Biochemical Pharmacology, Vol. 28, pp. 3441–3449.

EFFECTS OF INDUCERS AND INHIBITORS ON THE METABOLISM OF AFLATOXIN B_1 BY RAT AND MOUSE*

HIRA L. GURTOO and RONALD P. DAHMS

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY 14263, U.S.A.

(*Received* 13 *September* 1978; *accepted* 9 *May* 1979)

Abstract—The microsomal metabolism of aflatoxin B_1 (AFB_1) via various pathways and the induction and inhibition specificities of these pathways were examined in Sprague–Dawley rats, inbred strains of mice, and recombinant inbred lines derived from AKR/J and C57L/J cross. The data suggests that the metabolism of aflatoxin B_1 is catalyzed by at least three different enzymes of the microsomal mixed function oxygenase system: one that mediates conversion to aflatoxin M_1 (AFM_1-hydroxylase) is cytochrome P-448-linked and is associated with the Ah locus at the level of regulatory genetic factors: the other two enzymes which are cytochrome P-450-linked, mediate the metabolism of aflatoxin B_1 to aflatoxin Q_1 (AFQ_1-hydroxylase) and to AFB_1-2,3-oxide (metabolic activation). Although AFQ_1-hydroxylase and metabolic activation, measured *in vitro* as the formation of DNA-alkylating metabolite(s), are not clearly distinguishable on the basis of induction and inhibition responses, the two activities can be distinguished on the basis of their kinetic parameters and genetic regulation: (a) the apparent K_m of the metabolic activation pathway is almost three orders of magnitude (19.7×10^{-4} M) higher than that of AFQ_1-hydroxylase (0.7×10^{-4}), [B. D. Roebuck and G. N. Wogan, *Cancer Res.* **37**, 1649 (1977)]; and (b) while both activities are induced by phenobarbital (PB) and depressed by 3-methylcholanthrene (3-MC), unlike the metabolic activation, differences in the 3-MC-induced depression of AFQ_1-hydroxylase activity were noted in Ah responsive and nonresponsive strains. Kinetic studies revealed that metabolic activation of AFB_1 is linear with the time of incubation up to 20 min, with up to 2 mg of microsomal protein in the incubation, and has a pH optimum of 7 to 7.4. While PB pretreatment, relative to control, enhanced the apparent V_{max} 3-fold, 3-MC depressed it about 60 per cent; however, the apparent K_m values of the three microsomal preparations were of the same order of magnitude (1.3 to 2×10^{-3} M). Under the conditions of the incubation, the minimum number of binding sites in DNA was estimated to correspond to a resultant specific activity of 8–9 nmoles AFB_1-metabolite bound/μmole DNA-P.

Microsomal mixed function oxygenase (MFO), an inducible enzyme complex responsible for the metabolism of more than 300 chemicals including drugs, carcinogens, steroids, insecticides and other chemicals, is present principally in the liver, but is also found in other mammalian tissues [1–7]. The substrate, induction and inhibition specificities of this enzyme complex have revealed at least two inducible forms of the enzyme: one which is inducible by phenobarbital (PB) and catalyzes the metabolism of many chemicals; and another which is inducible by polycyclic aromatic hydrocarbons, e.g. 3-methylcholanthrene (3-MC), and preferentially catalyzes the metabolism of a limited number of substrates such as polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene). β-Diethylaminoethyl diphenylpropylacetate (SKF–525A) and metyrapone preferentially inhibit the phenobarbital-inducible enzyme system, whereas α- and β-naphthoflavones preferentially inhibit the 3-methylcholanthrene-inducible system [1, 8–10]. In addition, purification and gel electrophoresis studies have revealed several forms of cytochrome P-450 [11–14] which are involved in the terminal oxidation step in the metabolism of the substrate. However, the control or phenobarbital-inducible MFO system differs from the 3-methylcholanthrene-inducible systems in various other properties: kinetic [13, 15, 16], spectral [12, 13, 17, 18], metabolite profiles form benzo[*a*]pyrene [19], immunochemical [20, 21] and electron paramagnetic [20] properties.

Aflatoxin B_1 (AFB_1), a mold metabolite produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*, is a potent hepatotoxic and a hepatocarcinogenic agent which contaminates several foods [22, 23] and has been implicated in the etiology of human liver disease [24–26]. Aflatoxin B_1 is also metabolized by the hepatic MFO (Fig. 1), and our recent genetics studies in mice have implicated phenobarbital-inducible cytochrome P-450 and 3-methylcholanthrene-inducible cytochrome P-448 systems in the preferential metabolism of aflatoxin B_1 to aflatoxin B_1-2,3-oxide (metabolic activation) and to aflatoxin M_1 (AFM_1) respectively [27]. In the present report we have studied the induction and inhibition of the metabolism of aflatoxin B_1 via various pathways in rats and mice and have also characterized the metabolic activation of aflatoxin B_1 (measured *in vitro* by the formation of DNA-alkylating metabolites) with respect to its enzymologic properties.

* This work was supported by USPHS Grants CA-13038 from the National Cancer Institute, ES-00993 from the National Institute of Environmental Health Sciences, and BC-303 from the American Cancer Society.

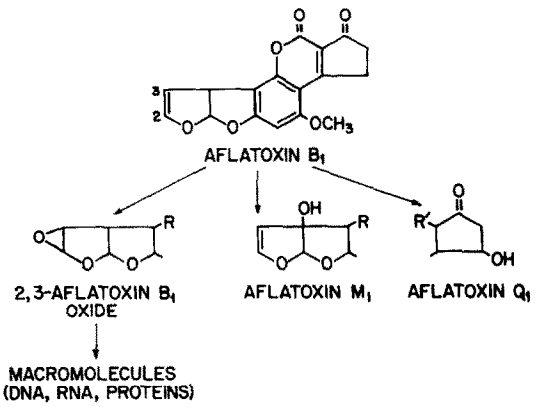

Fig. 1. Some hepatic microsomal mixed function oxygenase-mediated metabolites of aflatoxin B_1. R and R′ are missing portions, respectively, of the aflatoxin B_1 molecule.

MATERIALS AND METHODS

Chemicals. Chromatographically pure aflatoxin B_1 was obtained from CalBiochem (San Diego, CA). Aflatoxin B_1 was tritiated by New England Nuclear (Boston, MA); it was purified by repeated thin-layer chromatography, as reported previously [28], and stored at −70°. Prior to use the labeled material was rechromatographed, diluted to the desired specific activity with unlabeled aflatoxin B_1 in dimethylsulfoxide, and stored in aliquots at −70°. Aflatoxins M_1 and Q_1 (AFQ_1) were a gift of Dr. G. N. Wogan of the Massachusetts Institute of Technology (Cambridge MA). NADP, DL-isocitrate and isocitrate dehydrogenase (type IV) were purchased from the Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography for quantitative studies on the metabolism of aflatoxin B_1 involved the use of 0.25 mm thick silica gel plates obtained from Merck (EM Laboratories, Elmsford, NY). SKF-525A was a gift of the Smith Kline & French Co. (Philadelphia, PA). Benzo[*a*]pyrene, metyrapone, and α- and β-naphthoflavones were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Other reagents were of the highest purity available and the sources have been reported elsewhere [28,29].

Treatment of animals. All animals received food and water *ad lib.* until the day of sacrifice, and were maintained under identical conditions. Male Sprague–Dawley rats (about 200 g) were injected either with PB or 3-MC as follows. 3-MC was dissolved in corn oil and injected intraperitoneally (30 mg/kg) for three consecutive mornings, and the rats were killed on the morning of day 4. The sodium salt of PB was diluted with water and injected for five consecutive mornings; the rats were killed on the morning of day 6. The amounts injected were as follows: first injection, 40 mg/kg; second 48 mg/kg; third, fourth and fifth, 74 mg/kg. Control animals received corn oil only. Our earlier and additional studies have demonstrated no differences in the metabolism of aflatoxin B_1 by microsomes from untreated, saline treated and corn oil-treated rats. This is consistent with the observations of other investigators who have studied other MFO activities [30].

The inducer treatment protocol for the mice of C57BL/6 and DBA/2 strains and AKXL recombinant inbred lines has been described previously [27].

Recombinant inbred lines. These lines, derived from C57L/J × AKR/J (AKXL)* cross, were supplied by Dr. B. A. Taylor of The Jackson Laboratory (Bar Harbor, ME).

In the development of recombinant inbred lines, two highly inbred but unrelated progenitor strains are used to produce F_2 progeny and the recombinant inbred lines are developed and maintained independently by strict brother–sister inbreeding for several subsequent generations [31, 32]. This procedure results in ever increasing chances of homozygosity at different loci, although different loci may be derived from one or the other progenitor strain. In the present studies all mice belonging to various recombinant inbred lines were selected after at least twenty generations of inbreeding since the F_2 generation.

Preparation of microsomes. Microsomes were prepared as described previously [27]. Livers were removed, perfused with cold saline (0.9% NaCl, w/v), and homogenized in 4 vol. of 0.25 M sucrose. The homogenates were centrifuged for 10 min at 10,000 *g*. The resulting supernatant fraction was centrifuged for 60 min at 105,000 *g* in a Beckman L3-50 ultracentrifuge. The microsomal pellets were washed once and finally suspended in 0.25 M sucrose for use in metabolism experiments. Protein concentration was determined as described by Lowry *et al.* [33].

Metabolism of AFB_1 to AFM_1 and AFQ_1. This activity of hepatic microsomes was assayed by the previously reported method [34]. Briefly, in a total volume of 250 μl, hepatic microsomes (about 1.0 mg protein) were incubated in a buffer (pH 7.4) mixture with [^{3}H]aflatoxin B_1 (0.57 mM) and an NADPH-generating system (prewarmed for 15 min at 37°) for 15 min at 37°. At the termination of the incubation the reaction was stopped with 200 μl of cold (−20°) methanol, followed by 1 ml of 0.1 M potassium phosphate buffer, pH 6.0. The mixture was extracted with chloroform and an aliquot of chloroform was evaporated to dryness under nitrogen. The residue was redissolved in ethyl acetate and spotted on a silica gel thin-layer chromatography plate (5 × 20 cm). The plate was developed in a solvent mixture of chloroform–isopropyl alcohol (95:5), dried, and viewed under ultraviolet light; the fluorescent bands corresponding to aflatoxins M_1 and Q_1 were demarcated, scraped and counted by scintillation spectrometry. Activity is expressed in terms of pmoles metabolite formed/mg of microsomal protein/15 min.

Although aflatoxin B_1 is hydroxylated at other sites in the molecule, for convenience, in this paper the metabolism of aflatoxin B_1 to aflatoxin M_1 is designated as AFM_1-hydroxylase, and that to aflatoxin Q_1 is designated as AFQ_1-hydroxylase.

Although in preliminary studies various concentrations of each inhibitor, ranging from 0.1 to 1 mM, were used, detailed studies were carried out with the selected concentrations (SKF-525A and metyra-

* The responsive AKXL-38A recombinant inbred line was separated from the nonresponsive AKXL-38 line after ten generations of brother–sister mating (see Ref. 32).

pone, 1.0 mM; α- and β-naphthoflavones, 0.5 mM) that were able to discriminate various enzyme activities involved in the metabolism of AFB_1 via various pathways. The data reported in the tables are means of at least two determinations.

Metabolism in vitro of AFB_1 to DNA-binding metabolite(s). The composition of the incubation mixture used to demonstrate DNA-binding was as follows: 0.08 M potassium phosphate buffer, pH 7.4; 3.3 mM magnesium chloride; 0.2 mM ethylenediaminetetracetic acid; NADPH-generating system containing 0.81 mM NADP, 17 mM DL-isocitrate, and isocitrate dehydrogenase Sigma type IV (200 μg protein); 2 mg native calf thymus DNA; hepatic microsomes (1 mg protein); and 0.44 mM [^{3}H]aflatoxin B_1 in 25 μl of dimethylsulfoxide (sp. act., 0.5 to 2.5 mCi/mmole). The buffer mixture containing the NADPH-generating system was incubated at 37° for 15 min to ensure the presence of an adequate amount of NADPH. The reaction was started with the addition of [^{3}H]aflatoxin B_1, and, unless stated otherwise, the incubation was carried out for 15 min. The total volume of the incubation mixture was 2.5 ml. At the termination of the incubation, the mixture was extracted two or three times with an equal volume of phenol (550 ml)–*m*-cresol (50 ml)–8-hydroxyquinoline (50 mg) mixture; the aqueous extract was washed several times with diethyl ether. The DNA in the aqueous phase was then precipitated, redissolved, treated with RNase, and subsequently deproteinized by repeated extraction with a chloroform–isoamyl alcohol (19:1) mixture. The final DNA preparation had a 260:280 nm value of 1.9 and contained less than 3 per cent of RNA. Details of this method have been reported previously [29]. Microsomes from phenobarbital-treated rats were used for investigations of the dependence of the metabolic activation of AFB_1 on the period of incubation, on the microsomal protein and DNA concentrations in the incubation, and for the determination of pH optimum. Each data point in Figs. 2–6 is a mean of two determinations.

Several investigators have demonstrated that the DNA-alkylating metabolite of aflatoxin B_1 arises from the oxidation of aflatoxin B_1 at the C_2–C_3 double bond [29, 35–39]. Swenson *et al.* [37, 38] isolated the 2,3-dihydrodiol of aflatoxin B_1 from the hydrolysates of aflatoxin B_1-DNA and aflatoxin B_1-RNA adducts formed *in vitro* as well as *in vivo*, indicating that the metabolite binding to DNA and RNA is, in all probability, aflatoxin B_1-2,3-oxide. Recent studies by Essigman *et al.* [39] and Lin *et al.* [40, 41] have demonstrated that at least 80–90 per cent of aflatoxin B_1 bound covalently to DNA *in vitro* in the microsome-mediated assay could be accounted for as a single component which was released as 2,3-dihydrodiol of aflatoxin B_1 upon hydrolysis of the aflatoxin B_1-DNA adduct. Therefore, microsome-mediated binding of aflatoxin B_1 to DNA essentially represents alkylation by a single metabolite of aflatoxin B_1 and will be designated by the term metabolic activation for the purposes of this paper.

RESULTS

Effects of inducers and inhibitors on the metabolism of AFB_1 to AFM_1 and AFQ_1 by the rat. In Table 1 are shown the effects of PB and 3-MC pretreatment on AFM_1-hydroxylase and AFQ_1-hydroxylase activities of rat liver microsomes. While PB enhanced AFM_1-hydroxylase activity an average of about 60 per cent, 3-MC caused about a 2.3-fold increase in the enzyme activity. On the other hand, PB enhanced AFQ_1-hydroxylase activity greater than 2-fold, whereas 3-MC resulted in a 20–30 per cent depression of the enzyme activity. These results suggest involvement of two different MFO activities in the metabolism of AFB_1 to AFM_1 and AFQ_1. To obtain additional evidence in support of this conclusion, the effects of combined pretreatment with PB and 3-MC were compared with the effects of independent treatment with either chemical (Table 1). The effects produced by the combination treatment on AFM_1-hydroxylase and AFQ_1-hydroxylase activities were essentially an expression of the additive effects of the independent treatments.

To further delineate enzyme multiplicity involved in the MFO-mediated metabolism of AFB_1, the effects of inhibitors of known specificities toward aryl hydrocarbon hydroxylase (AHH) [1, 8–10] were studied; these included SKF-525A, metyrapone, α- and β-naphthoflavones, and acetone (Table 2).

While SKF-525A produced no inhibition of AFM_1-hydroxylase from the control rats, it produced a strong inhibition of the enzyme from PB-treated rats (67 per cent) and a relatively weaker inhibition of

Table 1. Effects of PB and 3-MC on rat liver microsomal metabolism of AFB_1 to AFM_1 and AFQ_1

			Activity (pmoles metabolite formed/mg protein/15 min)			
			AFM_1		AFQ_1	
Expt.	Treatment	Number of rats (n)	Mean ± SE	Activity as % of control	Mean ± S.E.	Activity as % of control
1	Control	9	3454 ± 226	100	2213 ± 177	100
	PB	9	4600 ± 586	133	4725 ± 645	213
	3-MC	9	8372 ± 775	242	1616 ± 227	73
2	Control	3	2875 ± 251	100	2824 ± 134	100
	PB	3	5329 ± 1368	185	6596 ± 1519	233
	3-MC	3	5994 ± 746	208	2359 ± 424	83
	PB + 3-MC	3	8303 ± 968	289	5833 ± 419	206

Table 2. Effects of inhibitors on the rat hepatic microsomal metabolism of AFB_1 to AFM_1 and AFQ_1

	% Inhibiton (−) or stimulation (+) of the activity[a]*					
	AFM_1			AFQ_1		
Inhibitor concn.	Control microsomes†	PB microsomes†	3-MC microsomes†	Control microsomes†	PB microsomes†	3-MC microsomes†
SKF-525A (1 mM)	+2	−67	−27	−57	−76	−68
Metyrapone (1 mM)	+4	−53	−60	−92	−98	−97
α-Naphthoflavone (0.5 mM)	−26	−16	−90	−78	−74	−86
β-Naphthoflavone (0.5 mM)	−56	−68	−95	−87	−78	−96
Acetone (0.3 mM)	−6	−2	+64	−45	−49	−9
(1.2 mM)	−5	−17	+47	−61	−82	−14

* Each value is a mean of two determinations. In most cases individual values varied less than 15 per cent from the mean.

† Activity in control microsomes in pmoles product formed/mg of microsomal protein/15 min was as follows: AFM_1, 3032; AFQ_1, 2369. The effect of PB and 3-MC on these activities is reported in Table 1.

the enzyme from 3-MC-treated rats (28 per cent). Metyrapone behaved analogously to SKF-525A: it had no effect on AFM_1-hydroxylase from control rats but inhibited the enzyme 55–60 per cent from PB or 3-MC-treated rats. On the other hand, α- and β-naphthoflavones were very strong (>90 per cent) inhibitors of AFM_1-hydroxylase from 3-MC-treated rats and were relatively weaker in producing inhibition of the enzyme from control or phenobarbital-treated rats, although in this respect β-naphthoflavone was stronger than ∝-napthoflavone.

Although metyrapone produced a stronger (>90 per cent) inhibition of the AFQ_1-hydroxylase than SKF-525A (60–75 per cent), both inhibitors, when used individually, produced a similar degree of inhibition of the enzyme from control, PB- or 3-MC-treated rats. Also, α- and β-naphthoflavones, which produced about 70–90 per cent inhibition of AFQ_1-hydroxylase, could not distinguish the enzyme from control, PB- and 3-MC-treated rats.

While 0.3 M and 1.2 M acetone caused a marginal inhibition of AFM_1-hydroxylase activity of control and PB-treated rats, it markedly stimulated (50–60 per cent) the enzyme activity of 3-MC-treated rats (Table 2). On the other hand, AFQ_1-hydroxylase of control and PB-treated rats showed concentration-dependent inhibition with acetone, while that from 3-MC-treated rats was inhibited marginally by either 0.3 or 1.2 M acetone.

Effects of inducers and inhibitors on the metabolic activation of AFB_1 and benzo[a]pyrene by the rat. Previously we have reported [42] that while PB pretreatment enhances the metabolic activation of both AFB_1 and benzo[*a*]pyrene (BP), 3-MC pretreatment enhances the metabolic activation of BP and somewhat depresses the metabolic activation of AFB_1 (see also footnote to Table 3). These data clearly suggest that the metabolic activations of AFB_1 and BP are catalyzed by different enzyme systems of the MFO. The data derived from the studies on the effects of inhibitors (Table 3) are in support of this conclusion. While SKF-525A and metyrapone strongly inhibited (65–90 per cent) metabolic activation of AFB_1 catalyzed by the hepatic microsomes

Table 3. Effects of inhibitors on the metabolic activation of AFB_1 and benzo[*a*]pyrene (BP) by rat hepatic microsomes

	% Inhibition*					
	AFB_1 activation			BP activation		
Inhibitor Concn.	Control micrsomes†	PB microsomes†	3-MC microsomes†	Control microsomes†	PB microsomes†	3-MC microsomes†
SKF-525A (1 mM)	75	86	69	58	69	10
Metyrapone (1 mM)	71	93	65	39	72	12
β-Naphthoflavone (0.5 mM)	60	69	51	32	44	81

* Each value is a mean of two determinations. In most cases individual values varied less than 10 per cent from the mean.

† Activity in pmoles AFB_1 metabolite bound to a μmole of DNA-P/mg of microsomal protein/15 min was as follows: AFB_1 activation: control, 610; PB, 2005 and 3-MC, 490. BP activation: control, 12.4; PB, 23.6; and 3-MC, 89.4.

Table 4. Effects of inducers on the metabolism of AFB_1 to AFM_1 and AFQ_1 catalyzed by mouse hepatic microsomes

	Activity (pmoles metabolite formed/mg protein/15 min)							
	C57BL/6 strain (n = 3)				DBA/2 strain (n = 3)			
	AFM_1		AFQ_1		AFM_1		AFQ_1	
Treatment	Activity (mean ± S.E.)	% Control	Activity (mean ± S.E.)	% Control	Activity (mean ± S.E.)	% Control	Activity (mean ± S.E.)	% Control
Control	4,027 ± 30	100	1,617 ± 78	100	2,149 ± 55	100	2,094 ± 114	100
PB	5,548 ± 242	138	4,718 ± 156	292	4,037 ± 322	188	5,249 ± 596	251
3-MC	27,585 ± 1,328	685	316 ± 118	19	2,382 ± 77	111	1,678 ± 29	80

from control, PB- and 3-MC-treated rats, β-naphthoflavone produced a moderate to strong inhibition (50–70 per cent) of the enzyme activity. In comparison to this, both SKF-525A and metyrapone, while producing a moderate to strong inhibition (40–70 per cent) of the metabolic activation of BP catalyzed by hepatic microsomes from control and PB-treated rats, very weakly inhibited (10 per cent) the enzyme activity of the 3-MC-treated rats. On the other hand, in accordance with the reports on the effects of these inhibitors on AHH [8–10], β-naphthoflavone was a potent inhibitor (81 per cent) of the metabolic activation of BP catalyzed by hepatic microsomes from 3-MC-treated rats and a weak to moderate inhibitor (30–40 per cent) of the activity of microsomes from control and PB-treated rats.

Metabolism of AFB_1 to AFM_1 and AFQ_1 by the mouse. Among inbred strains of mice, some strains are responsive to induction by 3-MC of several hepatic MFO activities, whereas other strains are nonresponsive [32, 43, 44]. However, some nonresponsive strains, e.g. AKR, are somewhat responsive to 3-MC induction of AHH in extrahepatic tissues [44]. Since our studies in rats on the inducers and inhibitors suggested enzyme multiplicity in the metabolism of AFB_1 to AFM_1, AFQ_1, and AFB_1-2,3-oxide (metabolic activation), we compared the metabolism of AFB_1 between C57BL/6 (an Ah responsive strain) and DBA/2 (an Ah nonresponsive strain) mice that were either control or treated with PB or 3-MC (Table 4). In C57BL/6, PB induced both AFM_1-hydroxylase (38 per cent) and AFQ_1-hydroxylase (292 per cent), whereas 3-MC, which induced AFM_1-hydroxylase (685 per cent), depressed AFQ_1-hydroxylase (81 per cent). On the other hand, in DBA/2, 3-MC had only a slight effect on AFM_1-hydroxylase or AFQ_1-hydroxylase, whereas phenobarbital induced both the activities [AFM_1-hydroxylase (88 per cent); AFQ_1-hydroxylase (251 per cent)] as in C57BL/6. Therefore, these studies have served to differentiate AFM_1-hydroxylase activity from AFQ_1-hydroxylase activity. A similar conclusion was derived from studies on the effects of β-naphthoflavone and metyrapone on AFM_1- and AFQ_1-hydroxylase activities of hepatic microsomes of DBA/2 mice. While β-naphthofla-

Table 5. Effects of inducers on the metabolic activation of Aflatoxin B_1 (AFB_1) and benzo[*a*]pyrene (BP) by hepatic microsomes from various inbred strains of mice and recombinant inbred (RI) lines*

	AFB_1 metabolic activation			BP metabolic activation			
	(nmoles metabolite/μmole DNA-P/incubation)			(nmoles metabolite/μmole DNA-P/incubation)			
Strain or RI line	Control	PB	3-MC	Control	PB	3-MC	AHH inducibility (3-MC/control)
C57BL/6J	7.0	20.7 (2.9)	4.0 (0.57)	52.6	241.8 (4.6)	143.8 (2.7)	3.2
DBA/2J	7.0	26.9 (3.8)	4.6 (0.66)	54.2	270.0 (5.0)	52.1 (1.0)	1.1
C57L/J	1.7	6.3 (3.7)	1.0 (0.59)	22.9	83.2 (3.6)	57.8 (2.5)	4.0
AKR/J	2.6	7.4 (2.8)	2.4 (0.92)	30.1	104.5 (3.5)	24.6 (0.82)	1.2
AKXL-8	4.1	10.7 (2.6)	1.3 (0.32)	21.6	106.5 (4.9)	67.2 (3.1)	2.7
AKXL-13	5.9	11.4 (1.9)	2.3 (0.39)	81.7	307.5 (3.8)	287.5 (3.5)	2.8
AKXL-19	4.8	19.5 (4.1)	2.6 (0.54)	45.2	158.9 (3.5)	140.5 (3.1)	2.5
AKXL-21	10.4	20.3 (2.0)	5.0 (0.48)	73.9	235.1 (3.2)	180.3 (2.4)	2.1
AKXL-37	4.1	10.4 (2.5)	1.6 (0.39)	49.4	135.4 (2.7)	76.5 (1.5)	2.4
AKXL-38A	3.3	18.7 (5.7)	2.5 (0.76)	64.4	217.8 (3.4)	163.9 (2.5)	2.9
AKXL-17	2.2	5.6 (2.5)	1.3 (0.59)	19.0	93.9 (4.9)	11.5 (0.60)	1.4
AKXL-38	9.2	16.8 (1.8)	4.7 (0.51)	246.2	515.2 (2.1)	190.2 (0.77)	0.9

* Assay conditions are described in detail in the text. However, these studies differed from other metabolic activation studies in that the incubation period was 1 hr and the incubation volume and the assay ingredients were doubled. Activities were related to the incubation, each of which contained 2 mg of microsomal protein. Each value is a mean of two or three determinations. AKR refers to AKR/J, an Ah nonresponsive strain, and L refers to C57L/J, an Ah responsive strain. Numbers in parentheses denote inducibility ratio, i.e. ratio of the induced activity/control activity. Background activity obtained in the absence of NADPH in the incubation mixture has been subtracted. The background activity for both AFB_1 and BP metabolic activation was negligible.

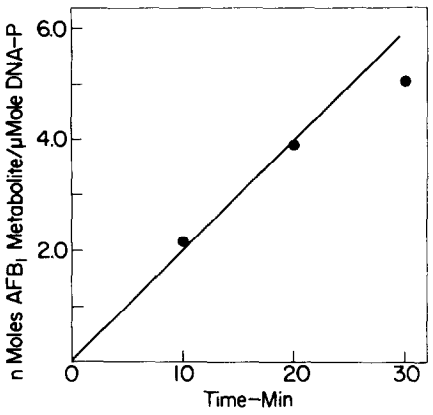

Fig. 2. Time-dependence of the metabolic activation of AFB_1. Each data print is a mean of two determinations.

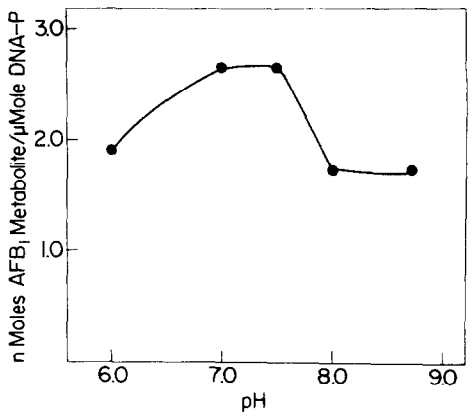

Fig. 4. pH Dependence of the metabolic activation of AFB_1. Each data point is a mean of two determinations.

vone inhibited both AFM_1- and AFQ_1-hydroxylase, metyrapone markedly inhibited AFQ_1-hydroxylase without inhibiting AFM_1-hydroxylase activity.

Effects of inducers on the metabolic activation of AFB_1 and BP by the mouse. Both Ah responsive and nonresponsive inbred strains of mice and recombinant inbred lines derived from progenitor strains AKR/J, an Ah nonresponsive strain, and C57L/J, an Ah responsive strain, were compared for the metabolic activation of AFB_1 and BP (Table 5). While PB enhanced metabolic activation of AFB_1 and BP in both Ah responsive and nonresponsive strains, 3-MC produced a differential effect. 3-MC depressed metabolic activation of AFB_1 in all strains of mice, whereas it induced metabolic activation of BP only in Ah responsive and not in Ah nonresponsive strains. These data demonstrate a strong positive correlation between metabolic activation of BP and AHH activity and a negative correlation between metabolic activation of AFB_1 and AHH or metabolic activation of BP. Although in these studies a 1 hr incubation period was used, our recent (unpublished) studies in eight inbred strains of mice, in which 15-min incubations were used, have produced qualitatively identical results.

Enzymologic properties of the metabolic activation of AFB_1. Of the various MFO-mediated metabolites of AFB_1, metabolite activation, leading to the formation of the putative AFB_1-2,3-oxide, is biologically very significant in terms of its involvement in carcinogenesis, mutagenesis and probably toxicity [35–38, 45]. Therefore, we have studied various enzymologic properties of this pathway.

The studies of the dependence of the rate of reaction on period of incubation and enzyme protein concentration revealed that the rate of reaction is linear with the period of incubation up to at least 20 min (Fig. 2) and with the microsomal protein concentration up to about 2 mg protein (Fig. 3). The reaction has a broad pH optimum of 7.0 to 7.4 (Fig. 4). Since DNA was used to trap the reactive metabolite, it was important to ascertain the minimal concentration of DNA that should be added to trap all the metabolite and to determine if DNA interfered with the formation of the reactive metabolite by causing substrate depletion from non-covalent interaction with unmetabolized substrate AFB_1 [46, 47]. Therefore, we studied the activity as a function of DNA concentration (Fig. 5). One mg DNA in the incubation provided enough sites for the binding of the reactive metabolite, and as the DNA concentration in the incubation increased, the specific activity of the binding decreased exponentially; however, the total concentration of the metabolite formed at

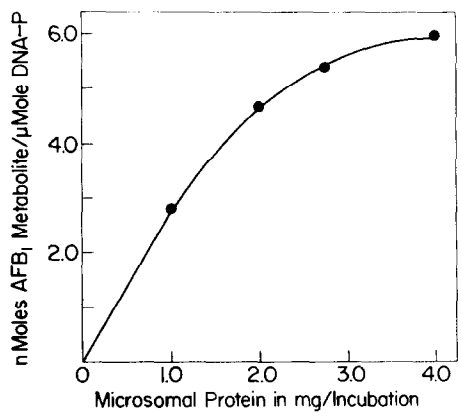

Fig. 3. Dependence of the metabolic activation of AFB_1 on the microsomal protein concentration. Each data point is a means of two determinations.

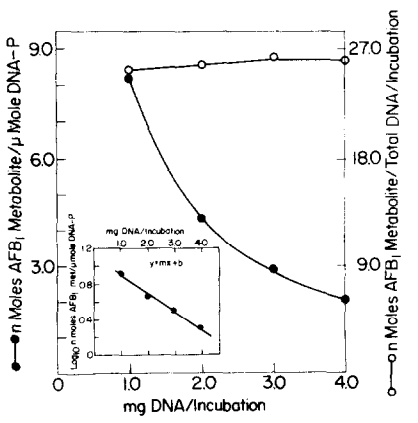

Fig. 5. Dependence of the metabolic activation of AFB_1 on the DNA concentration in the incubation. Each data point is a mean of two determinations.

various DNA concentrations remained unchanged. These data clearly show that 1 mg DNA was sufficient for trapping all the metabolite and that DNA did not interfere with the metabolic activation process. Although it is very likely that at limiting DNA concentrations the specific activity would be a function of the DNA concentration, because of the difficulties associated with the recovery of the DNA from the incubation, following its purification to ensure the removal of non-covalently bound AFB_1, DNA concentrations less than 1 mg were not used. Figure 6 shows the Lineweaver–Burk plot of the initial velocity versus the substrate concentration. The apparent K_m values of the microsomes from control, PB- and 3-MC-treated rats are similar, i.e. 19.7×10^{-4} M for control and PB, and 12.4×10^{-4} M for 3-MC. On the other hand, relative to control (apparent V_{max}, 4.44 nmoles metabolite bound/μmole DNA-P), the apparent V_{max} of the microsomes from 3-MC-treated rats is decreased (1.43 units), whereas that of the microsomes from PB-treated rats is considerably higher (13.3 units).

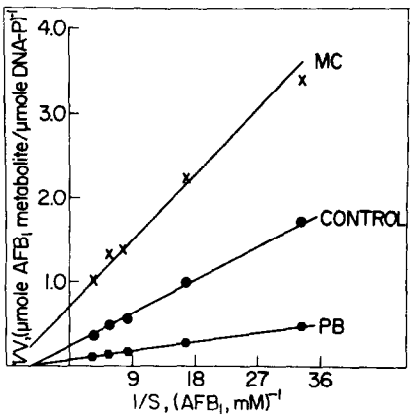

Fig. 6. Lineweaver–Burk plot of the hepatic microsome-mediated metabolic activation of AFB_1. Each data point is a mean of two determinations.

DISCUSSION

Since the discovery of aflatoxins in the early 1960s [48], extensive studies have been carried out on the metabolism of AFB_1, and in a few studies the effect of pretreatment of animals on some biotransformation pathways of AFB_1 have been studied [34, 49]. However, no systematic studies have been carried out to evaluate the possible enzyme multiplicity involved in the metabolism of AFB_1, the most potent of all the known aflatoxins [50]. Nor has the induction of AFB_1 metabolism by polycyclic aromatic hydrocarbons been evaluated sufficiently. Using various inducers and inhibitors as probes to study the microsomal metabolism of AFB_1 to AFM_1, AFQ_1, and AFB_1-2,3-oxide, our data suggest involvement of different MFO activities in the microsomal metabolism of AFB_1. AFM_1-hydroxylase is preferentially induced by 3-MC, whereas AFQ_1-hydroxylase and metabolic activation are preferentially induced by PB. This suggests involvement of at least two different enzyme activities of MFO in the metabolism of AFB_1: one, which is cytochrome P-450-linked, mediates preferentially the metabolism of AFB_1 to AFQ_1 and to AFB_1-2,3-oxide, and the other, which is cytochrome P-448 (P_1-450)-linked, mediates the metabolism of AFB_1 to AFM_1. The data on the effects of inhibitors—notably SKF-525A, metyrapone, and the α- and β-naphthoflavones—are consistent with the suggestion of the involvement of several enzyme activities of MFO in the metabolism of AFB_1. Although both AFQ_1-hydroxylase and metabolic activation possess similar induction and inhibition specificities and, therefore, are not clearly distinguishable, these two activities can be distinguished on the basis of their kinetic parameters and genetic regulation. The apparent K_m of the metabolic activation pathway is three orders of magnitude higher (19.7×10^{-4}, calculated from Fig. 6) than that

Table 6. Induction and "repression" by 3-MC of the metabolism of AFB_1 to AFM_1 and AFQ_1 in various inbred mouse strains

Mouse strain*	AFM_1 inducibility Ratio: 3-MC-induced / Control	AFQ_1 formation (pmoles AFQ_1 formed/mg microsomal protein/15 min) Control (mean ± S.E.)	3-MC (mean ± S.E.)	% Increase or decrease relative to control
DBA/2J	1.00†	1351 ± 136	1030 ± 115	−23
DBA/2CR	0.73†	809 ± 115	1125 ± 102	+38
AKR/J	1.30†	1017 ± 79	1037 ± 115	+2
ST/bJ	1.50†	2461 ± 191	3301 ± 120	+34
C57BL/6	5.2‡	1725 ± 255	674 ± 77	−61
C57L/J	3.6‡	1665 ± 99	817 ± 82	−51
BALB/cJ	3.6‡	1316 ± 175	377 ± 15	−71
SM/J	4.9‡	3520 ± 94	1563 ± 123	−56

* Eight to twelve mice of each strain were studied.
† Non-inducible with 3-MC for AHH and the metabolism of AFB_1 to AFM_1 [27].
‡ Inducible with 3-MC for AHH and the metabolism of AFB_1 to AFM_1 [27].

of AFQ_1-hydroxylase (0.7×10^{-4}, see Ref. 51). Genetic studies with inbred and recombinant inbred strains of mice revealed that, while 3-MC depresses metabolic activation 10–70 per cent in the tested inbred strains and recombinant inbred lines of mice [27], whether Ah responsive or nonresponsive (Table 5), it depresses AFQ_1-hydroxylase much more in Ah responsive than in Ah nonresponsive strains (Tables 4 and 6). However, these differences do not exclude the possibility of one enzyme with two catalytic sites for the metabolism of the different portions of the AFB_1 molecule, i.e. formation of AFQ_1 and AFB_1-2,3-oxide.

The observation that AFM_1-hydroxylase is induced more by 3-MC than by PB and is inhibited more by α- and β-naphthoflavones than by SKF-525A or metyrapone suggests that AFM_1 formation, like AHH activity, is preferentially mediated by a cytochrome P-448-linked system. This interpretation is consistent with recent genetic studies [27] which have shown that, in 700 mice of various inbred strains, recombinant inbred lines, and progenies of selected matings, AFM_1-hydroxylase induction cosegregates with the induction of AHH. However, those genetic studies also suggested that the association between the AFM_1-hydroxylase and AHH induction is operational at the regulatory gene level and not at the structural gene level, as data on the recombinant inbred lines suggested that AHH enzyme is different from AFM_1-hydroxylase. The inhibition with acetone further distinguishes AFM_1-hydroxylase from AHH. Acetone, which slightly inhibited AFM_1-hydroxylase in control and phenobarbital-treated rat liver microsomes (Table 2), caused a marked stimulation of the enzyme in 3-MC-treated rat liver microsomes. Similar concentrations of acetone had no effect on AHH in control rat liver microsomes but inhibited (50–70 per cent) AHH in microsomes from PB- or 3-MC-treated rat liver [9].

The studies on the inducers and inhibitors further serve to distinguish the metabolic activation of AFB_1 from the metabolic activation of BP and AHH activity. Both metabolic activation of BP [42] and AHH activity [9] are preferentially induced by 3-MC. SKF-525A and metyrapone preferentially inhibit AHH activity [1, 8–10] and, as shown here (Table 3), metabolic activation of BP in control PB-treated rat liver microsomes, whereas β-naphthoflavone preferentially inhibits AHH activity [8–10] and, as shown here (Table 3), metabolic activation of BP in microsomes from 3-MC-treated rats. In the present studies metabolic activation of AFB_1 was preferentially induced by PB and inhibited nearly equally by SKF-525A, metyrapone or β-naphthoflavone. These data distinguishing the metabolic activation of AFB_1 from that of BP are consistent with the results of genetic studies obtained on various inbred strains and recombinant inbred lines of mice (Table 5), but are in discordance with the report of Alexandrov and Frayssinet [52] who have suggested that metabolic activation of both AFB_1 and BP is catalyzed by the same MFO activity, namely AHH.

Using various inducers and different animal species, several investigators have isolated multiple forms, at least eight forms, of cytochrome P-450 having different substrate sensitivities, immunological properties, and electrophoretic mobilities [11–14, 53, 54]. The present studies suggest possible involvement of different cytochromes in the metabolism of AFB_1 via various pathways. This suggestion underlines the difficult and interesting nature of the task involved in understanding the complex nature of AFB_1 metabolism.

From the linear relation between the DNA concentration in the incubation (in the range of 1–4 mg) and the log specific activity of the metabolic activation (inset Fig. 5), two conclusions are possible: (a) 1–4 mg of DNA in the incubation is a suitable concentration range for metabolic activation studies, and (b) under the conditions of the incubation, the minimum number of binding sites in DNA in the incubation corresponds to a specific activity of 8–9 nmoles metabolite/μmole DNA-P. Although the apparent K_m values of the enzyme from control and PB-treated rat liver microsomes are the same, the apparent K_m of 3-MC-treated rat liver microsomes is of the same order of magnitude and, therefore not distinguishable on the basis of this kinetic parameter. However, a 68 per cent decrease in apparent V_{max} suggests decreased enzyme concentration in microsomes from 3-MC-treated rats, assuming of course, that the rate-limiting steps in the V_{max} term are not affected. By the same argument, a 3-fold enhancement in the apparent V_{max} of microsomes from PB-treated rats suggests an increased enzyme content of these microsomes. PB pretreatment of rats enhances the apparent V_{max} about 3-fold but not the apparent K_m of the rat liver AHH, whereas 3-MC pretreatment, in addition to enhancing the V_{max} about 4-fold, decreases the apparent K_m 80 per cent [15]; these data have been interpreted to suggest that 3-MC pretreatment induces a new AHH enzyme [15, 17]. These diverse effects of inducers on the kinetic parameters of AHH and AFB_1 metabolic activation are consistent with the interpretation derived from induction and inhibition studies that AHH and AFB_1 metabolic activation are catalyzed by different activities of the MFO system.

REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. W. F. Benedict, N. Considine and D. W. Nebert, *Molec. Pharmac.* **6**, 266 (1973).
3. L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.* **10**, 412 (1962).
4. R. M. Welch, Y. E. Harrison, A. H. Conney, P. J. Poppers and M. Finster, *Science* **160**, 541 (1968).
5. D. W. Nebert, L. L. Bausserman and R. R. Bates, *Int. J. Cancer* **6**, 470 (1970).
6. W. Levin, A. H. Conney, A. P. Alvares, I. Merkatz and A. Kappas, *Science* **176**, 419 (1972).
7. J. P. Whitlock, Jr., H. L. Cooper and H. V. Gelboin, *Science* **177**, 618 (1972).
8. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
9. F. J. Weibel, J. C. Leutz, L. Diamond and H. V. Gelboin, *Archs. Biochem. Biophys.* **144**, 78 (1971).
10. J. T. Ahokas, O. Pelkonen and N. T. Karki, *Cancer Res.* **37**, 3737 (1977).
11. A. P. Alvares and P. Siekevitz, *Biochem. biophys. Res. Commun.* **54**, 923 (1973).
12. M. T. Huang, S. B. West and A. Y. H. Lu, *J. biol. Chem.* **251**, 4659 (1976).

13. A. Y. H. Lu, D. Ryan, J. Kawalek, P. Thomas, S. B. West, M. T. Huang and W. Levin, *Biochem. Soc. Trans.* **4**, 169 (1976).
14. D. A. Haugen, M. J. Coon and D. W. Nebert, *J. biol. Chem.* **251**, 1817 (1976).
15. A. P. Alvares, G. R. Schilling and R. Kuntzman, *Biochem. biophys. Res. Commun.* **30**, 588 (1968).
16. H. L. Gurtoo and T. C. Campbell, *Biochem. Pharmac.* **19**, 1729 (1970).
17. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. biophys. Res. Commun.* **29**, 521 (1967).
18. W. Levin, A. Y. H. Lu, D. Ryan, S. West, R. Kuntzman and A. H. Conney, *Archs. Biochem. Biophys.* **153**, 543 (1972).
19. R. E. Rasmussen and I. Y. Wang, *Cancer Res.* **34**, 2290 (1974).
20. D. W. Nebert, J. R. Robinson and H. Kon, *J. biol. Chem.* **248**, 7637 (1973).
21. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).
22. R. C. Shank, G. N. Wogan and J. B. Gibson, *Fd Cosmet. Toxic.* **10**, 51 (1972).
23. M. E. Alpert, G. N. Wogan and C. S. Davidson, *Gastroenterology* **54**, 149 (1968).
24. R. N. M. MacSween and A. R. Scott, *J. clin. Path.* **26**, 936 (1973).
25. A. F. Bagshawe, A. M. Parker and A. Jindani, *Br. med. J.* **1**, 88 (1971).
26. C. L. Vogel, P. P. Anthony, N. Mody and L. F. Barker, *Lancet* **2**, 621 (1970).
27. H. L. Gurtoo, R. P. Dahms, P. Kanter and J. B. Vaught, *J. biol. Chem.* **253**, 3952 (1978).
28. H. L. Gurtoo and T. C. Campbell, *Molec. Pharmac.* **10**, 776 (1974).
29. H. L. Gurtoo and C. V. Dave, *Cancer Res.* **35**, 382 (1975).
30. R. K. Abramson, B. A. Taylor, D. Tomlin and J. J. Hutton, *Biochem. Genet.* **15**, 723 (1977).
31. B. A. Taylor and H. Meier, *Genet. Res.* **26**, 307 (1976).
32. S. A. Atlas, B. A. Taylor, B. A. Diwan and D. W. Nebert, *Genetics*, **83**, 537 (1976).
33. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
34. R. P. Dahms and H. L. Gurtoo, *Res. Commun. Chem. Path. Pharmac.* **15**, 11 (1976).
35. R. C. Garner, E. C. Miller and J. A. Miller, *Cancer Res.* **32**, 2058 (1972).
36. H. L. Gurtoo and C. V. Dave, *Res. Commun. Chem. Path. Pharmac.* **5**, 635 (1973).
37. D. H. Swenson, J. A. Miller and E. C. Miller, *Biochem. biophys. Res. Commun.* **53**, 1260 (1973).
38. D. H. Swenson, E. C. Miller and J. A. Miller, *Biochem. biophys. Res. Commun.* **60**, 1036 (1974).
39. J. M. Essigman, R. G. Croy, A. M. Nadzan, W. F. Busby, Jr., V. N. Reinhold, G. Buchi and G. N. Wogan, *Proc. natn. Acad. Sci. U.S.A.* **74**, 1870 (1977).
40. J.-K. Lin, K. A. Kenman, E. C. Miller and J. A. Miller, *Cancer Res.* **38**, 2424 (1978).
41. J.-K. Lin, J. A. Miller and E. C. Miller, *Cancer Res.* **37**, 4430 (1977).
42. H. L. Gurtoo and N. Bejba, *Biochem. biophys. Res. Commun.* **61**, 735 (1974).
43. D. W. Nebert, J. R. Robinson, A. Niwa, K. Kumaki and A. P. Poland, *J. cell. Physiol.* **85**, 393 (1975).
44. K. Bürki, A. G. Liebelt and E. Bresnick, *Biochem. Genet.* **13**, 417 (1975).
45. H. L. Gurtoo, R. P. Dahms and B. Paigen, *Biochem. biophys. Res. Commun.* **81**, 965 (1978).
46. M. B. Sporn, C. W. Dingham, H. L. Phelps and G. N. Wogan, *Science* **151**, 1539 (1966).
47. H. S. Black and B. Jergensons, *Pl. Physiol.* **42**, 731 (1967).
48. K. Sargeant, A. Sheridan, J. O'Kelly and R. B. A. Carneghan, *Nature, Lond.* **192**, 1096 (1961).
49. D. S. P. Patterson and B. A. Roberts, *Biochem. Pharmac.* **20**, 3377 (1971).
50. G. N. Wogan, G. S. Edwards and P. M. Newberne, *Cancer Res.* **31**, 1936 (1971).
51. B. D. Roebuck and G. N. Wogan, *Cancer Res.* **37**, 1649 (1977).
52. K. Alexandrov and C. Frayssinet, *Cancer Res.* **34**, 3289 (1974).
53. W. L. Dean and M. J. Coon, *J. biol. Chem.* **10**, 3255 (1977).
54. F. P. Guengerich, *J. biol. Chem.* **252**, 3970 (1977).