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Genetic Expression of Aflatoxin B_1 Metabolism: Effects of 3-Methylcholanthrene and 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin on the Metabolism of Aflatoxins B_1 and B_2 by Various Inbred Strains of Mice

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SUMMARY

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Hepatic microsomal metabolism of aflatoxin B_1 to aflatoxins M_1 (aflatoxin B_1-4-hydroxylase), Q_1 (aflatoxin B_1-9-hydroxylase) and to B_1-2,3-oxide (aflatoxin B_1-2,3-oxygenase) and of aflatoxin B_2 to aflatoxin M_2 (aflatoxin B_2-4-hydroxylase) was studied in inbred strains of mice pretreated with phenobarbital (PB), 3-methylcholanthrene (MC), or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In all strains tested (C57BL/6 Jacobs, BALB/cCr, CBA/J, C57L/J, DBA/2HaD, AKR/Sn, RF/J, and 129/J), PB induced aflatoxin B_1-2,3-oxygenase (2.4- to 6.4-fold) and, with the exception of C57L/J, aflatoxin B_1-9-hydroxylase (1.8- to 4.6-fold), whereas PB either had no effect or slightly induced aflatoxin in B_1-4-hydroxylase. In comparison to this, MC either had no effect or caused depression of aflatoxin B_1-2,3-oxygenase and of aflatoxin B_1-9-hydroxylase activities. On the other hand, MC induced aflatoxin B_1-4-hydroxylase (2.5- to 3.6-fold) and also aflatoxin B_2-4-hydroxylase (3.1- to 4.8-fold) only in Ah responsive strains and not in Ah nonresponsive strains. Dose–response studies with TCDD indicated that both aflatoxin B_1-4-hydroxylase and aflatoxin B_2-4-hydroxylase are induced by TCDD in the Ah nonresponsive strain (DBA/2) provided a dose 9-fold higher than that used for the Ah responsive strain (C57BL/6) is administered. The ED_{50} value for aflatoxin B_1-4-hydroxylase and aflatoxin B_2-4-hydroxylase induction in DBA/2 strain (1.3 μg/kg) was accordingly 9-fold higher than that in C57BL/6 (0.14 μg/kg). In contrast, TCDD dose–response curves for aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase were essentially similar in both strains, i.e., DBA/2 and C57BL/6. At the lowest dose, both activities increased slightly but as the dose increased, the activities either were inhibited or remained unaffected. The data indicate that (a) several cytochrome *P*-450s of the mixed function oxygenase participate in the metabolism of aflatoxin B_1 via various pathways, (b) these pathways of aflatoxin B_1 metabolism are under different regulatory controls, (c) the regulation of aflatoxin B_1-4-/B_2-4-hydroxylase and aryl hydrocarbon hydroxylase induction is controlled by the same or closely linked genetic factors at the regulatory gene level and not at the structural gene level, and (d) the regulation in (c) is most likely mediated by an induction-specific receptor which apparently has lower affinity for the inducer in the nonresponsive strain DBA/2.

INTRODUCTION

Metabolism of more than 300 chemicals including drugs, carcinogens, insecticides, and steroids is mediated by the microsomal mixed function oxygenase complex which is principally located in the liver; however, its presence has also been reported in most other mammalian tissues. By means of various biochemical and immunochemical techniques liver microsomes have been found to contain several forms of cytochrome *P*-450 (1). The relative proportions of various forms are determined by the type of inducer and the species and strain of animal used in these studies (1).

Benzo[*a*]pyrene, an environmental carcinogen, is preferentially metabolized by one or a few of these cyto-

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chrome *P*-450 forms and the resulting activity, measured as the formation of some phenolic metabolites, is commonly referred to as aryl hydrocarbon hydroxylase activity. This activity is preferentially induced by 3-methylcholanthrene in certain strains of mice (Ah responsive strains) but not in other strains (Ah nonresponsive) (2, 3). In conjunction with epoxide hydrase, another microsomal enzyme, mixed function oxygenase complex converts benzo[*a*]pyrene to phenols, quinones, dihydrodiols, arene oxides, and diol epoxides (4, 5). Some of these metabolites, especially arene oxides and diol epoxides, bind avidly to DNA and may be responsible for the induction of cancer. Strain differences in mice have also been reported (2, 6, 7) in the induction by 3-methylcholanthrene of DNA- and protein-binding metabolites of benzo[*a*]pyrene: Ah responsive strains are responsive, whereas Ah nonresponsive strains are nonresponsive. However, Ah nonresponsiveness is not absolute as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),[1] a potent inducer, evokes the same degree of induction of aryl hydrocarbon hydroxylase in Ah nonresponsive strains of mice at a 10 to 18 times higher dose than that required for the induction in Ah responsive strains (8). These data and other studies suggest the existence of an induction-specific receptor which is defective in its affinity for the inducer in Ah nonresponsive strains (9–11).

Aflatoxin B_1, an environmental hepatocarcinogen produced by the mold *Aspergillus flavus*, is also metabolized by the hepatic microsomal mixed function oxygenase (Fig. 1a). Activation of aflatoxin B_1 involves formation of aflatoxin B_1-2,3-oxide by the microsomal mixed function oxygenase (12–14), while the same enzyme complex deactivates it via conversion to aflatoxins Q_1, M_1, and P_1 (15, 16). Our recent genetic studies in mice have demonstrated that aflatoxin M_1 formation is mediated by the cytochrome *P*-448 (P_1-450)-linked oxygenase system, as its induction cosegregated with Ah responsiveness in over 700 mice of different strains, recombinant inbred lines, and offsprings of selected matings (17). On the other hand, cytochrome *P*-450-linked oxygenase system is involved in the preferential metabolism of aflatoxin B_1 to the 2,3-oxide (17). These and subsequent studies (18) suggest that the genetic regulation of the induction of aryl hydrocarbon hydroxylase and the metabolism of aflatoxin B_1 to aflatoxin M_1 occurs at the regulatory gene level rather than at the structural gene level. Since genetic regulation of Ah responsiveness has been elucidated with the use of TCDD as an inducer, we have employed TCDD as a probe to understand the genetic regulation of aflatoxin B_1 metabolism via various pathways, e.g., formation of aflatoxin B_1-2,3-oxide and aflatoxins M_1 and Q_1. In addition, we have also compared the effects of phenobarbital and 3-methylcholanthrene pretreatment on various pathways of aflatoxin B_1 metabolism in eight inbred strains of mice.

Aflatoxin B_2, another metabolite of *A. flavus*, is structurally identical to aflatoxin B_1 except for the absence of the C2–C3 double bond (Fig. 1b). Aflatoxin B_2 exhibits very weak metabolism-mediated binding to DNA and is also very weakly carcinogenic and mutagenic (15, 16, 19, 20). However, aflatoxin B_2 is metabolized by the hepatic microsomal mixed function oxygenase to aflatoxin M_2 (21), a metabolite analogous to aflatoxin M_1 (Fig. 1b). Since the absence of the C2–C3 double bond considerably affects the activation, detoxification, and biological activity of aflatoxins (15, 16, 19, 20), it was of interest to evaluate its effect on the genetic regulation of aflatoxin metabolism. Therefore, in the present paper we also report on strain differences in mice in the induction of the metabolism of aflatoxin B_2 to aflatoxin M_2, as well as the dose response of this induction, elicited by TCDD in C57BL/6 and DBA/2 strains of mice.

MATERIALS AND METHODS

Chemicals and animals. Tritium-labeled aflatoxins B_1 and B_2, obtained from Moravek Biochemicals, City of Industry, California, were of greater than 97% purity as determined by thin-layer chromatography. However, prior to use, the labeled material was rechromatographed and diluted to the desired specific activity with the unlabeled aflatoxin obtained from Calbiochem, San Diego, California. The prepared material was dissolved in dimethylsulfoxide and stored in aliquots at −70°C. Other chemicals were obtained as follows: 3-methylcholanthrene, NADP, DL-isocitrate, and isocitrate dehydrogenase (type IV) from Sigma Chemical Company, St. Louis, Missouri; thin-layer chromatograms, made up of silica gel (0.25-mm thickness) on glass plates, from Merck (EM Laboratories), Elmsford, New York; TCDD, aflatoxins M_1 and Q_1, and 3-hydroxybenzo[*a*]pyrene were generous gifts from Dr. Alan Poland, McArdle Laboratories, University of Wisconsin, Madison, Dr. G. N. Wogan, Masschusetts Institute of Technology, Cambridge, and Dr. Harry Gelboin of the National Cancer Institute, Bethesda, Maryland, respectively.

Mice were obtained either from the Jackson Laboratory, Bar Harbor, Maine, or from the animal breeding facilities of this institute.

Treatment with inducers. Mice of eight inbred strains were treated with either phenobarbital or 3-methylcholanthrene, whereas TCDD treatment in different doses was administered to the mice of only two strains (C57BL/6 Jacobs and DBA/2J). Control mice received either corn oil which was the vehicle for 3-methylcholanthrene or *p*-dioxane which was the vehicle for TCDD. TCDD in different doses, ranging from 0.15 to 15 μg/kg body wt, and 3-methylcholanthrene in a single dose of 1.5 mg/mouse were given intraperitoneally 48 h before sacrifice. Phenobarbital was given in drinking water at a dose of 0.8 mg/ml for 7 days prior to sacrifice. Mice were caged in groups of six per cage and precautions were taken to prevent exposure to any known pesticides. No significant differences in activity were observed between oil-treated and untreated controls (18). Female mice ranging in age from 6 to 8 weeks were used in the present studies. No sex differences have been previously observed (22) in the metabolism of aflatoxin B_1 by hepatic microsomes from C57BL/6 mice.

Preparation of microsomes. For studies on strain differences in induction by phenobarbital and 3-methylcholanthrene of the metabolism of aflatoxin B_1 to aflatoxin

[1] Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PB, phenobarbital; MC, 3-methylcholanthrene.

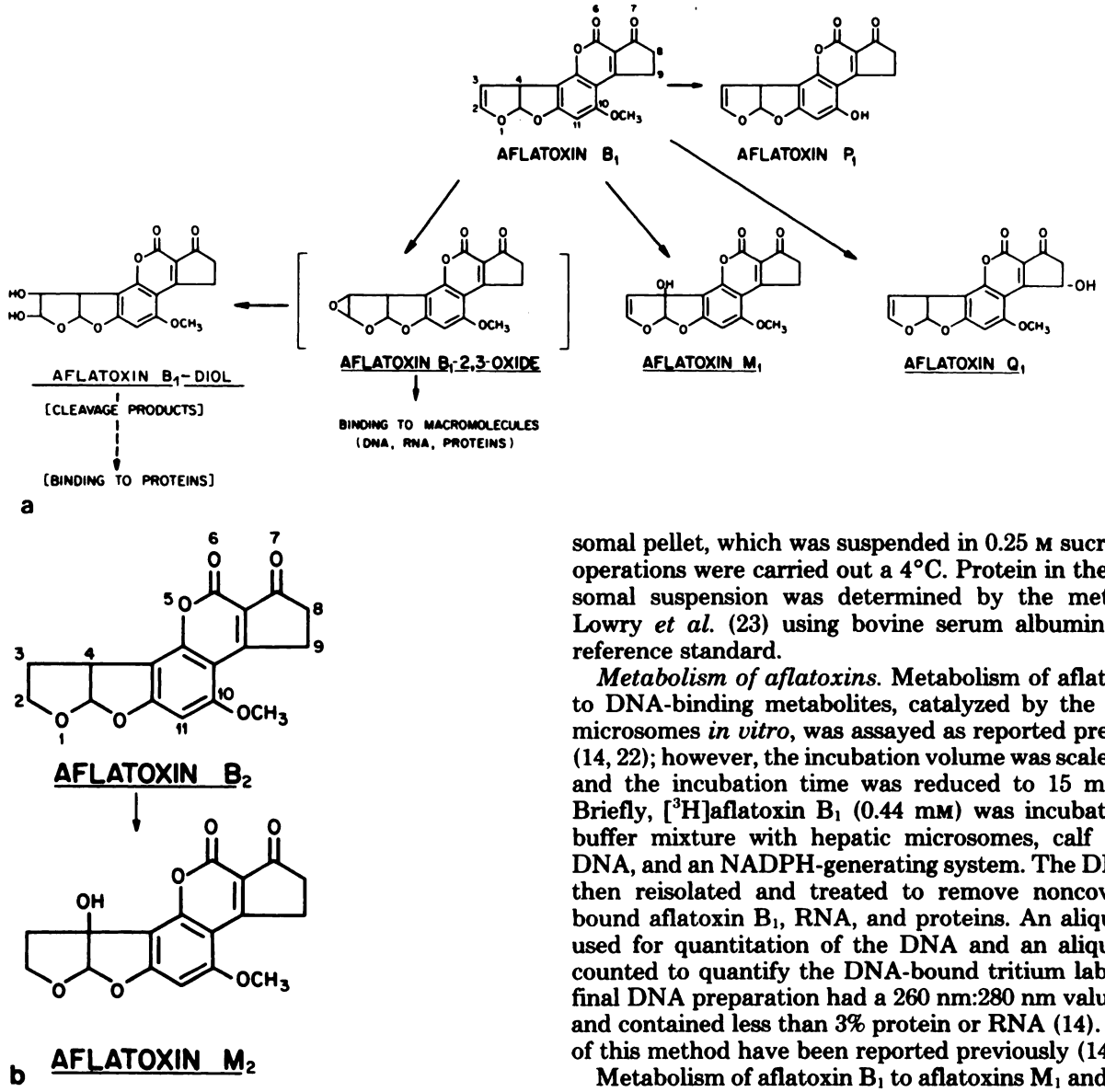


FIG. 1. *Hepatic microsomal mixed function oxygenase-mediated metabolism of aflatoxin B_1 to aflatoxins B_1-2,3-oxide, M_1, Q_1, and P_1 (A), and of aflatoxin B_2 to aflatoxin M_2 (B)*

B_1-2,3-oxide and to aflatoxins M_1 and Q_1, four groups of mice with two or three mice per group were used for each treatment and the livers of the mice of each group were pooled. For studies on TCDD, each dose of TCDD was administered to five mice which were assayed individually. In addition, in the TCDD experiments, mice treated with 3-methylcholanthrene or *p*-dioxane were used, respectively, as positive and negative controls. Animals received food and water *ad libitum* until the day of sacrifice and were maintained under identical conditions. Animals were killed by cervical dislocation, and the liver was excised from each animal, cut into several pieces, rinsed with cold 0.85% sodium chloride solution, and homogenized (w/w, 1:8) in ice-cold sucrose (0.25 M) solution. The homogenate was centrifuged at 15,000*g* for 15 min and the resulting supernatant was collected and centrifuged at 105,000*g* for 90 min to obtain the microsomal pellet, which was suspended in 0.25 M sucrose. All operations were carried out a 4°C. Protein in the microsomal suspension was determined by the method of Lowry *et al.* (23) using bovine serum albumin as the reference standard.

Metabolism of aflatoxins. Metabolism of aflatoxin B_1 to DNA-binding metabolites, catalyzed by the hepatic microsomes *in vitro*, was assayed as reported previously (14, 22); however, the incubation volume was scaled down and the incubation time was reduced to 15 min (18). Briefly, [^{3}H]aflatoxin B_1 (0.44 mM) was incubated in a buffer mixture with hepatic microsomes, calf thymus DNA, and an NADPH-generating system. The DNA was then reisolated and treated to remove noncovalently bound aflatoxin B_1, RNA, and proteins. An aliquot was used for quantitation of the DNA and an aliquot was counted to quantify the DNA-bound tritium label. The final DNA preparation had a 260 nm:280 nm value of 1.9 and contained less than 3% protein or RNA (14). Details of this method have been reported previously (14).

Metabolism of aflatoxin B_1 to aflatoxins M_1 and Q_1 was carried out as described elsewhere (24). A similar procedure was used to assay the metabolism of aflatoxin B_2 to aflatoxin M_2. Briefly, [^{3}H]aflatoxin B_1 (0.45 mM) or B_2 (0.25 mM) was incubated with hepatic microsomes and an NADPH-generating system for 15 min. The reaction was terminated and the mixture was extracted with chloroform, an aliquot of which after suitable preparation was analyzed by thin-layer chromatography, using silica gel thin-layer chromatograms and chloroform:isopropanol (95:5) as the developing solvent. The plates were viewed under uv light and fluorescent bands corresponding to authentic samples of aflatoxins M_1 and Q_1 were scraped and counted. No authentic sample of aflatoxin M_2 was available; therefore, tentative identification of aflatoxin M_2 is based on its fluorescence characteristics as well as its mobility on thin-layer chromatograms relative to aflatoxin M_1 (21). One unit of activity represents the amount of microsomal protein catalyzing the formation from aflatoxin B_1 or B_2 of 1 pmol of the metabolite/min.

Although aflatoxins B_1 and B_2 are metabolized via

various pathways involving oxidation, reduction, and conjugation reactions, for convenience, in this paper, the metabolism of aflatoxin B_1 to aflatoxin B_1-2,3-oxide, and to aflatoxins M_1 and Q_1 is designated as aflatoxin B_1-2,3-oxygenase, aflatoxin B_1-4-hydroxylase, and aflatoxin B_1-9-hydroxylase activity, respectively. For the sake of consistency, metabolism of aflatoxin B_2 to aflatoxin M_2 is designated as aflatoxin B_2-4-hydroxylase activity.

Aryl hydrocarbon hydroxylase. Hepatic microsomal metabolism of benzo[*a*]pyrene was measured by the fluorometric method reported previously from another laboratory (25). Activity is expressed in units per milligram of microsomal protein; 1 unit of activity represents the amount of microsomal protein catalyzing the formation from benzo[*a*]pyrene, in 1 min, of the phenolic metabolites having fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[*a*]pyrene.

RESULTS

Effects of phenobarbital and 3-methylcholanthrene pretreatment on the metabolism of aflatoxin B_1. The effects of phenobarbital and 3-methylcholanthrene on aflatoxin B_1-2,3-oxygenase, aflatoxin B_1-4-hydroxylase, and aflatoxin B_1-9-hydroxylase activities are shown in Table 1 and the corresponding inducibility ratios are shown in Table 2.

Among the strains of mice tested, C57BL/6, BALB/cCR, CBA/J, and C57L/J are Ah responsive with aryl hydrocarbon hydroxylase inducibility ratio ranging from 3.5 to 5.2, whereas DBA/2 HaD, AKR/Sn, RF/J, and 129/J are Ah nonresponsive strains with aryl hydrocarbon hydroxylase inducibility ratio ranging from 0.9 to 1.5 (Table 2). These results on the Ah responsiveness of these strains are consistent with published reports (26).

As shown in Tables 1 and 2, phenobarbital caused induction of aflatoxin B_1-2,3-oxygenase in all the eight strains, increasing the activity 2.4- to 6.2-fold. On the other hand, 3-methylcholanthrene decreased the activity 30 to 50% in five strains (C57BL/6, BALB/cCr, CBA/J, CBA/J, C57L/J, and DBA/2HaD) but it had no effect (AKR/Sn) or slightly increased the activity (RF/J and 129/J) in other strains. These data are consistent with previously reported (17) results on C57BL/6J, C57L/J, DBA/2J, and AKR/J. The additional data provided here for DBA/2HaD, AKR/Sn, and especially for BALB/cCr, CBA/J, RF/J, and 129/J, further confirm that aflatoxin B_1-2,3-oxygenase activity in various strains of mice is either depressed or not affected by 3-methylcholanthrene pretreatment. This depression is independent of Ah responsiveness, as aflatoxin B_1-2,3-oxygenase activity was equally (30–40%) depressed in Ah nonresponsive (DBA/2J or DBA/2 HaD) and Ah responsive C57BL/6J strains (17).

Phenobarbital pretreatment increased aflatoxin B_1-9-hydroxylase activity 1.8- to 4.6-fold in Ah responsive and Ah nonresponsive strains, the only exception being the C57L/J strain. On the other hand, 3-methylcholanthrene depressed this activity 60–75% in all four Ah responsive strains. In Ah nonresponsive strains 3-methylcholanthrene depressed the activity about 30 and 8% in DBA/2HaD and RF/J, respectively, but increased the activity 44 and 39% respectively, in AKR/Sn and 129/J. Clearly, the effect of 3-methylcholanthrene was more specific in that it depressed aflatoxin B_1-9-hydroxylase activity in Ah responsive strains and less so or not at all in Ah nonresponsive strains.

Compared with the effects of phenobarbital on aflatoxin B_1-2,3-oxygenase and on aflatoxin B_1-9-hydroxylase activity, phenobarbital produced a mixed effect on aflatoxin B_1-4-hydroxylase activity in Ah responsive and nonresponsive strains, ranging from mild depression (20%) to mild enhancement (47%). In contrast to this, 3-methylcholanthrene either caused induction (2.5 to 3.6 fold) or had essentially no effect on aflatoxin B_1-4-hydroxylase activity. The interesting observation, consistent with the earlier report (17), was that all four Ah

TABLE 1

Effect of phenobarbital (PB) and 3-methylcholanthrene (MC) pretreatment on the metabolism of Aflatoxin B_1 and Aflatoxin B_2 by hepatic microsomes from various inbred strains of mice

In addition to control groups, four groups of mice of each strain were pretreated with either phenobarbital or 3-methylcholanthrene. Each group comprised two or three mice. Livers from each group were pooled and processed for the isolation of microsomes by differential centrifugation. The hepatic microsomes were assayed for the metabolism of aflatoxin B_1 to aflatoxin B_1-2,3,-oxide and to aflatoxins Q_1 and M_1 and of aflatoxin B_2 to aflatoxin M_2. Details have been described in the text.

Strain of mice	Aflatoxin B_1-2,3-oxygenase[a, b]			Aflatoxin B_1-9-hydroxylase (Q_1)			Aflatoxin B_1-4-hydroxylase(M_1)			Aflatoxin B_2-4-hydroxylase(M_2)[c]		
	Cont.	PB	MC	Cont.	PB	MC	Cont.	PB	MC	Cont.	PB	MC
	Activity: pmol metabolite formed/min/mg microsomal protein											
C57BL/ 6 Jacobs	418	1757	276	221 ± 45	448 ± 60	74 ± 30	673 ± 155	530 ± 100	2390 ± 309	225	231	850
BALB/cCr	463	2084	239	149 ± 31	685 ± 41	47 ± 16	575 ± 103	671 ± 76	2075 ± 90	195	350	944
CBA/J	697	2079	343	267 ± 37	591 ± 37	113 ± 19	410 ± 106	606 ± 112	1021 ± 172	169	526	583
C57L/J	396	2470	282	340 ± 42	389 ± 8	84 ± 42	634 ± 166	617 ± 135	2362 ± 394	360	1096	1123
DBA/2HaD	570	2239	391	262 ± 67	621 ± 70	168 ± 42	518 ± 161	766 ± 166	472 ± 94	296	607	255
AKR/Sn	749	1792	739	251 ± 55	532 ± 40	364 ± 72	514 ± 118	719 ± 135	884 ± 197	206	337	290
RF/J	668	2486	762	169 ± 63	299 ± 24	156 ± 31	325 ± 84	461 ± 79	468 ± 134	104	287	101
129/J	415	2133	500	234 ± 84	512 ± 113	327 ± 91	556 ± 163	694 ± 270	785 ± 121	Not done		

[a] Each value is the mean of two determinations on two different groups of mice. Although each incubation contained 1 mg microsomal protein and 2 mg calf thymus DNA (18), activity calculations are based on the amount of AFB_1 metabolite trapped by 1 mg DNA.

[b] Cont., PB, and MC denote hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated mice, respectively.

[c] Each value is the mean of two determinations on two different groups of mice.

TABLE 2

Comparison of the induction of the metabolism of aflatoxin B_1 to aflatoxins M_1, Q_1, and B_1 -2,3-oxide, and of aflatoxin B_2 to aflatoxin M_2 with Ah responsiveness of the inbred strains of mice

The data of Table 1 were used to obtain the respective inducibility ratios. In these calculations mean activities of hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats were used.

Strain of mice	Aflatoxin B_1-2,3-oxygenase		Aflatoxin B_1-9-hydroxylase (Q1)		Aflatoxin B_1-4-hydroxylase (M_1)		Aflatoxin B_2-4-hydroxylase (M_2)		AHH inducibility ratio
	PB/cont.	MC/cont.	PB/cont.	MC/cont.	PB/cont.	MC/cont.	PB/cont.	MC/cont.	
C57BL/6 Jacobs	4.20	0.66	2.00	0.33	0.80	3.50	1.03	3.77	5.2
BALB/cC-r	4.50	0.52	4.60	0.31	1.17	3.60	1.79	4.84	3.5
CBA/J	2.98	0.49	2.20	0.42	1.47	2.50	3.10	3.45	4.2
C57L/J	6.24	0.71	1.14	0.25	0.97	3.70	3.04	3.12	3.6
DBA/2HaD	3.93	0.68	2.37	0.66	1.47	0.91	2.05	0.86	0.9
AKR/Sn	2.39	0.98	2.12	1.44	1.40	1.69	1.63	1.40	1.3
RF/J	3.72	1.14	1.77	0.92	1.42	1.43	2.75	0.97	1.5
129/J	5.14	1.20	2.19	1.39	1.24	1.40	NOT DONE		1.0

responsive strains (C57BL/6, BALB/cCr, CBA/J, and C57L/J) were responsive to the induction of aflatoxin B_1-4-hydroxylase, whereas Ah nonresponsive strains (DBA/2HaD, AKR/Sn, RF/J, 129/J) were essentially nonresponsive to the induction of aflatoxin B_1-4-hydroxylase by 3-methylcholanthrene.

Effects of phenobarbital and 3-methylcholanthrene pretreatment on the metabolism of aflatoxin B_2. In Table 1 are shown the effects of phenobarbital and 3-methylcholanthrene pretreatment on the metabolism of aflatoxin B_2 to aflatoxin M_2. While phenobarbital was without effect in the C57BL/6 strain, it caused a 1.6- to 3.0-fold induction of aflatoxin B_2-4-hydroxylase in six other strains of mice. On the other hand, 3-methylcholanthrene caused a 3.5- to 4.8-fold induction of aflatoxin B_2-4-hydroxylase in Ah responsive strains but only a 0.9- to 1.5-fold induction in Ah nonresponsive strains. The correlations of aflatoxin B_1-4-hydroxylase versus aflatoxin B_2-4-hydroxylase responsiveness ($r = 0.91$) to 3-methylcholanthrene and that of aflatoxin B_2-4-hydroxylase responsiveness versus Ah responsiveness ($r = 0.86$) were high.

Effect of TCDD on the metabolism of aflatoxin B_1. The effects of TCDD on the induction of aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase activities in DBA/2 and C57BL/6 are shown in Fig. 2. For comparison log dose–response curves for the induction of aryl hydrocarbon hydroxylase by TCDD are also included. In DBA/2 (Fig. 2a), an Ah nonresponsive strain, aflatoxin B_1-9-hydroxylase activity was induced an average of about 25% at the lowest dose of 0.15 μg/kg body wt, but at higher doses the activity decreased, falling below the control level at the highest dose of 15 μg/kg body wt. Aflatoxin B_1-2,3-oxygenase activity also exhibited dose dependence, except that the average activity at 0.15 μg/kg was not different from the control activity, but at 1.5 μg/kg it was about 20% higher and fell below the control level at the highest dose of 15 μg/kg. In the C57BL/6 strain (Fig. 2b), aflatoxin B_1-9-hydroxylase activity increased slightly at 0.15 μg/kg dose and fell rapidly as TCDD dose increased, reaching less than 50% of the control level at the highest dose of 15 μg/kg. On the other hand, at the lowest dose (0.15 μg/kg) aflatoxin B_1-2,3-oxygenase activity increased an average of about 20% and stayed at the same level at higher doses of TCDD.

Aryl hydrocarbon hydroxylase activity was inducible severalfold in both strains of mice, except for the differences in dose-response curves. While in C57BL/6 aryl hydrocarbon hydroxylase activity increased rapidly with the increasing dose of TCDD, reaching a plateau level at the 1.5 μg/kg dose, higher doses of TCDD were required

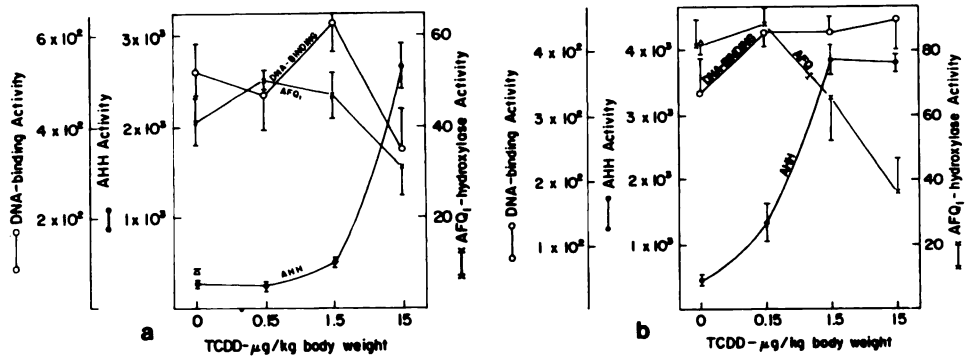


FIG. 2. *Effects of TCDD on aryl hydrocarbon hydroxylase activity and the metabolism of aflatoxin B_1 to aflatoxin B_1-2,3-oxide (DNA-binding metabolite) and to aflatoxin Q_1 catalyzed by hepatic microsomes of DBA/2J (a) and C57BL/6 Jacobs (b) strains of mice*

The livers from control and TCDD-treated mice were removed 48 h later for the isolation of hepatic microsomes which were assayed for aryl hydrocarbon hydroxylase activity, and for the formation of aflatoxin B_1-2,3-oxide (DNA-binding) and aflatoxin Q_1. Each dose treatment was applied to five mice and microsomes from each mouse were assayed individually. Bars represent mean ± SE. For the definition of DNA-binding activity see footnote *a* in Table 1.

to evoke the same degree of induction in DBA/2, i.e., while the 1.5 μg/kg dose produced optimal induction in C57BL/6, a similar degree of induction in DBA/2 was achieved with a 10-fold higher dose of 15 μg/kg. These results on aryl hydrocarbon hydroxylase are similar to those reported previously by other investigators (2).

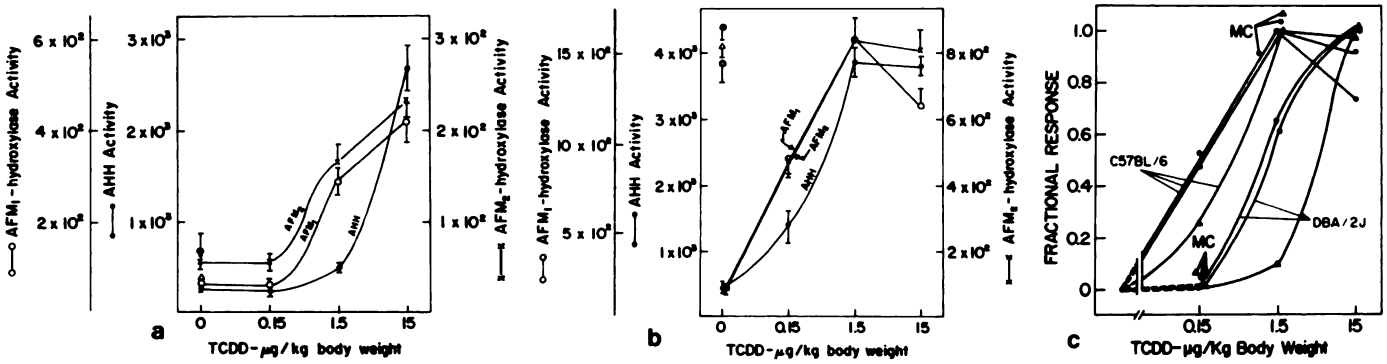
In Fig. 3 are compared the dose–response curves for the induction of aryl hydrocarbon hydroxylase, aflatoxin B_1-4-hydroxylase, and aflatoxin B_2-4-hydroxylases in DBA/2 (Fig. 3a) and C57BL/6 (Fig. 3b) strains. Although these dose–response curves in each strain are comparable, the shapes of the aflatoxin B_1-4- and aflatoxin B_2-4-hydroxylase curves are considerably different from those of aryl hydrocarbon hydroxylase.

To eliminate strain differences in basal enzyme activity, maximally induced activity and other variables related to pharmacokinetic parameters for TCDD, data in Fig. 3 (a and b) have been converted to fractional responses and are presented in Fig. 3c. This conversion was accomplished by equating the mean control activity to zero and the mean maximally TCDD-induced activity to 1.0 and the fractional response of other values for each strain was calculated. For comparison, values for each activity obtained in 3-methylcholanthrene-treated mice are also indicated. From these plots, the dose of TCDD (ED_{50}) which elicits 50% of the maximal induction of each activity in each strain was calculated. In the Ah responsive strain C57BL/6, the ED_{50} for aflatoxin B_1-4- and aflatoxin B_2-4-hydroxylase(s) is about 0.14 μg/kg body wt, whereas it is about ninefold higher, i.e., 1.30 μg/kg, in the Ah nonresponsive strain DBA/2. Similarly, the ED_{50} for aryl hydrocarbon hydroxylase in the C57BL/6 is about 0.7 μg/kg compared to 7.8 μg/kg in the DBA/2 strain. The latter values for aryl hydrocarbon hydroxylase are similar to those reported previously by others (2).

In an earlier report (17), 14 strains including C57BL/6J and DBA/2J were classified as responsive or nonresponsive to the induction of aflatoxin B_1-4-hydroxylase by 3-methylcholanthrene. Present data, as demonstrated with C57BL/6 and DBA/2, suggest that it should be possible to classify mice strains as responsive or nonresponsive on the basis of their response to 0.14 μg/kg body wt of TCDD. Responsive strains like C57BL/6, most likely, will elicit induction of aflatoxin B_1-4-hydroxylase activity when pretreated with this dose of TCDD, whereas nonresponsive mice like DBA/2 may require a 10-fold higher dose. The present data also demonstrate strain differences in the induction of aflatoxin B_2-4-hydroxylase by either 3-methylcholanthrene (Table 2) or TCDD (Fig. 3).

DISCUSSION

In the present report we have compared induction in eight inbred strains by phenobarbital and 3-methylcholanthrene of the metabolism of aflatoxin B_1 to aflatoxin B_1-2,3-oxide and to aflatoxins M_1 and Q_1, as well as the metabolism of aflatoxin B_2 to aflatoxin M_2 in seven of these strains. While phenobarbital induced (2.4- to 6.2-fold) aflatoxin B_1-2,3-oxygenase activity in all strains, it either failed to induce or induced slightly aflatoxin B_1-4-hydroxylase activity (0.8- to 1.5-fold). On the other hand, aflatoxin B_1-9-hydroxylase was induced (1.8- to 4.6-fold) in all strains, except in C57L/J. These data clearly suggest that aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase, but not aflatoxin B_1-4-hydroxylase, activities are preferentially catalyzed by cytochrome *P*-450. These results and the correlation coefficients obtained for the phenobarbital induction of aflatoxin B_1-2,3-oxygenase versus aflatoxin B_1-9 hydroxylase ($r = -0.13$), aflatoxin B_1-2,3-oxygenase versus aflatoxin B_1-4-hydroxylase ($r = -0.61$), and aflatoxin B_1-9-hydroxylase versus aflatoxin B_1-4-hydroxylase ($r = 0.09$) suggest that at least three enzyme forms of the cytochrome *P*-450 system are involved in the metabolism of aflatoxin B_1; two of these, i.e., aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase, are inducible by phenobarbital and the third one, i.e., aflatoxin B_1-4-hydroxylase, is not preferentially induced by phenobarbital. Our recent studies on the *in vitro* inhibition of these activities in rat hepatic microsomes are in agreement with these data and indicate that



FIGS 3a,b. *Effects of TCDD on aryl hydrocarbon hydroxylase activity and the metabolism of aflatoxins B_1 and B_2 to aflatoxins M_1 and M_2, respectively, catalyzed by hepatic microsomes of DBA/2J (a) and C57BL/6 Jacobs (b) strains of mice*

Experimental details have been described in the text and in the legend of Fig. 2.

FIG. 3C. *Fractional response of the induction of aflatoxin M_1/M_2 hydroxylase(s) and aryl hydrocarbon hydroxylase by TCDD*

Data of Fig. 2 were used to construct this figure. This was accomplished by equating the mean control activity to zero and the mean maximally TCDD-induced activity to 1.0 and the fractional response of other values for each strain was calculated. For comparison, values for each activity obtained in 3-methylcholanthrene (MC)-treated mice are also included, and the symbols representing these values have been placed arbitrarily with respect to the abscissa. ○, Aflatoxin M_1 hydroxylase; ●, aflatoxin M_2 hydroxylase; and ▲, aryl hydrocarbon hydroxylase.

while phenobarbital induces both aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase, the enzyme forms responsible for these activities are different (18).

The correlations obtained for 3-methylcholanthrene induction clearly dissociate aflatoxin B_1-4-hydroxylase from aflatoxin B_1-2,3-oxygenase ($r = -0.60$) and from aflatoxin B_1-9-hydroxylase ($r = -0.74$). On the other hand, an excellent correlation ($r = 0.87$) was obtained when aflatoxin B_1-4-hydroxylase induction was correlated to aryl hydrocarbon hydroxylase induction. The correlations for 3-methylcholanthrene induction of aflatoxin B_1-4-hydroxylase versus aflatoxin B_2-4-hydroxylase ($r = 0.91$) and that for aflatoxin B_2-4-hydroxylase versus aryl hydrocarbon hydroxylase ($r = 0.86$) were also excellent. These data suggest that aflatoxin B_1-4- and B_2-4-hydroxylase(s) and aryl hydrocarbon hydroxylase are under the control of the same genetic factors. These data are in agreement with the previous report (17) in that Ah responsive strains are also responsive for the induction of aflatoxin B_1-4-hydroxylase by 3-methylcholanthrene, whereas Ah nonresponsive strains are nonresponsive for this induction. Present data have also demonstrated this type of response for aflatoxin B_2-4-hydroxylase.

In the present study, we have also demonstrated that while TCDD caused a dose-dependent increase in aryl hydrocarbon hydroxylase activity in both the Ah responsive mouse strain C57BL/6 and the Ah nonresponsive mouse strain DBA/2, it failed to produce a similar effect on aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase activities in either of these strains. On the contrary, it produced a slight elevation of these activities at low doses and inhibition as the TCDD dose increased. These data further support different enzyme identity and/or regulation of aryl hydrocarbon hydroxylase from the other two activities of aflatoxin B_1 metabolism. The dissimilar characteristics of TCDD dose–response curves for aflatoxin B_1-2,3-oxygenase and aflatoxin B_1-9-hydroxylase in C57BL/6 and DBA/2 further suggest, in agreement with the data on mouse strains, that these two activities either are catalyzed by different enzyme forms of cytochrome *P*-450 or are under different regulatory controls.

In comparison to the effect on aflatoxin B_1-9 hydroxylase and aflatoxin B_1-2,3-oxygenase, but similar to the effect on aryl hydrocarbon hydroxylase, TCDD produced a dose-dependent increase in aflatoxin B_1-4- and B_2-4-hydroxylase activities in both C57BL/6 and DBA/2. However, the profiles of the dose–response curves, while identical for aflatoxin B_1-4- and B_2-4-hydroxylase(s), were different for aflatoxin B_1-4-/B_2-4-hydroxylase(s) and aryl hydrocarbon hydroxylase in both C57BL/6 and DBA/2 strains. These data are consistent with the suggestion derived from studies on mouse strains (17) that aflatoxin B_1-4-hydroxylase and aryl hydrocarbon hydroxylase are probably different enzymes. This interpretation is supported by the recent report (11) in which evidence was provided for the existence of multiple forms of 3-methylcholanthrene-induced cytochrome P-450. The identical characteristics of dose–response curves for aflatoxin B_1-4- and aflatoxin B_2-4-hydroxylases strongly suggest that the two activities are the function of the same genetic factors. Although producing induction of aflatoxin B_1-4-/B_2-4-hydroxylase in C57BL/6 and DBA/2, TCDD was less effective in DBA/2 than in C57BL/6, as the dose–response curve for DBA/2 was shifted to the right and on the average a nine times higher dose of TCDD was required to produce the same degree of induction as that produced in C57BL/6. This difference was clearly reflected in a ninefold difference in ED_{50} values.

Now what is the evidence that suggests that aflatoxin B_1-4-hydroxylase and aryl hydrocarbon hydroxylase activities are catalyzed by different enzyme forms, most likely different cytochrome *P*-450s? The evidence includes: (a) trimodal distribution when aflatoxin B_1-4-hydroxylase activity of induced and uninduced mice of several BXD and AKXL recombinant inbred strains is plotted against the corresponding aryl hydrocarbon hydroxylase activity, as against the bimodal distribution when induction ratios alone were plotted (17); (b) differences in *in vitro* inhibition and/or stimulation by α- and β-naphthoflavones and acetone of aflatoxin B_1-4-hydroxylase and aryl hydrocarbon hydroxylase in hepatic microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats (18); and (c) dissimilar characteristics of TCDD dose–response curves for aflatoxin B_1-4-hydroxylase and aryl hydrocarbon hydroxylase induction in C57BL/6 and DBA/2 strains. These differences between the two activities also suggest that the strong association demonstrated previously (17) and here between Ah responsiveness and aflatoxin B_1-4-hydroxylase induction is not occurring at the structural gene level but is most likely operational at the regulatory gene level. Indeed, TCDD induction of aryl hydrocarbon hydroxylase in Ah responsive and nonresponsive strains is believed to be mediated via an interaction with a specific cytosolic receptor (9–11). Experimental evidence suggests that TCDD occupies the same sites on the receptor as occupied by polycyclic aromatic hydrocarbons, e.g., 3-methylcholanthrene (27). Higher potency of TCDD, compared to 3-methylcholanthrene, has been attributed to a higher affinity of the receptor for TCDD. A similar explanation has been invoked to explain the higher sensitivity of the Ah responsive strains, e.g., C57BL/6J, to TCDD induction of aryl hydrocarbon hydroxylase than the Ah nonresponsive strains, e.g., DBA/2J. Similar receptor mutation most likely explains the lower sensitivity of the DBA/2 strain, compared to C57BL/6, to the induction of aflatoxin B_1-4-/B_2-4-hydroxylases by TCDD. Poland *et al.* (28) have reported that following intraperitoneal administration of [^{14}C]TCDD, hepatic accumulation of the radiolabel was greatest in C57BL/6J mice, intermediate in hybrid B6D2F_1/J mice, and least in DBA/2J mice, a pattern which paralleled the sensitivity of these strains to aryl hydrocarbon hydroxylase induction by TCDD (C57BL/6J>B6D2F_1/J>DBA/2J). Recent reports (9–11) suggests that TCDD interacts with the cytosolic receptor and the "complex" then is translocated to the nucleus, and this phenomenon represents an early step in the sequence of events leading to the increased synthesis of cytochrome P_1-450 (*P*-448).

Based on the rate of hepatic uptake of [^{14}C]TCDD, the number of cells per gram of liver (29), and the ED_{50} value

of TCDD for the induction of aryl hydrocarbon hydroxylase (27), it has been estimated that at the ED_{50} dose there are 2.5×10^4 molecules of TCDD per cell (27). Similar calculations with cell cultures have suggested that aryl hydrocarbon hydroxylase is half-maximally induced when 2.1×10^5 molecules per H4-IIE cell are present. Because of this sensitivity it has been recommended that aryl hydrocarbon hydroxylase induction be used as a bioassay for detecting small amounts of TCDD (30). Since aflatoxin B_1-4-hydroxylase is about five times more sensitive to induction by TCDD than aryl hydrocarbon hydroxylase (ED_{50}: 0.14 versus 0.70 μg/kg), the sensitivity of the bioassay for TCDD could be improved fivefold by substituting aflatoxin B_1 for benzo[a]pyrene as the substrate.

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