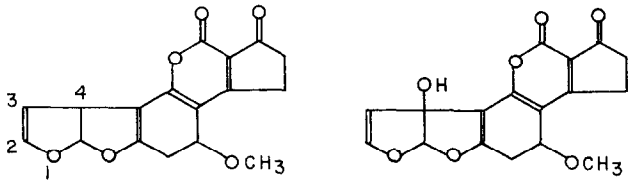

FIG. 1. Structures of aflatoxins B_1 (I) and M_1 (II).

possibility that the liver is the site for the conversion of aflatoxin B_1 to M_1 which is a toxic and carcinogenic compound.* The purpose of this investigation was to study the possibility of an enzyme-catalyzed conversion of aflatoxin B_1 to M_1 by rat liver preparations as well as the substrate and phenobarbital inducibility of an anticipated aflatoxin-4-hydroxylating system.

EXPERIMENTAL

Materials

Male albino Wistar rats bred in this laboratory and originally obtained from the Veterinary Research Laboratories, Onderstepoort, Pretoria, were used in these studies. The aflatoxins were available in this laboratory. NADPH, NADH, NADP, nicotinamide, sodium isocitrate and isocitrate dehydrogenase were obtained from Boehringer, Germany. All other reagents used were of analytical grade quality.

Methods

Administration of phenobarbital and aflatoxin B_1 to rats and fractionation of rat livers. Intraperitoneal doses of 75 mg phenobarbital per kg per day and 500 μg aflatoxin B_1 per kg per day were given for 5 days to two separate groups of three rats weighing 200 $\pm$ 5 g each. One group of three rats, used as control, was dosed i.p. with the solvent viz., 33·3% dimethysulfoxide in water (0·3ml).

All the rats were killed by decapitation after 5 days, their livers removed and washed with a cold 0·9% NaCl solution. The livers were homogenized in a Dounce homogenizer in an ice bath in three volumes of 0·02 M Tris-HCl buffer (pH 7·4) containing 3mM $MgCl_2$ and 0·25 M sucrose. Mitochondrial, microsomal and 105,000 *g* supernatant fractions were prepared essentially as described by Mahler and Cordes,[8] employing differential centrifugation in Rotor No. 40 of a Spinco ultracentrifuge. The absence of contamination of the microsomal fractions by mitochondria was confirmed by electron microscopy.

The mitochondrial and microsomal fractions were suspended in 15 ml of 0·02M Tris-HCl buffer (pH 7·4) containing 3mM $MgCl_2$ and 0·25 M sucrose per liver and used as such in further studies.

Assay of aflatoxin-4-hydroxylase activity

The concentrations of the different components of the incubation mixtures for the determination of hydroxylating activity were: aflatoxin B_1, B_2 or G_1 in a final concentration of 8% methanol (v/v) (64·3μM); NADPH (2mM); Tris-HCl buffer (pH 7·4) 18·4mM); $MgCl_2$ (2·8mM) and 0·5 ml liver fraction of varying protein content in a total volume of 5·0 ml. The final sucrose concentration was 25mM. The reaction

*I.F.H. Purchase, personal communication.

was initiated by the addition of aflatoxin. The reaction mixtures were incubated in cotton wool plugged test tubes (in the presence of atmospheric oxygen) at 37·5° in a waterbath equipped with a shaking device and shaken gently. Higher hydroxylating activity was not observed at higher NADPH concentrations up to 20 mM. In some experiments a NADPH generating system was employed in which case NADP (1 mM), nicotinamide (20 mM), sodium isocitrate (20 mM) and 5 units of isocitrate dehydrogenase were included in the incubation mixture of 5 ml which gave results similar to that obtained from incubations mixtures using NADPH directly. The use of NADPH directly has also been described by Ernster and Orrenius.[9] In all cases the rate of formation of aflatoxin-4-hydroxylated derivatives decreased rapidly after 60 min. The reaction rate was constant for the first 60 min. Oxygenated microsomal preparations and incubations in the presence of an oxygen atmosphere did not show higher hydroxylating activities than the incubations in the presence of air. Saturation of the incubation mixtures with toluene to avoid bacterial contamination did not produce different results from incubations without toluene even for incubation times up to 20 hr.

Aliquots of 0·8 or 1·0 ml were taken at certain time intervals from the reaction mixtures. These aliquots were shaken with 15 to 20 ml of acetone which stopped the reaction. Ten ml distilled water were added and the aflatoxins were extracted from this with three portions of 20 ml chloroform. The combined chloroform extracts were dried with anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator. The extract was then dissolved in 2·0 ml chloroform and the toxins separated and identified by thin-layer chromatography. Good results were also obtained by evaporating the acetone samples to dryness and then dissolving the toxins in 2·0 ml chloroform.

Aflatoxin M_1 and also M_2 and "GM_1" (most probably the 4-hydroxylated derivative of aflatoxin G_1) extracted from reaction mixtures, were identified by thin-layer chromatography. Thin-layer chromatoplates were prepared from kieselgel (Camag D5), to a wet layer thickness of 0·3 mm and activated by heating to 105° for 1 hr before use. Extracts were spotted in chloroform and the chromatoplates were developed in acetone–chloroform (1:4, v/v) or in methanol–chloroform 1:19, v/v).

The concentrations of aflatoxins M_1, M_2, B_1, B_2 and G_1 were determined quantitatively by comparing the fluorescence in ultraviolet light of the different toxin spots with that of known standards. For the quantitative determination of aflatoxin "GM_1" aflatoxin M_1 was used as standard. A Photovolt model 530 densitometer for solid-state fluorescence measurements[10] was employed using filter number 465 (about 465 mμ) to record the fluorescent intensity corresponding to the different spots e.g. of aflatoxins B_1, M_1 and also aflatoxin B_{2a} in cases where aflatoxin B_1 was the substrate. [Aflatoxin B_{2a} and G_{2a} have recently been identified as two hydroxy derivatives of aflatoxins B_2 and G_2, respectively, hydroxylated at position 2 of the terminal furane ring[11,12] (see Fig. 1.).] The areas under the peaks, a measure of the toxin concentration, were measured by the integrator, and the absolute concentration determined by comparison with results obtained for the standards. The amounts of aflatoxins used as standards on the chromatoplates were 9·68 $\mu\mu$moles for aflatoxins M_1 and M_2 and 1·61 mμmoles for aflatoxin B_1, B_2 and G_1. This method has a possible experimental error of approximately 3 per cent. The recovery of aflatoxins in the extracts was approximately 96 per cent.

Determination of protein

The method of Lowry *et al.*[13] was used for protein determination using bovine serum albumin as standard.

RESULTS

Identification and quantitative determination of aflatoxins in reaction mixtures

Aflatoxin M_1 was identified on thin-layer chromatograms as illustrated in Figs. 2(a) and (b) employing two different developing solvents.

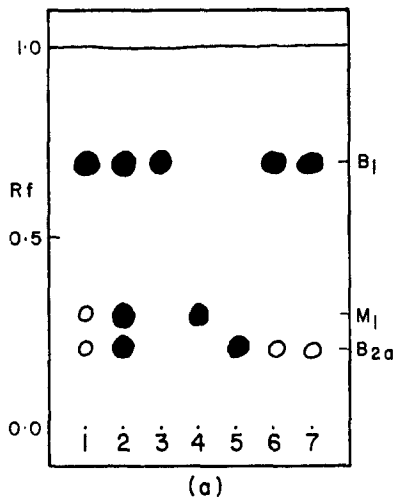

FIG. 2(a). Thin-layer chromatographical identification of aflatoxins M_1, B_1 and B_{2a}. Developing solvent: acetone–chloroform (1:4, v/v). Fifty μl of 2·0 ml extracts of different reaction mixtures obtained (as described in the Experimental section) after 1 hr of incubation at 37°, were spotted on the chromatoplate prepared from kieselgel (Camag D_5) as described in the text. Numbers 1, 2, 6 and 7 refers to reaction mixtures containing mitochondrial, microsomal, 105,000 *g* supernatant and blank [0·02 M Tris-HCl buffer (pH 7·4) plus 3 mM $MgCl_2$ plus 0·25 M sucrose instead of a liver fraction] fractions, respectively. Aflatoxin B_1 was used as substrate. Numbers 3, 4 and 5 are aflatoxin B_1, M_1 and B_{2a} standards ($1{\cdot}6$, $9{\cdot}7\times10^{-3}$ and $9{\cdot}7\times10^{-3}$ mμmoles, respectively).

Aflatoxin M_2 was identified in a similar manner by means of TLC. The identification of aflatoxins M_1 and M_2 was also confirmed by paper chromatography employing the method described by Holzapfel *et al.*[3] This method was also used to show that the only 4-hydroxylated aflatoxins formed when aflatoxins B_1 or B_2 were used as substrates, were aflatoxins M_1 or M_2, respectively. The results obtained indicated that under the experimental conditions used, conversions of aflatoxins $B_1 \rightarrow B_2$ and $M_1 \rightarrow M_2$ and thus $B_1 \rightarrow M_2$ and $B_2 \rightarrow M_1$ could not be catalyzed by rat liver microsomes. When acetone–chloroform (1:4, v/v) was used as developing solvent, B_2 had a smaller R_f value than B_1 while G_1 and "GM_1" had smaller R_f values than B_2 and B_{2a}, respectively, as illustrated in Fig. 2(c).

Under the reaction conditions of the assay for aflatoxin B_1 -4-hydroxylase, aflatoxin B_{2a} was a major product formed even in the absence of any liver fraction. Its concentration was in many cases higher than that of aflatoxin M_1. The rate of formation of aflatoxin B_{2a} was however enhanced in the presence of rat liver microsomal

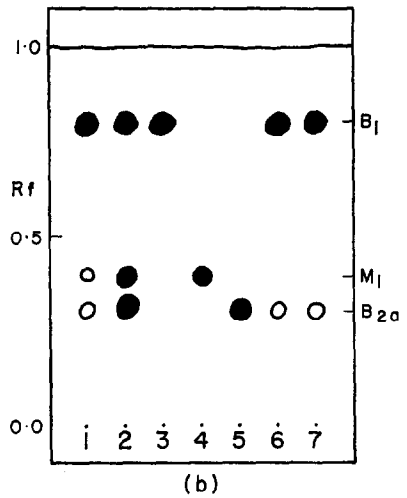

FIG. 2(b). Thin-layer chromatographical identification of aflatoxins M_1, B_1 and B_{2a}. Developing solvent : methanol–chloroform (1:19, v/v). Numbers 1 to 7 as in Fig. 2(a). Similar amounts of extracts (obtained as described under methods) and standards were spotted as indicated in the legend to Fig. 2(a).

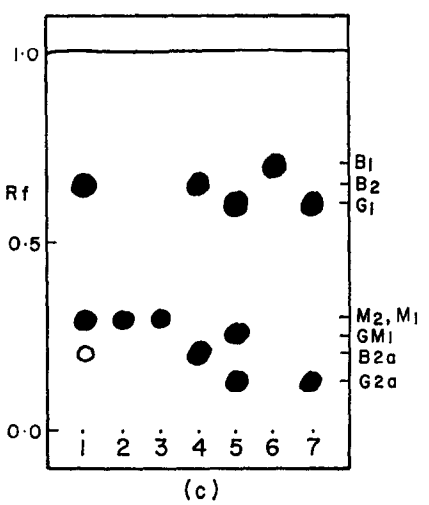

FIG. 2(c). Schematical representation of the R_f values of aflatoxins B_1, B_2, G_1, M_1, M_2, "GM_1", B_{2a} and G_{2a} obtained from extracts of incubation mixtures or standards. Developing solvent:acetone–chloroform (1:4, v/v). Numbers 1 to 7 refer to: (1) reaction mixture where aflatoxin B_2 was used as substrate; (2) M_2 standard; (3) M_1 standard; (4) combined B_2 and B_{2a} standards; (5) reaction mixture where G_1 was used as substrate; (6) B_1 standard, and (7) a combined G_1 and G_{2a} standard. 50 μl of the reaction mixture extracts obtained after 8 hr of incubation at 37° (using rat liver microsomal fractions) were spotted. The composition of the incubation mixtures and the method of extraction are given in the text. The amounts of aflatoxin B_2 and G_1 in their standard spots were similar to that of B_1 and those of aflatoxins M_1, M_2, B_{2a} and G_{2a} similar to that of M_1 as given in the legend to Fig. 2(a).

preparations. When aflatoxin B_2 was used as substrate (in the presence of microsomal fractions) only a small quantity of B_{2a} was formed but aflatoxin M_2 was formed readily. Aflatoxin G_{2a} was formed when aflatoxin G_1 was used as substrate and its production was also enhanced in the presence of rat liver microsomes.

An example of a diagram by a Photovolt model 530 densitometer from a thin-layer chromatoplate which was used for the quantitative determination of aflatoxins M_1 and B_1 is illustrated in Fig. 3.

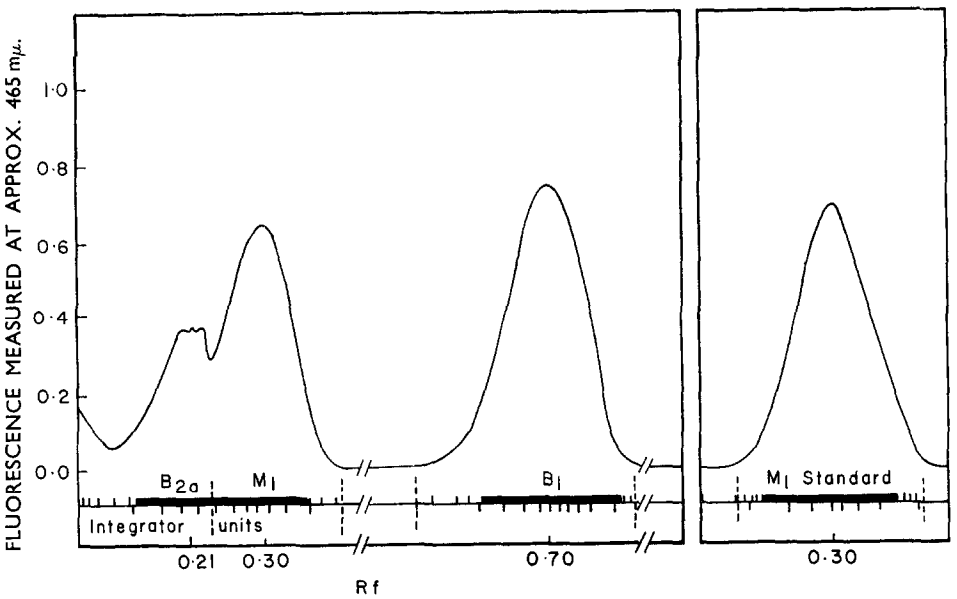

FIG. 3. A schematic representation of a diagram produced by a Photovolt model 530 densitometer from a thin-layer chromatoplate. fifty μl of 2·0 ml extract of a 1 ml aliquot taken from a 5 ml incubation mixture (containing rat liver microsomes of a phenobarbital treated rat) after 1 hr of incubation, was spotted on the chromatoplate and developed in acetone–chloroform (1:4, v/v). A much higher sensitivity was employed for scanning the aflatoxin M_1 (from the extract) and aflatoxin M_1 standard spots (25/25) than for the aflatoxin B_1 spot (250/250) due to their lower concentrations. At a sensivity of 25/25 this method was sensitive enough to determine 1×10^{-4} μg aflatoxin M_1.

The integrator units for the aflatoxin M_1 standard and the aflatoxin M_1 (obtained from the incubation mixture mentioned in the legend to Fig. 3) were 72 and 64, respectively. (Fig. 3) The amount of aflatoxin M_1 formed and thus the specific activity in μμmoles/min/mg protein (μμmoles aflatoxin M_1 formed/min/mg protein) can be calculated from these values. The aflatoxin M_1 standard (1μl) was equivalent to 9·68 μμmoles aflatoxin M_1. In this case the specific activity was 11·15 which corresponds to 0·67 mμmoles of aflatoxin M_1 formed per hr per mg protein in 5 ml of incubation mixture. The protein concentration of the microsomal fraction used in this case was 6·6 mg/ml which gave a final protein concentration in the reaction mixture of 0·66 mg/ml. Except where indicated otherwise, hydroxylase activity was determined after 10, 30 and 60 min of incubation.

The concentration of aflatoxins M_2, B_2, G_1 and also B_{2a} and G_{2a} were determined in a similar manner.

Localization of aflatoxin B_1-4-hydroxylase activity

As illustrated in Table 1, the hydroxylating activity was found to be localized in the microsomal fractions of rat livers. Some activity was, however, also found in mitochondrial fractions but not in the 105,000 *g* supernatants.

Induction of aflatoxin-4-hydroxylase activity by phenobarbital and aflatoxin B_1

Results obtained from the livers of aflatoxin B_1 and phenobarbital treated rats indicated an induction of the hydroxylating enzyme of 3·5 and 2·3, respectively, when aflatoxin B_1 was used as substrate (Table 2). The inductive effect of both compounds was however restricted to the microsomal fraction as indicated in Table 2.

TABLE 1. LOCALIZATION OF AFLATOXIN-4-HYDROXYLASE ENZYME SYSTEM*

Fraction	Activity for three different rats ($\mu\mu$moles/min/mg protein)			Average activity ($\mu\mu$moles/min/mg protein)
Mitochondria	0·70,	0·75,	0·95	0·80
Microsomes	3·50,	4·10,	4·55	4·05
105,000 *g* supernatant	0·00,	0·00,	0·00	0·00

* The preparation of the different rat liver cellular fractions and the assay method for the hydroxylating system were the same as that described in the text.

TABLE 2. INDUCTION OF AFLATOXIN B_1-4-HYDROXYLASE ACTIVITY BY PHENOBARBITAL AND AFLATOXIN B_1

Inducer	Activity for three different rats ($\mu\mu$moles/min/mg protein)		Average activity ($\mu\mu$moles/min/mg protein)	
	microsomes	mitochondria	microsomes	mitochondria
Phenobarbital	8·20, 8·10, 11·15	0·90, 1·10, 1·15	9·15	1·05
Aflatoxin B_1	12·40, 14·20, 15·35	0·80, 0·90, 1·00	13·95	0·90
Controls	3·55, 3·90, 4·45	0·70, 0·75, 0·95	3·95	0·80

* Two groups of three rats weighing 200 ± 5 g each were dosed intraperitoneally with 75 mg phenobarbital and 500 μg of aflatoxin B_1 per kg per day for 5 days respectively. The control group of three rats was dosed intraperitoneally with a corresponding volume of the solvent, viz., 33·3% dimethylsulfoxide in water (0·3 ml). The rats were killed by decapitation after 5 days. The rat liver fractions and the assay method for hydroxylating activity were similar to that described in the Experimental section.

The 4-hydroxylation of aflatoxin B_2 and G_1 by rat liver microsomal fractions was also induced by phenobarbital and aflatoxin B_1 as indicated in Table 3. The results given in Table 3 indicate that aflatoxin B_2 and G_1 can also act as substrates for the aflatoxin-4-hydroxylating system. The specific activities were, however, much lower than that of aflatoxin B_1 (Table 3).

A linear relationship between protein concentration and the aflatoxin B_1-4-hydroxylating activity of rat liver microsomes was found.

Effect of pH on aflatoxin B_1-4-hydroxylase activity

A pH optimum of about 7·4 was found for the hydroxylation of aflatoxin B_1 as illustrated in Fig. 4.

TABLE 3. INDUCTION OF MICROSOMAL 4-HYDROXYLATION OF AFLATOXIN B_2 AND G_1 BY PHENOBARBITAL AND AFLATOXIN B_1*

Inducer	Substrate	Activity for three different rat ($\mu\mu$ moles/min/mg protein)	Average
Phenobarbital	Aflatoxin B_2	4·95, 5·10, 5·85	5·30
	Aflatoxin G_1	5·05, 5·85, 6·20	5·70
Aflatoxin B_1	Aflatoxin B_2	6·80, 8·20, 8·25	7·75
	Aflatoxin G_1	7·50, 7·85, 9·25	8·20
Control	Aflatoxin B_2	2·90, 3·15, 3·55	3·20
	Aflatoxin G_1	2·45, 2·80, 2·85	2·70

* The experimental procedure was similar to that described in Table 2 except that aflatoxins B_2 and also G_1 replaced aflatoxin B_1 as substrate in the reaction mixtures used for the assay of hydroxylating activity.

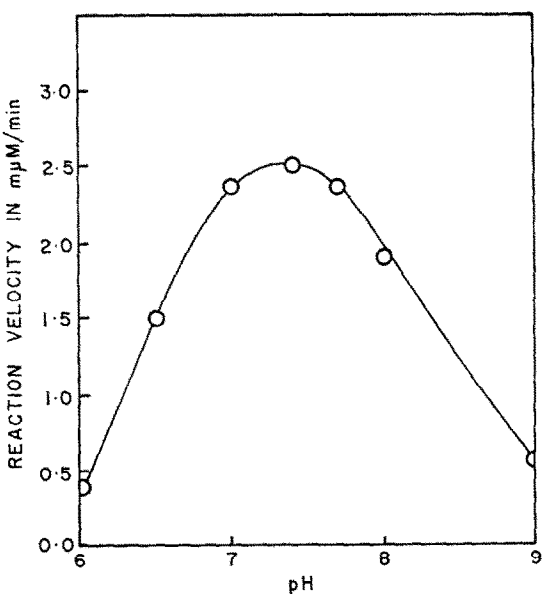

FIG. 4. Effect of pH on aflatoxin B_1-4-hydroxylase activity. Enzyme activities were determined in rat-liver microsomal preparations as described in the Experimental Section. Sodium phosphate buffers were used at pH 6·0 to 7·4 and Tris-HCl buffers at pH 7·4 to 9·0.

Pyridine nucleotide requirement

NADPH and NADH were compared as cofactors for aflatoxin B_1-4-hydroxylation by rat liver microsomes. A requirement for NADPH was established. Very low activity was found with NADH and ascorbate. In the absence of an electron donor a slight activity was observed which was probably due to small quantities of NADPH present in the microsomal preparations. Where microsomal fractions of aflatoxin B_1 treated rats were used, the specific activity with NADPH as electron donor was 12·1 $\mu\mu$moles/min/mg in comparison with about 1·5 $\mu\mu$moles/min/mg for NADH, ascorbate and no electron donor.

Breakdown of aflatoxin B_1 by rat liver microsomal fractions

From studies of the effect of the duration of incubation on the reduction of aflatoxin B_1 concentration in the reaction mixture, it was evident that rat liver microsomes can metabolize aflatoxin B_1 very effectively. After 20 hr of incubation, less than 20 per cent of the original amount of aflatoxin B_1 was left in the incubation mixture. This was not due to instability of the toxin in the reaction mixture because it was found that aflatoxin B_1 was stable for at least 1 week in aqueous solutions under similar conditions of pH and temperature. The observed breakdown (not hydroxylation, but hydroxylated derivatives may act as intermediates) of aflatoxin B_1 by rat liver microsomes was induced by aflatoxin B_1 but not by phenobarbital (Fig. 5).

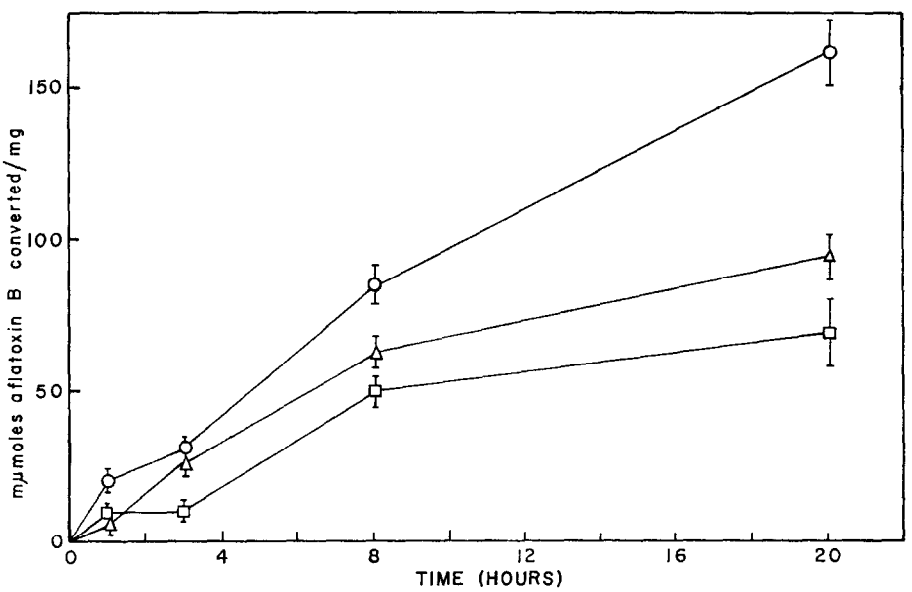

FIG. 5. Effect of incubation time on the amount of aflatoxin B_1 metabolized (catabolized) by rat liver microsomes from rats pretreated with phenobarbital (□) and aflatoxin B_1 (0) and also control rats (△). The assay method for the hydroxylase activity is given in the Experimental Section. The values in mμmoles of aflatoxin B_1 converted/mg protein indicated here, were the averages of values obtained from the microsomal fractions of three rats in each group and were calculated per 5 ml reaction mixture and the vertical lines indicate the range in which the values fall.

Although the amount of aflatoxin B_1 converted per mg protein after 20 hr was lower in the case of phenobarbital treated rats compared with that of the controls, the total activities were similar due to the higher protein content of the microsomal preparations from phenobarbital treated rats. A slight decrease in microsomal protein was found in the case of aflatoxin B_1 treated rats.

The concentrations of the first products formed from aflatoxin B_1 in the incubation mixtures, e.g. aflatoxins M_1 and B_{2a}, also seemed to decrease after 3 and 7 hr of incubation, respectively. No new fluorescent spots occurred on thin-layer chromatograms. These facts support the conclusion that the aflatoxins can be broken down by

rat liver microsomes. The catabolism of aflatoxins B_2 and G_1 showed a similar pattern to that of B_1.

It was evident that after 3 hr of incubation, aflatoxin M_1, M_2 and "GM_1" concentrations reached a maximum (B_1, B_2 and G_1 used as substrates, respectively) and then decreased (Fig. 6). According to these results there is a possibility that aflatoxins M_1, M_2 and "GM_1" are intermediates in the catabolism of aflatoxins B_1, B_2 and G_1 by rat liver microsomes.

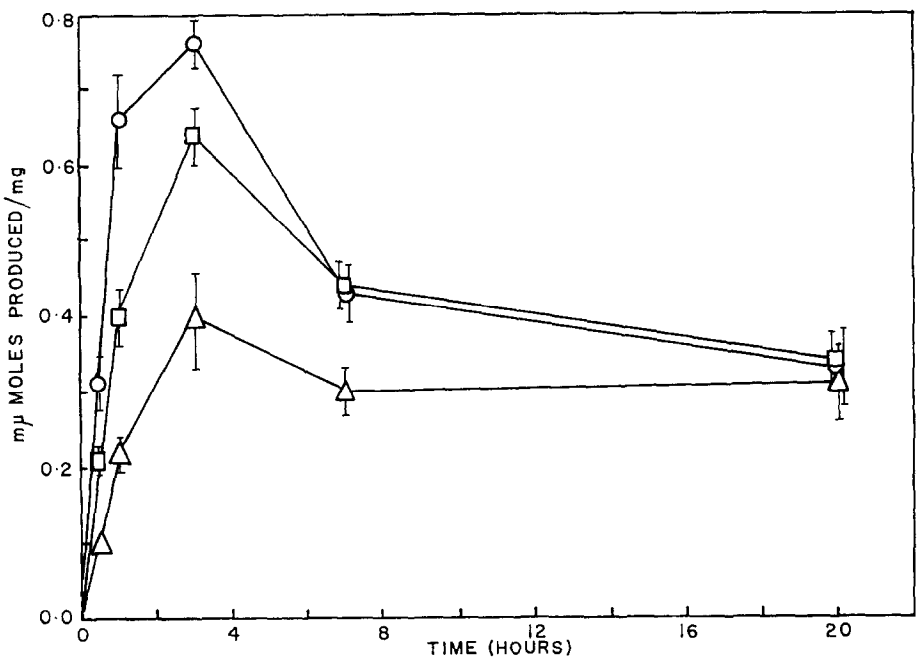

FIG. 6. Effect of incubation time on the production of aflatoxins M_1 (○), M_2 (□) and "GM_1" (△) where aflatoxins B_1, B_2 and G_1 were used as substrates, respectively. The mμmoles of aflatoxin produced per mg protein indicated here, were the averages of values obtained from the liver microsomal fractions of a group of three phenobarbital treated rats and the vertical lines indicate the range in which the values fall. The assay method for the hydroxylase activity is given in the Experimental Section. Similar patterns were obtained with aflatoxin B_1 treated and control rats.

DISCUSSION

Experimental evidence has been presented for the aflatoxin-4-hydroxylating activity of rat liver microsomes. It was found that aflatoxins can also be metabolized by rat liver microsomes. It appears very likely, therefore, that the aflatoxins represent another series of substrates which can be degraded by the drug metabolizing system of liver microsomes. It is interesting to note that breakdown of aflatoxins by microorganisms has recently been described by Ciegler *et al.*[14]

A reliable assay method for aflatoxin-4-hydroxylase activity, based on the quantitative determination of the fluorescence of the different aflatoxins concerned on thin-layer chromatoplates, was developed. NADPH was found to be a cofactor and the

pH optimum was about 7·4. Aflatoxins B_1, B_2 and G_1 were found to be substrates for the aflatoxin-4-hydroxylase system which most possibly includes a NADPH-cytochrome reductase and a CO-binding pigment (P450) as found in the drug-hydroxylating system.[9]

It is known that aflatoxin B_1 inhibits the induction of other enzymes such as tryptophan pyrrolase and tyrosine transaminase by hydrocortisone[15,16] as well as the induction of zoxazolamine hydroxylase[17] and 3,4-benzpyrene hydroxylase by 3,4-benzpyrene.[18] However, in the present investigation it was found to induce its own hydroxylation more than three-fold. Similar substrate induction of liver microsomal hydroxylating systems was reported by Ernster and Orrenius.[9] Pretreatment with aflatoxin B_1 was found to slightly decrease microsomal protein which is consistent with earlier observations concerning its ability to inhibit protein synthesis.[19] Since the effect of aflatoxin B_1 on the aflatoxin-4-hydroxylase enzyme system was a marked increase, it can be concluded that a specific induction of protein synthesis occurred. The fact that the induction of the other hydroxylases, mentioned above, was inhibited by aflatoxin B_1 may indicate that these hydroxylases (including aflatoxin-4-hydroxylase) are in fact different enzyme systems that differ in their inducibility, the structural requirements for their inducers and in the properties of their protein components. The inductive effect of phenobarbital was similar to its effect on other hydroxylation reactions catalyzed by liver microsomes.[18]

During this investigation it was also found that the formation of aflatoxins B_{2a} and G_{2a} was enhanced in the presence of rat liver microsomal preparations from aflatoxins B_1 and G_1, respectively. Aflatoxin B_{2a}, however, was not readily formed from aflatoxin B_2 under similar reaction conditions. The possible role of a rat liver microsomal enzyme system in the conversion of aflatoxins B_1 and G_1 to B_{2a} and G_{2a}, respectively, is under investigation in this laboratory.

The apparent inductive effect of aflatoxin B_1 on the metabolism of aflatoxin B_1, B_2 and G_1 by rat liver microsomes and the probability that aflatoxin M_1, M_2 and "GM_1" are intermediates in the catabolism may provide a basis for further studies on the metabolism of aflatoxins in rat liver. The possibility that one or more unknown metabolites of aflatoxin B_1 are more toxic than the parent compound has been reported recently by Portman *et al.*[7]

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