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CYTOCHROME P-450 INDUCTION BY 3-METHYLCHOLANTHRENE AND ITS ANTAGONISM BY 2,2-DIMETHYL-5-*t*-BUTYL-1,3-BENZODIOXOLE

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Abstract—Previous studies in this laboratory have shown 2,2-dimethyl-5-*t*-butyl-1,3-benzodioxole (DBBD) to antagonize 3-methylcholanthrene induction of cytochrome P-450 in Dub:ICR mice yet have no effect on phenobarbital induction. In the present experiments, C57BL/6 mice, an Ah responsive strain, produced a similar response under the same experimental conditions. The hypothesis that DBBD, although not a cytochrome P-450 inducer, competes with 3-methylcholanthrene for binding to the Ah receptor was tested. Using sucrose density gradients, the Ah receptor was measured in hepatic cytosol from Dub:ICR and C57BL/6 male mice. DBBD was unable to displace either 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 3-methylcholanthrene from the Ah receptor, *in vitro*. However, in *in vivo* experiments, DBBD treatment of Dub:ICR mice caused Ah receptor depression at 6 and 24 hr with complete recovery in between, while 3-methylcholanthrene treatment caused a 2-fold Ah receptor reduction at 2 hr followed by complete recovery after 12 hr. When 3-methylcholanthrene and DBBD were coadministered, the depression of the Ah receptor was additive. DBBD-pretreated mice had a 2.25-fold reduction in Ah receptor level, effectively blocking the ability of 3-methylcholanthrene to increase the cytochrome P-450 content and either benzo[*a*]pyrene hydroxylase or ethoxyresorufin *O*-deethylase activities. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis confirmed that 3-methylcholanthrene induction of cytochrome P-450 was inhibited by DBBD pretreatment. Hence, although DBBD does not displace 3-methylcholanthrene from the Ah receptor *in vitro*, it does antagonize 3-methylcholanthrene induction of cytochrome P-450 and also reduces the amount of available receptor *in vivo*. This interaction may be due either to antagonism or to downregulation of the Ah receptor.

5-*t*-Butyl-1,3-benzodioxole (BBD) is a methylenedioxyphenyl (MDP) compound, and 2-methyl-5-*t*-butyl-1,3-benzodioxole (MBBD) and 2,2-dimethyl-5-*t*-butyl-1,3-benzodioxole (DBBD) are analogs with one or both of their methylene hydrogens replaced by methyl groups respectively. MDP compounds have a biphasic effect on cytochrome P-450 and monooxygenase activity, namely, inhibition followed by induction [1]. Inhibition is related to the formation of a stable MDP metabolite–cytochrome P-450 complex which is also responsible for the appearance of a Type III double Soret optical difference spectrum [2, 3]. The mechanism for MDP induction of cytochrome P-450 is unknown. Substitution of BBD at the methylene carbon with methyl groups prevented the analogs, MBBD and DBBD, from forming an MDP metabolite–cytochrome P-450 complex, either *in vivo* or *in vitro*, and from inducing cytochrome P-450 [4, 5]. However, it should be noted that DBBD does induce other proteins, namely, the NADPH–cytochrome P-450 reductase and epoxide hydrolase [5].

Based upon the reported ability of isosafrole to displace [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from the Ah receptor [6], we suggested that substitution of the methylene hydrogens by methyl groups may prevent MBBD and DBBD from binding to the Ah receptor, thus blocking cytochrome P-450 induction [4]. The mechanism for aromatic hydrocarbon induction has been the subject of numerous reviews (e.g. Refs. 7 and 8). The hypothesis enjoying the most support includes the following events: the aromatic hydrocarbon moves passively across the cell membrane [9]; once inside it binds to a cytosolic Ah receptor [10, 11] coded for by the regulatory gene. Translocation of the ligand–receptor complex into the nucleus [12–14] and its interaction with structural genes [15] stimulate transcription of those structural genes [16, 17]. Recently, the initial location of the Ah receptor has been challenged. Whitlock and Galeazzi [18] have presented evidence that the Ah receptor is located entirely within the nucleus; however, Denison and coworkers [19] believe that the apparent nuclear localization seen at low buffer volumes is due to contamination of the nuclear pellet by entrapped cytosol.

When the outbred stock Dub:ICR mice were treated with 3-methylcholanthrene (3-MC) and DBBD at the same time, the cytochrome P-450 content and benzo[*a*]pyrene hydroxylase and ethoxyresorufin *O*-deethylase activities were all lower than in those mice treated with 3-MC alone [5]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) confirmed that 3-MC induction of cytochrome P-450 was reduced by DBBD, while no interaction of DBBD with phenobarbital induction was seen [5]. Although no compound had yet been shown to be an antagonist of the Ah receptor [7], competition between 3-MC and DBBD for binding to the Ah receptor was proposed for the attenuation

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of cytochrome P-450 induction, suggesting that DBBD was an Ah receptor antagonist [5].

Recently, a cytosolic 4S protein, which binds [^{3}H]-3-MC [20] and [^{3}H]benzo[*a*]pyrene [21, 22], has been demonstrated in C57BL/6J and DBA/2J mice. This 4S protein does not appear to be a receptor involved in the induction of aromatic hydrocarbon hydroxylase [21] but may be a binding protein involved in some other, as yet unknown, aspect of cellular regulation [21, 22].

To establish whether DBBD is an Ah receptor antagonist, we have examined the effect of coadministration of DBBD with either phenobarbital or 3-MC in C57BL/6 mice, an inbred Ah responsive strain. Since the 4S binding protein is present in both C57BL/6 and Dub:ICR mice [23], it, as well as the Ah receptor, were investigated for a possible role in DBBD antagonism. To test these hypotheses, the abilities of BBD, MBBD, and DBBD to displace [^{3}H]TCDD and [^{3}H]3-MC from the Ah receptor and the 4S peak were measured via sucrose density gradient analysis.

MATERIALS AND METHODS

Experimental animals and treatment protocol. Seven-week-old Dub:ICR and C57BL/6 male mice were obtained from Dominion Laboratories, Dublin, VA, and Charles River, Kingston, NY, respectively. The animals were caged in groups of five or less, housed in a facility with a 12-hr light/dark cycle, and were allowed Wayne Feed Blox F-6 and tap water *ad lib*. The mice were held for 1 week before preparation of cytosol or dosing. In the coadministration experiments, C57BL/6 mice received the following doses i.p. daily for 3 days: sodium phenobarbital, 80 mg/kg in corn oil; 3-MC, 20 mg/kg in corn oil; and DBBD, 500 mg/kg in corn oil. Control groups received corn oil. In the time course experiments, Dub:ICR mice received a single i.p. dose of the following compounds: 3-MC, 80 mg/kg in corn oil; DBBD, 500 mg/kg in corn oil; or 3-MC, 80 mg/kg, and DBBD, 500 mg/kg, in corn oil. Animals were killed at 0, 2, 6, 12, 24, and 48 hr after i.p. injection. The 0 hr group received an i.p. injection of corn oil immediately before decapitation. In the DBBD pretreatment experiments, Dub:ICR mice received a daily i.p. dose of 500 mg/kg DBBD in corn oil or corn oil alone for 3 days. On day 4, mice from each group either were killed and hepatic cytosol was prepared or they were treated with a single i.p. dose of 40 mg/kg 3-MC in corn oil or corn oil alone. Those mice who received the additional oil or 3-MC treatment on day 4 were killed 24 hr later, and hepatic microsomes were prepared.

Preparation of microsomes. The animals were killed on day 4 or 5 by decapitation, and microsomes were prepared as previously described [4].

Preparation of cytosol. Mice were killed by decapitation and the livers were perfused, *in situ*, until blanched, with ice-cold 1.15% (w/v) KCl–MDEG [25 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7.4; 1 mM dithiothreitol; 1.5 mM EDTA; and 10% (v/v) glycerol] via a 23-gauge needle inserted into the hepatic portal vein. After perfusion, the gall bladder was separated from the liver, and the pooled livers were minced and washed three times with MDEG buffer. The minced livers were suspended in 3 vol. (w/v) of MDEG buffer and homogenized with a polytron. All procedures were performed at 4° unless otherwise stated. The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant fraction was filtered through glass wool and centrifuged at 105,000 *g* for 60 min. Following each centrifugation the turbid layer was removed with a Pasteur pipet. After the last centrifugation the cytosol was removed using a pipet, without disturbing the microsomal pellet. The cytosol was divided into aliquots, bubbled with nitrogen, and stored at −80°. Cytosol was used within 2 weeks of preparation; however, no loss of binding occurred under these conditions for at least 2 months. Protein was determined by the method of Lowry *et al.* [24], using bovine serum albumin (BSA) fraction V as the standard.

Microsomal enzyme assays. 7-Ethoxycourmarin *O*-deethylase activity was determined by the method of Aitio [25]. Benzo[*a*]pyrene hydroxylase activity was determined by the method of Yang and Kicha [26] as modified by Denison *et al.* [27]. Ethoxyresorufin *O*-deethylase activity was determined fluormetrically by the method of Pohl and Fouts [28]. NADPH was used for the benzo[*a*]pyrene hydroxylase assay. The following NADPH-regenerating system was used in other assays: 25 mM $NADP^+$, 200 mM glucose-6-phosphate, and 40 units/ml glucose-6-phosphate dehydrogenase. Fifty microliters of NADPH-regenerating system was used per ml of reaction volume. Time course experiments were run for each assay to ensure first-order kinetics.

Spectral analysis. All spectra were recorded with an SLM Aminco DW-2C UV/VIS spectrophotometer at room temperature. Cytochrome P-450 content was measured according to the method of Omura and Sato [29].

Electrophoresis. SDS–PAGE [30] was performed as previously described except that a 3% stacking gel was included [4].

Sucrose gradient analysis. Procedures used were similar to those of Okey *et al.* [11] with the following modifications: (1) a protein concentration of 8 mg/ml was used instead of 5 mg/ml; (2) an incubation temperature of 20° was used instead of 4°; and (3) MDEG buffer was substituted for HDEG buffer [25 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4; 1 mM dithiothreitol; 1.5 mM EDTA; and 10% (v/v) glycerol]. One milliliter of cytosol (8 mg/ml) was incubated with either 10 nM [^{3}H]TCDD or [^{3}H]3-MC and 5 μl dioxane (total binding), or [^{3}H]TCDD or [^{3}H]3-MC and the test compound (nonspecific binding) for 1 hr at 20°. The labeled ligand and the test compound were added simultaneously to the cytosol. The final incubation concentrations of the test compounds are given in the tables. [^{3}H]TCDD, [^{3}H]3-MC, and the test compounds were each added in 5 μl of solvent. [^{3}H]TCDD, TCDD, BBD, MBBD, and DBBD were dissolved in dioxane, while [^{3}H]3-MC and 3-MC were dissolved in ethanol. Phenobarbital was dissolved in distilled water. All glassware was silanized with Prosil-28 to reduce ligand adsorption. [^{14}C]-BSA, 1000 dpm, was added to each gradient as an internal sedimentation marker (4.4S); sedimentation

coefficients were calculated by the method of Martin and Ames [31]. Gradients were centrifuged at 2° for 15 hr at 48,500 rpm in a Sorval AH-650 rotor ($g_{av} = 219{,}000$). After centrifugation, fractions (7 drops/fraction) were collected through the bottom of each tube using a Hoefer gradient tube fractionator (San Francisco, CA). Radioactivity in each fraction was determined by liquid scintillation counting using dual isotope analysis. Percentage displacement (%D) was determined by the following equation:

$$\%D = 100 \times \frac{\text{Total binding of } [^3H]\text{TCDD} - \text{nonspecific binding of } [^3H]\text{TCDD} + \text{test compound}}{\text{Total binding of } [^3H]\text{TCDD} - \text{nonspecific binding of } [^3H]\text{TCDD} + \text{TCDD}}$$

TCDD produced 100% displacement. A similar equation was used for [^{3}H]3-MC except that the test compound, 3-MC, was defined as producing 100% displacement.

Materials. [1,6-^{3}H]TCDD (52 Ci/mmole) was obtained from the ICN Chemical and Radioisotope Division (Irvine, CA) and Drs. C. F. Wilkinson and M. S. Denison, Cornell University. Both samples were purified by the method of Gasiewicz and Neal [32]. [G-^{3}H]3-MC (34 Ci/mmole) was purchased from the Amersham Corp., Arlington Heights, IL. [*Methyl*-^{14}C]Methylated BSA was bought from New England Nuclear, Boston, MA. BBD was synthesized by the method of Clark *et al.* [33]. MBBD and DBBD were synthesized by the method of Cole *et al.* [34]. BBD, MBBD, and DBBD were further purified using a silica gel 60 (35–70 mesh) column (American Scientific Products, Charlotte, NC) developed with 95% (v/v) hexane:5% (v/v) ethyl acetate. No contaminants were detected using thin-layer chromatography, NMR spectroscopy, and elemental analysis. The results of the elemental analysis are as follows: BBD (Calcd: C, 74.13; H, 7.92. Found: C, 74.04; H, 7.95), MBBD (Calcd: C, 74.97; H, 8.39. Found: C, 75.03; H, 8.43), and DBBD (Calcd: C, 75.69; H, 8.79. Found: C, 75.76; H, 8.84). Benzo[*a*]pyrene, bovine liver catalase, bovine liver L-glutamic dehydrogenase, BSA (fraction V), dextran (industrial grade), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-(*N*-morpholino)-propanesulfonic acid, NADP$^+$, NADPH, ovalbumin, porcine heart fumarase, and sucrose (grade I) were purchased from the Sigma Chemical Co., St. Louis, MO. Ammonium persulfate, Coomassie Brillant Blue R-250, dithiothreitol, and SDS were obtained from Bio-Rad Laboratories, Richmond, CA. Other chemicals and sources are as follows: charcoal (neutral Norit), glycerol, and Scintiverse II, Fisher Scientific Co., Fair Lawn, NJ; *p*-dioxane (Gold label), 7-ethoxycoumarin, and 7-hydroxycoumarin, Aldrich Chemical Co., Milwaukee, WI; 3-MC, Eastman Kodak Co., Rochester, NY; TCDD, KOR Inc., Cambridge, MA; sodium phenobarbital, Gaines Chemical Inc., Pennsville, NJ; corn oil, ICN Nutritional Biochemicals, Cleveland, OH; Prosil-28, PCR Inc., Gainesville, FL; and 7-ethoxyresorufin, Pierce Chemical Co.,

Table 1. Effects of simultaneous treatment with various inducing agents on hepatic microsomal monooxygenase variables in C57BL/6 mice

Treatment	Cytochrome P-450 (nmoles/mg protein)	Benzo[*a*]pyrene hydroxylase (nmoles/min/mg protein)	Ethoxyresorufin *O*-deethylase (nmoles/min/mg protein)	Ethoxycoumarin *O*-deethylase (nmoles/min/mg protein)
Oil	1.02	1.72	0.27	2.22
2,2-Dimethyl-5-*t*-butyl-1,3-benzodioxole (DBBD)	1.01	1.68	0.18	2.10
Phenobarbital in oil	1.76*	5.12*†	0.82	6.6*†‡
Phenobarbital + DBBD in oil	1.72*	5.21*†	0.48	5.18*
3-Methylcholanthrene	1.86*§	14.42*§	7.59*§	5.46*§
3-Methylcholanthrene + DBBD	1.44*	7.72*	3.51*	3.96*

Values are expressed as a mean of three experiments. The average S.E. per compound was 0.10, 0.45, 1.33, and 0.41 for cytochrome P-450, benzo[*a*]pyrene hydroxylase, ethoxyresorufin *O*-deethylase, and ethoxycoumarin *O*-deethylase respectively.

* $P < 0.01$, compared to control value.

† $P < 0.01$, compared to 3-methylcholanthrene and 3-methylcholanthrene + DBBD.

‡ $P < 0.01$, compared to phenobarbital + DBBD.

§ $P < 0.01$, compared to 3-methylcholanthrene + DBBD.

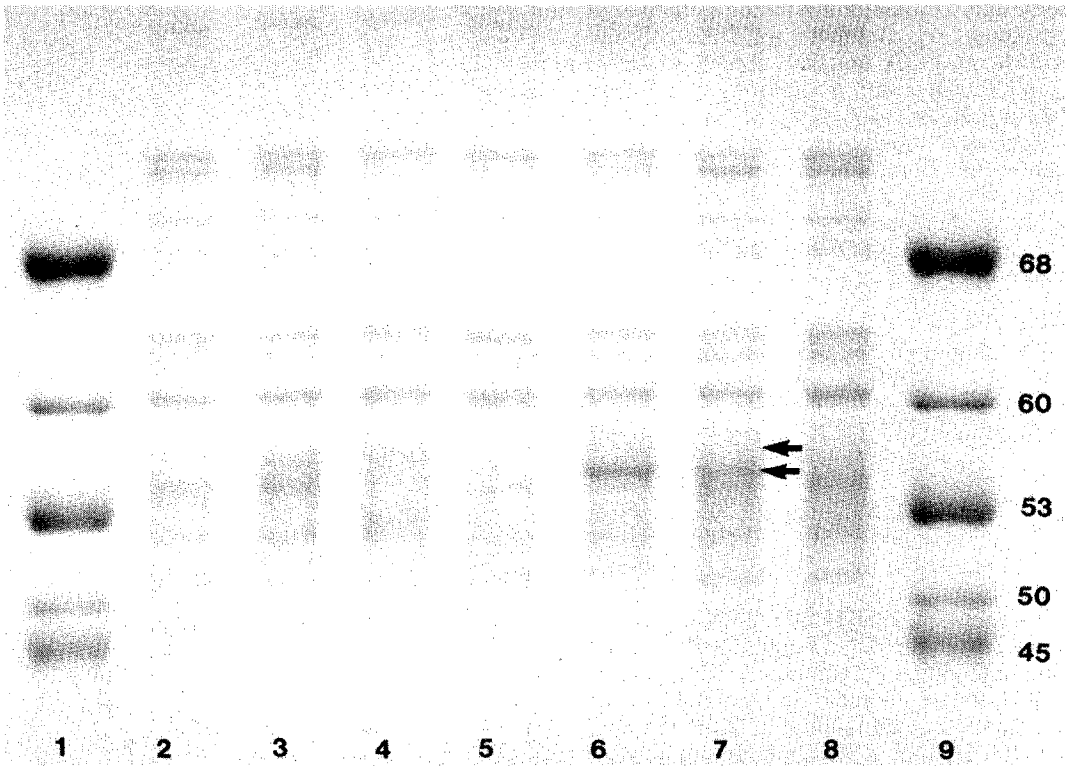

Fig. 1. SDS–PAGE of hepatic microsomes from C57BL/6 mice treated with various inducing agents. A mixture of molecular weight standards was applied to wells 1 and 9. Microsomes (18 μg protein) from treated mice were applied as indicated: (2) DBBD, (3) phenobarbital + DBBD, (4) phenobarbital, (5) oil, (6) 3-MC, (7) 3-MC + DBBD, and (8) DBBD. Migration is from the top of the gel to the bottom. Figures on the right represent molecular weight × 10^3. The arrows point to the 53,000 and 54,000 molecular weight bands. Note the decreased intensity of the 3-MC + DBBD 53,000 and 54,000 molecular weight bands compared to 3-MC.

Rockford, IL. All other chemicals were of at least reagent grade quality and were obtained from either the Sigma Chemical Co. or the Aldrich Chemical Co.

Statistical analysis. Data are expressed as a mean of three experiments. Statistical significance was determined using an analysis of variance to compute the least significant difference with $P < 0.01$ used as a criterion for significance [35].

RESULTS

2,2-Dimethyl-5-t-butyl-1,3-benzodioxole antagonism of 3-methylcholanthrene induction of cytochrome P-450 in C57BL/6 mice. The DBBD-treated mice had a cytochrome P-450 content and monooxygenase activities similar to those of the control (Table 1). No interaction of DBBD with phenobarbital induction was seen except for a slight decrease in the 7-ethoxycoumarin *O*-deethylase activity. In contrast, the 3-MC-treated mice had a higher cytochrome P-450 content and higher benzo[*a*]pyrene hydroxylase, ethoxyresorufin *O*-deethylase, and 7-ethoxycoumarin *O*-deethylase activities than those mice treated with 3-MC + DBBD (Table 1).

The gel in Fig. 1 shows that microsomes from the treated mice had the following molecular weight bands induced: phenobarbital—50,000, 53,000, and 54,000; DBBD—53,000, 70,000, and 78,000; phenobarbital + DBBD—50,000, 53,000, 53,000, 54,000, 70,000 and 78,000; 3-MC—53,000 and 54,000; and 3-MC + DBBD—53,000, 53,000, 54,000, 70,000 and 78,000. DBBD induced a 53,000 molecular weight band which is just below the phenobarbital and 3-MC molecular weight bands. Both bands for the 3-MC + DBBD-treated mice had a decreased staining intensity compared to the 3-MC 53,000 and 54,000 molecular weight bands. DBBD had no effect on the phenobarbital induction.

Sucrose density gradient analysis of [3H]TCDD and [3H]3-MC binding in hepatic cytosol from C57BL/6 and Dub:ICR mice. Figure 2 shows the [^{3}H]TCDD and [^{3}H]3-MC binding profile in cytosol for C57BL/6 and Dub:ICR mice. In C57BL/6 mice, an Ah responsive inbred strain, the Ah receptor can be measured with either [^{3}H]TCDD or [^{3}H]3-MC. Under the experimental conditions used, the Ah receptor had an 8-9S sedimentation value (fractions 10–12). A 3-4S peak (fractions 20–22) was also seen in both strains with [^{3}H]TCDD and [^{3}H]3-MC but was 12–20 times greater when measured with [^{3}H]3-MC (Fig. 2, Tables 2 and 3). In Dub:ICR mice, an outbred stock, the Ah receptor was detectable only with [^{3}H]TCDD and contained about one-third less receptor than C57BL/6 mice (Fig. 2, Tables 2 and 3).

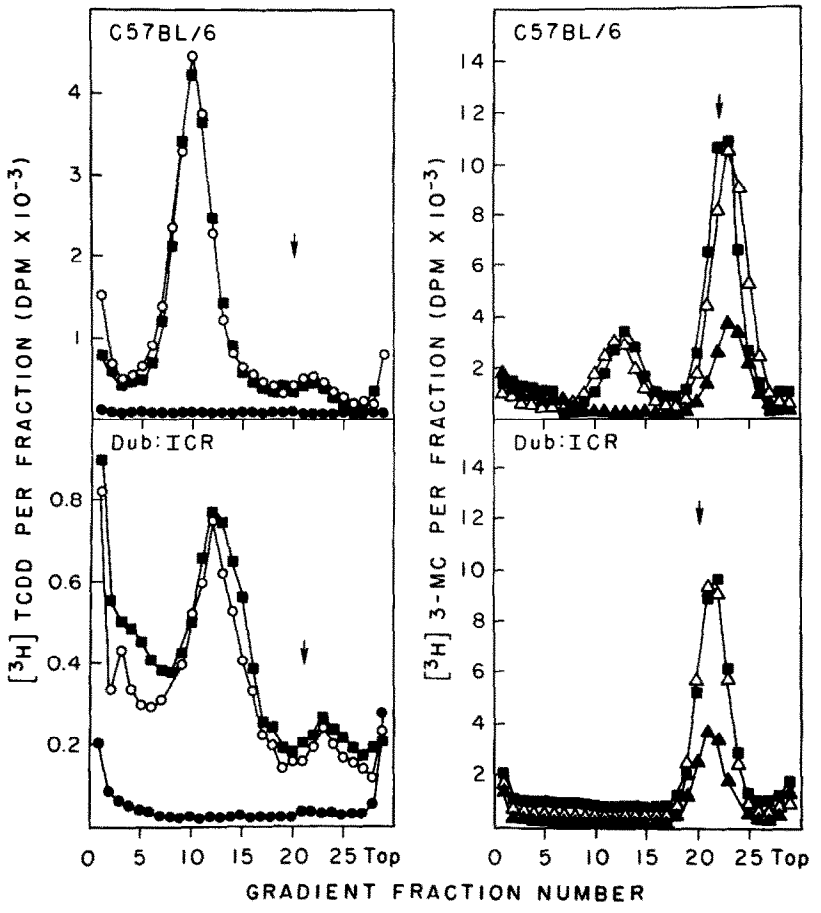

Fig. 2. Binding of [^{3}H]TCDD (left column) and [^{3}H]3-MC (right column) in hepatic cytosol from C57BL/6 and Dub:ICR mice following sucrose gradient analysis. Cytosol was incubated with 10 nM [^{3}H]-TCDD (○——○), 10 nM [^{3}H]TCDD + 1.0 μM TCDD (●——●), or 10 nM [^{3}H]TCDD + 10 μM DBBD (■——■) (left column). Cytosol was incubated with 10 nM [^{3}H]3-MC (△——△), 10 nM [^{3}H]3-MC + 1.0 μM 3-MC (▲——▲), or 10 nM [^{3}H]3-MC + 10 μM DBBD (■——■) (right column). The arrow indicates the sedimentation peak of [^{14}C]BSA. For further details, see sucrose gradient analysis in Materials and Methods.

Table 2. Displacement of [^{3}H]TCDD from the Ah receptor and the 3-4S peak among various aromatic compounds in C57BL/6 and Dub:ICR mice

	% Displacement of [^{3}H]TCDD			
	C57BL/6 mice		Dub:ICR mice	
Test compound-final concentration	Ah receptor	3-4S peak	Ah receptor	3-4S peak
1.0 μM 2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)*	100† (58 ± 8)‡	100† (14 ± 3)‡	100† (19 ± 1)‡	100† (7 ± 1)‡
1.0 μM 3-Methylcholanthrene (3-MC)*	88†	12	84†	18
10.0 μM 5-*t*-Butyl-1,3-benzo-dioxole (BBD)	0§	0	0	0
10.0 μM 2-Methyl-5-*t*-butyl-1,3-benzodioxole (MBBD)	0	5	0	2
10.0 μM 2,2-Dimethyl-5-*t*-butyl-1,3-benzodioxole (DBBD)	0	0	0	0
10.0 μM Phenobarbital*	0	6	0	0

Values are expressed as a mean of three experiments. The average S.E. per compound was 7 (Ah receptor) and 17 (3-4S peak) in C57BL/6 mice and 16 (Ah receptor) and 14 (3-4S peak) in Dub:ICR mice.

* To provide continuity, these control data also appeared in Cook and Hodgson [23].

† $P < 0.01$, compared to zero displacement.

‡ Specific binding in fmoles per mg cytosolic protein, expressed as the mean ± S.E.

§ The level of detection of this assay is approximately ±2.0%.

Table 3. Displacement of [^{3}H]3-MC from the Ah receptor and the 3-4S peak among various aromatic compounds in C57BL/6 and Dub:ICR mice

Test compound-final concentration	% Displacement of [^{3}H]3-MC			
	C57BL/6 mice		Dub:ICR mice	
	Ah receptor	3-4S peak	Ah receptor	3-4S peak
1.0 μM 3-Methylcholanthrene (3-MC)*	100† (50 ± 6)‡	100† (175 ± 20)‡	ND§ (ND)‡	100†‖ (144 ± 9)‡
1.0 μM 2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)*	103†	46†	ND	55†
10.0 μM 5-*t*-Butyl-1,3-benzo-dioxole (BBD)	0¶	0	ND	0
10.0 μM 2-Methyl-5-*t*-butyl-1,3-benzodioxole (MBBD)	3	0	ND	0
10.0 μM 2,2-Dimethyl-5-*t*-butyl-1,3-benzodioxole (DBBD)	5	3	ND	0
10.0 μM Phenobarbital*	0	0	ND	3

Values are expressed as a mean of three experiments. The average S.E. per compound was 6 (Ah receptor) and 10 (3-4S peak) in C57BL/6 mice and 10 (3-4S peak) in Dub:ICR mice.

* To provide continuity, these data also appeared in Cook and Hodgson [23].

† $P < 0.01$, compared to zero displacement.

‡ Specific binding in fmoles per mg cytosolic protein expressed as the mean ± S.E.

§ Not detectable.

‖ $P < 0.01$, compared to TCDD.

¶ The level of detection of this assay is approximately ±2.0%.

Neither BBD nor its methyl substituted analogs, MBBD and DBBD, were able to displace [^{3}H]-TCDD or [^{3}H]3-MC from the Ah receptor or the 3-4S peak in C57BL/6 and Dub:ICR mice, *in vitro* (Fig. 2, Tables 2 and 3). Similar results were obtained with phenobarbital. In contrast, 3-MC and TCDD displaced [^{3}H]3-MC and [^{3}H]TCDD, respectively, from the Ah receptor and the 3-4S peak in both mice strains (Fig. 2, Tables 2 and 3). Both [^{3}H]TCDD and [^{3}H]3-MC showed similar levels of the Ah receptor in C57BL/6 mice but [^{3}H]3-MC could not be used to detect the Ah receptor in Dub:ICR mice (Fig. 2, Tables 2 and 3). Both TCDD and 3-MC displaced either [^{3}H]TCDD or [^{3}H]3-MC from the Ah receptor to the same extent (Tables 2 and 3). This was not the case for the 3-4S peak. 3-MC was unable to displace [^{3}H]TCDD from the 3-4S peak (Table 2), while TCDD displaced about one-half of the [^{3}H]3-MC from the 3-4S peak when compared to 3-MC (Table 3).

The *in vivo* effects of Dub:ICR mice treated with 3-MC, DBBD, and 3-MC + DBBD on the Ah receptor and the 3-4S peak are shown in Fig. 3. [^{3}H]TCDD was the radioligand used to measure the Ah receptor and the 3-4S peak. Mice treated with a single 80 mg/kg i.p. dose of 3-MC showed a 2-fold reduction in the Ah receptor level at 2 hr, followed by complete recovery at 12 hr. The 3-4S peak was reduced less than 1.5-fold 2 hr after 3-MC treatment and recovered fully at 12 hr. In contrast, the 3-4S peak was unaffected in mice treated with DBBD (500 mg/kg), while the Ah receptor was depressed at 6 hr, recovered at 12 hr, followed by a 2-fold reduction at 24 hr with recovery at 48 hr. When mice were simultaneously given 3-MC (80 mg/kg) and DBBD (500 mg/kg), an additive response was seen (Fig. 3). The Ah receptor was depressed more than 3-fold at 2 hr and 2-fold at 24 hr. The 3-4S peak was depressed slightly at 2 hr but recovered fully at 6 hr.

Effects of 2,2-dimethyl-5-t-butyl-1,3-benzodioxole pretreatment on the Ah receptor and 3-methylcholanthrene induction of cytochrome P-450. Dub:ICR mice were pretreated with either corn oil or 500 mg/kg DBBD for 3 days. On day 4 mice either were killed and the Ah receptor levels measured, or they were treated with 40 mg/kg 3-MC or oil alone. Those mice who received the oil or 3-MC treatment on day 4 were killed 24 hr later (day 5), and the cytochrome P-450 content and monooxygenase activities were measured. The DBBD-pretreated mice had an Ah receptor level of 12 fmoles/mg protein on day 4 compared to 27 fmoles/mg protein for the oil-pretreated mice, which represents a 2.25-fold reduction in the Ah receptor (Table 4). For the mice killed on day 5, the oil–3-MC-treated mice had a greater cytochrome P-450 content and greater benzo[*a*]pyrene hydroxylase and ethoxyresorufin *O*-deethylase activities than either the DBBD–3-MC-treated mice or oil–oil-treated control mice (Table 4). The DBBD–3-MC-treated mice had a similar cytochrome P-450 content and similar benzo[*a*]pyrene hydroxylase and ethoxyresorufin *O*-deethylase activities when compared to the oil–oil-treated control mice.

The gel in Fig. 4 shows that microsomes from the treated mice had the following molecular weight bands induced: oil–3-MC–53,000 and 54,000; and DBBD–3-MC–53,000; 53,000, 54,000, 70,000 and 78,000. DBBD induced a 53,000 molecular weight band which is just below the 3-MC molecular weight band. This gel demonstrates that the DBBD pretreatment reduced the 3-MC induction of the 54,000 molecular weight band.

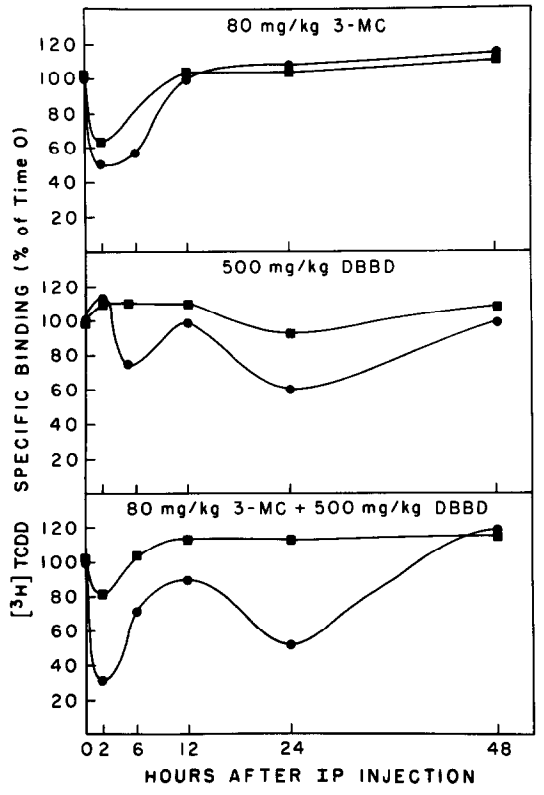

Fig. 3. Effects of 3-MC, DBBD, and 3-MC + DBBD treatment, *in vivo*, on the Ah receptor (●——●) and 3-4S peak (■——■) in Dub:ICR mice. Mice received i.p. injections of 80 mg/kg 3-MC, 500 mg/kg DBBD, or 80 mg/kg 3-MC and 500 mg/kg DBBD and were killed at the indicated times; then cytosol was prepared. Three animals were used for each time point. The 0 hr group received an i.p. injection of corn oil immediately before decapitation. Cytosol was incubated with 10 nM [^{3}H]TCDD. Specific binding was expressed as the percentage of the 0 hr group. For further details, see sucrose density gradient analysis in Materials and Methods.

DISCUSSION

DBBD, when administered simultaneously with 3-MC, has been shown to attenuate 3-MC induction of cytochrome P-450 yet has no effect on phenobarbital induction of cytochrome P-450 in Dub:ICR mice, an outbred stock [5]: Coadministration of DBBD with either phenobarbital or 3-MC produced a similar effect in C57BL/6 mice, an Ah responsive inbred strain (Table 1). Mice treated with phenobarbital or phenobarbital + DBBD had similar cytochrome P-450 contents and monooxygenase activities, although the 7-ethoxycoumarin *O*-deethylase activity induced by phenobarbital was slightly lower in the presence of DBBD. This decrease is small compared to the large decreases seen for the 3-MC + DBBD-treated mice. In contrast, the cytochrome P-450 content and the benzo[*a*]pyrene hydroxylase, ethoxyresorufin *O*-deethylase, and 7-ethoxycoumarin *O*-deethylase activities for the 3-MC + DBBD-treated mice were all lower than those for mice treated with 3-MC alone (Table 1). SDS–PAGE confirmed that 3-MC induction of cyto-

Table 4. Effect of DBBD pretreatment on the Ah receptor and 3-MC induction of cytochrome P-450 in Dub:ICR mice

Treatment (days 1–3)	Ah receptor level on day 4 (fmoles/mg protein)	Treatment (day 4)	Cytochrome P-450 on day 5 (nmoles/mg protein)	Benzo[*a*]pyrene hydroxylase on day 5 (nmoles/min/mg protein)	Ethoxyresorufin *O*-deethylase on day 5 (nmoles/min/mg protein)
Oil		Oil	1.07	1.52	0.37
Oil	27*	3-Methylcholanthrene in oil	1.31†	3.33†	1.45†
DBBD in oil	12	3-Methylcholanthrene in oil	1.07	1.66	0.58

Values are expressed as a mean of three experiments. The average S.E. per compound was 2, 0.07, 0.42, and 0.31 for the Ah receptor, cytochrome P-450, benzo[*a*]pyrene hydroxylase, and ethoxyresorufin *O*-deethylase respectively. [^{3}H]TCDD was used as the radiolabeled ligand to measure the Ah receptor.

* $P < 0.01$, compared to DBBD.

† $P < 0.01$, compared to either the oil–oil control or DBBD–3-methylcholanthrene.

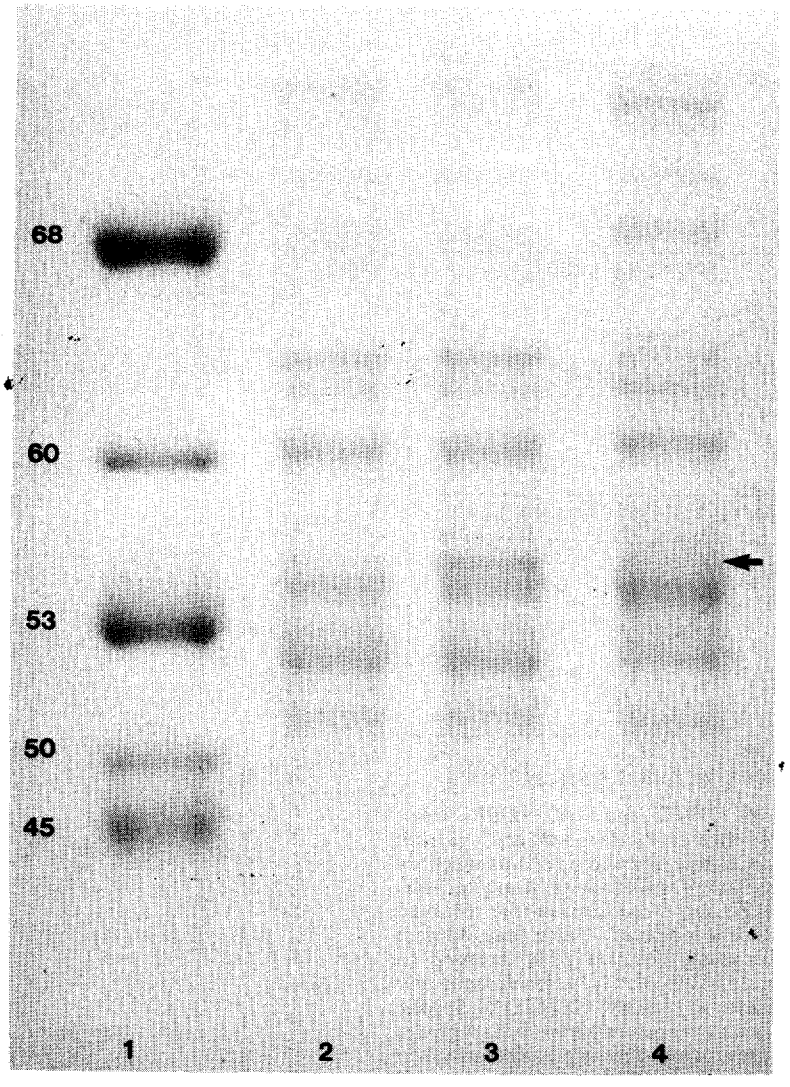

Fig. 4. SDS–PAGE of hepatic microsomes from Dub:ICR mice treated with various inducing agents. A mixture of molecular weight standards was applied to well 1. Microsomes (15 μg protein) from treated mice were applied as indicated: (2) oil–oil, (3) oil–3-MC, and (4) DBBD–3-MC. Migration is from the top of the gel to the bottom. Figures on the left represent molecular weight × 10^3. The arrow points to the 54,000 molecular weight band region. Note the decreased intensity of the DBBD–3-MC 54,000 molecular weight band compared to the oil–3-MC band.

chrome P-450 was reduced by DBBD. Both the 53,000 and 54,000 molecular weight bands in the 3-MC + DBBD-treated mice had a decreased staining intensity compared to the same bands in the 3-MC-treated mice (Fig. 1). In Dub:ICR mice, only the 54,000 molecular weight band was decreased [5]. No such reduction was seen for phenobarbital- and phenobarbital + DBBD-treated mice. Hence, these data demonstrate that DBBD antagonized 3-MC induction of cytochrome P-450 in C57BL/6 mice, as was seen with Dub:ICR mice [5].

Competition between 3-MC and DBBD for binding to the Ah receptor has been suggested as a possible mechanism for DBBD attenuation of cytochrome P-450 induction by 3-MC [5]. To test this hypothesis, the ability of DBBD to displace [^{3}H]-TCDD and [^{3}H]3-MC from the Ah receptor was examined. DBBD as well as its analogs, BBD and MBBD, were unable to displace [^{3}H]TCDD or [^{3}H]-3-MC from the Ah receptor or the 3-4S peak in C57BL/6 and Dub:ICR mice, *in vitro* (Fig. 2, Tables 2 and 3). These data suggest that DBBD does not compete with [^{3}H]3-MC or [^{3}H]TCDD for binding to the Ah receptor.

Dub:ICR mice were treated with 3-MC, DBBD, and 3-MC, DBBD, *in vivo*, and the Ah receptor and 3-4S peak levels were measured over a 48-hr period using [^{3}H]TCDD (Fig. 3). Mice treated with 3-MC had a 2-fold reduction in the Ah receptor level at 2 hr followed by complete recovery at 12 hr. Okey *et al.* [11] reported a similar decrease in the Ah receptor level after treatment of mice, *in vivo*, with TCDD, 3-MC, or β-naphthoflavone. This decrease in the cytosolic Ah receptor level can be interpreted as either Ah receptor occupation by the ligand [11], translocation of the receptor into the nucleus [11],

or binding of the ligand–receptor complex with chromatin which reduces receptor redistribution into the cytosol [18]. Whatever the interpretation, the attenuation of the Ah receptor level demonstrates that the test compound either interacts with the receptor or affects receptor synthesis or degradation. In addition, the 3-4S peak was decreased by *in vivo* treatment of mice with 3-MC which is consistent with the *in vivo* displacement data (Fig. 2, Tables 2 and 3). The role of the 3-4S peak as a possible carrier protein for polycyclic aromatic hydrocarbons has been discussed in detail [22, 23, 36].

Treatment of Dub:ICR mice with DBBD resulted in an early Ah receptor depression at 6 hr and a later Ah receptor depression at 24 hr with recovery in between (Fig. 3). This second Ah receptor depression has not been observed with other compounds [11, 12]. The 3-4S peak was unaffected throughout the 48 hr of measurement. Both *in vitro* and *in vivo* data (Tables 2 and 3, Fig. 3) demonstrate that the 3-4S peak played no role in DBBD antagonism of cytochrome P-450 induction by 3-MC. However, unlike the *in vitro* data, the *in vivo* data suggest that DBBD interacts with the Ah receptor or affects receptor synthesis or degradation (Fig. 3). This contradiction may be due either to inadequate optimization of the *in vitro* assay conditions or to a DBBD metabolite formed, *in vivo*, which competes for the Ah receptor. The use of sucrose gradients assumes that the radiolabeled ligand concentration is saturating, there is minimal complex dissociation during centrifugation, and the *in vivo* treatments which affect this complex alter the receptor level and not its binding affinity for the ligand. In the present studies, preliminary experiments (data not shown) established that the [^{3}H]TCDD and [^{3}H]3-MC concentrations were saturating under the conditions used.

When 3-MC and DBBD were coadministered, the depression of the Ah receptor and the 3-4S peak was additive (Fig. 3). To examine the possibility that the 24 hr Ah receptor depression is physiologically significant, mice were pretreated with DBBD, and the Ah receptor and 3-MC induction of cytochrome P-450 were measured. Table 4 shows that DBBD pretreatment produced a 2.25-fold reduction in the Ah receptor when compared to the oil control. This DBBD pretreatment blocked 3-MC from increasing the cytochrome P-450 content and benzo[*a*]pyrene hydroxylase and ethoxyresorufin *O*-deethylase activities (Table 4). SDS–PAGE confirmed that 3-MC induction of cytochrome P-450 was inhibited by DBBD pretreatment (Fig. 4). This experiment correlated the reduction in the Ah receptor level produced by DBBD with the inability of 3-MC to induce cytochrome P-450. Therefore, the 24-hr Ah receptor depression appears to be physiologically significant and may result in the diminished capacity of 3-MC to induce cytochrome P-450.

Mice were also treated with a single i.p. injection of 3-MC or 3-MC + DBBD, killed 24 hr later, and their cytochrome P-450 content and benzo[*a*]pyrene hydroxylase and 7-ethoxyresorufin *O*-deethylase activities measured (data not shown). As we have seen before, the 3-MC-treated mice had a greater cytochrome P-450 content and greater monooxygenase activities than those mice treated with 3-MC + DBBD. These data suggest that the early Ah receptor depression also contributes to the ability of DBBD to antagonize cytochrome P-450 induction by 3-MC. Tukey *et al.* [14] have shown a dose–response relationship between the amount of [^{3}H]TCDD–Ah receptor complex appearing in the nucleus and the amount of P_1-450 mRNA induced. Under these circumstances a reduction in the amount of the 3-MC–Ah receptor complex reaching the nucleus could reduce cytochrome P-450 induction.

The later Ah receptor depression at 24 hr can be explained by either DBBD interacting with the Ah receptor or a DBBD-induced downregulation of the Ah receptor, particularly since DBBD is not an effective competitor *in vitro*. No other compound as yet has been shown to be an Ah receptor antagonist [7] or produce receptor downregulation. DBBD antagonism of cytochrome P-450 induction by 3-MC may be due to a combination of direct competition as evidenced by the early Ah receptor depression followed by downregulation as seen by the later Ah receptor depression (Fig. 3). However, the data (Fig. 3, Table 4) suggest that the later Ah receptor depression occurring at 24 hr plays a more important role in DBBD antagonism. Since TCDD toxicity is correlated with binding to the Ah receptor [37], DBBD may be a useful tool in elucidating the mechanism of TCDD toxicity.

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