0026-895X/010113-08$02.00/0

MOLECULAR PHARMACOLOGY, 36:113–120

[^{125}I]2-Iodo-3,7,8-trichlorodibenzo-*p*-dioxin-Binding Species in Mouse Liver Induced by Agonists for the Ah Receptor: Characterization and Identification

ALAN POLAND, PHILLIP TEITELBAUM, EDWARD GLOVER

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706 (A.P., E.G.) and Syntex Corporation, Palo Alto, California (P.T.)

Received November 28, 1988; Accepted March 8, 1989

SUMMARY

The administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to C57BL/6J mice produces a dose-related increase in the hepatic uptake of [^{125}I]2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin ([^{125}I]Cl_3D*p*D) *in vivo* and the binding of the radioligand to liver homogenate *in vitro* [*Mol. Pharmacol.* **36**: 121–127 (1989)]. The TCDD-induced hepatic binding species was found to be predominantly in the microsomal fraction and was inactivated by heating at 60°, trypsin, and mercurials. The TCDD-induced binding species was found to have an apparent equilibrium dissociation constant, K_D, ([^{125}I]Cl_3D*p*D) of 56 ± 16 nM and a pool size, B_{max}, of 22 ± 5 nmol/g of liver. A number of halogenated dibenzo-*p*-dioxins, biphenyls, and polycyclic aromatic hydrocarbons compete with [^{125}I]Cl_3D*p*D binding to this species; all are aromatic and planar. The distinctive profile of this binding species, a protein of large pool size induced in the microsomal fraction of liver but not other tissues and induced by agonists for the Ah receptor, suggested that this moiety might be cytochrome P_3-450. The coincidence of the major microsomal species covalently labeled with the photoaffinity ligand [^{125}I]2-iodo-3-azido-7,8-dibromodibenzo-*p*-dioxin and that immunochemically stained with polyclonal antiserum that binds to cytochrome P_3-450 confirms this hypothesis. This is a novel role for a cytochrome P-450 isozyme, as an induced sequestration site that enhances the hepatic localization of the agonist drug.

TCDD serves as the prototype for a large group of toxic environmental contaminants, the halogenated aromatic hydrocarbons (i.e., certain chlorinated or brominated congeners of dibenzo-*p*-dioxins, dibenzofurans, azo(*xy*)benzenes, and biphenyls), which are thought to exert most or all of their biological effects by stereospecific binding to a soluble protein, the Ah receptor, and the altered gene expression initiated by this ligand-receptor complex (1). These compounds share (a) isosterism, which accounts for receptor binding and (b) lipophilicity and chemical inertness, which account for their persistence in the environment, accumulation in the biological food chain, and generally long biological half-lives in animals. The relative toxic potency of these compounds on chronic administration can be largely accounted for by two factors, their binding affinity for the Ah receptor (K_D) and their rate of elimination.

The pharmacokinetics of TCDD have been investigated in rats (2, 3) mice (4), hamsters (5), guinea pigs (6, 7) nonhuman primates (8), and humans (9, 10). In the rodent species, TCDD is (a) primarily concentrated in liver and adipose tissue, (b) slowly metabolized to polar products that are eliminated in urine and feces (with some elimination of unmetabolized TCDD in guinea pigs and hamsters), and (c) eliminated by first-order kinetics, with a whole body half-life that varies from 10 to 35 days for these species (see Ref. 7 for exception). Of particular note is the relatively high concentration of TCDD in liver in rats and mice, where the hepatic concentration is equivalent to 20–40% of the total dose administered, higher than in other species (11) and higher than that seen with other comparably lipophilic compounds, which are primarily stored in adipose tissue.

Teitelbaum (12) observed that TCDD administration to C57BL/6J mice produced a dose-related stimulation of (a) the hepatic uptake of a tracer dose of [^{3}H]TCDD *in vivo* and (b) the binding of radioligand by liver homogenate from these mice *in vitro*. In the accompanying paper, we have reproduced these studies using [^{125}I]Cl_3D*p*D as a radioligand (13). These observations suggest that TCDD (and other Ah receptor agonists) induce a hepatic binding species that is responsible for the hepatic localization of these compounds.

This work was supported in part by the National Institute of Environmental Health Science Grant ES-01884 and the National Cancer Institute Core Grant 07175.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [^{125}I]Cl_3D*p*D, [^{125}I]2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin; [^{125}I]N_3Br_2D*p*D, [^{125}I]2-iodo-3-azido-7,8-dibromodibenzo-*p*-dioxin; BSA, bovine serum albumin; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; MOPS, 3-[*N*-morpholino] propanesulfonic acid.

In this report, we examine the properties of this TCDD-induced hepatic binding species, its subcellular localization, structure-binding relationship, binding affinity and pool size, and from this characterization we identify this moiety as cytochrome P_3-450.

Experimental Procedures

Materials. Trypsin (bovine pancreas Type I), *p*-hydroxymercuribenzoate, Tween 20, BSA (No. A-7638, globin-free for blotting, or No. A-4503, for incubation in the binding assay), 2-mercaptoethanol, and 3,3′-diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal goat anti-rabbit immunoglobulin G antisera (61-003-2) and peroxidase/rabbit antiperoxidase (PAP-61-242-2) were purchased from ICN, Immunobiologicals Division (Lisle, IL). GF/A glass filters (2.4 cm diameter) were obtained from Whatman, Inc. (Clifton, NJ), and nitrocellulose sheets (0.45 μm) were bought from Bio-Rad Laboratories (Richmond, CA). The halogenated dibenzo-*p*-dioxins and dibenzofurans were synthesized in our laboratory or were gifts of other investigators, as previously reported. The chlorinated biphenyls were obtained from Analabs, Inc. (New Haven, CT) or Ultra Scientific (Hope, RI), and the polycyclic aromatic hydrocarbons were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Eastman Kodak Chemicals (Rochester, NY).

Rabbit polyclonal antiserum to rat cytochrome P-450c was kindly provided by Dr. Colin Jefcoate (Dept. of Pharmacology, University of Wisconsin Medical School). Details of cytochrome P-450 purification (14), the method of immunization (15), and immunoglobulin purification (16) have been published previously.

[^{125}I]Cl_3D*p*D and [^{125}I]N_3Br_2D*p*D. [^{125}I]Cl_3D*p*D was synthesized and purified as reported previously (13), with an initial specific activity of 217 Ci/mmol. The photoaffinity ligand [^{125}I]N_3Br_2D*p*D was synthesized and purified as previously reported (17), with an initial specific activity of 2176 Ci/mmol.

Animals. C57BL/6J mice, originally obtained from The Jackson Laboratory (Bar Harbor, ME), were bred and maintained in our animal quarters. Congenic C57BL/6J (Ah^d/Ah^d) mice were bred as previously described (13).

Standard binding assay. [^{125}I]Cl_3D*p*D binding to liver homogenate was determined as previously reported (13) unless noted otherwise. The standard assay conditions consisted of a 1-ml incubation that contained 0.25 mg of liver homogenate from TCDD-treated C57BL/6J mice in MEN buffer (25 mM MOPS, 1 mM EDTA, 0.02% NaN_3, pH 7.5), 10 mg of BSA (Sigma No. A-4503), and 0.3 pmol of [^{125}I]Cl_3D*p*D (initial specific activity, 272 Ci/mmole or ~1.44 × 10^5 dpm) added in dimethyl sulfoxide (≤10 μl). The mixture was incubated at 20° for 45 min, and the bound ligand was separated by filtration on GF/A glass filters (2.4-cm diameter, washed, and quantified by γ-scintillation counting (Minaxi 5000; Packard-United Technologies, Des Plaines, IL). Nonspecific binding was determined as binding to heat-inactivated homogenate (60° for 10 min). [^{125}I]Cl_3D*p*D binding is reported as either total ligand binding or specific ligand binding (total minus nonspecific binding).

Competitive binding assay. Competitive binding was determined by incubation of varying concentrations of unlabeled ligand with [^{125}I]Cl_3D*p*D, as outlined above. Nonspecific binding (i.e., binding to heat-inactivated homogenate) was determined and subtracted from each value of total binding to give specific binding. Specific binding was plotted as a function of the log of the competing ligand concentration, and the concentration that reduced maximal (uncompeted) binding by 50%, EC_{50}, was estimated graphically.

Gel electrophoresis, transfer to nitrocellulose, immunochemical staining, and autoradiography. The photoaffinity-labeled microsomal fractions (see legend to Fig. 3) were collected on GF/A filters (2.4-cm diameter), washed, and eluted with 2% lithium dodecyl sulfate. The samples were resolved by denaturing electrophoresis on a discontinuous polyacrylamide slab gel using the method of Laemmli (18) (acrylamide/bisacrylamide, 30:0.8; 4% stacking gel; 9% separating gel; 1.5-mm thick), with 15 mA current. The proteins were transferred to a 9 × 15 cm sheet of nitrocellulose, by the general procedure of Towbin *et al.* (19) and Glass *et al.* (20), in a Trans-blot apparatus (Bio-Rad) using 200 mA for 2 hr. The nitrocellulose blot was briefly rinsed with PBST buffer (20 mM potassium phosphate, 150 mM potassium chloride, 0.3%, w/v, Tween 20, pH 7.4) at 20° and was then shaken overnight with PBST buffer that contained 1% BSA (Sigma No. A-7638, globin-free) and 0.02% NaN_3, at 4°, for blocking. After rinsing, the blot was incubated with the primary polyclonal antiserum (rabbit anti-rat cytochrome P-450c) which was diluted 1:1000 in PBST buffer, in a sealed polyethene bag for 2 hr at 20°. The blot was rinsed, incubated with the secondary antibody (goat anti-rabbit immunoglobulin G), which was diluted 1:200 in PBST buffer, for 30 min at 20°, and rinsed. The blot was incubated with peroxidase/rabbit antiperoxidase (ICN No. 61-242-2) complex, which was diluted 1:1500 in PBST buffer, for 30 min and rinsed. Finally, the peroxidase stain was developed by placing the blot in a solution of 0.8 mM 3,3′-diaminobenzidine tetrahydrochloride, 1.4 mM H_2O_2, and 50 mM Tris, pH 7.6, for 10 min. The blot was then rinsed, dried, and exposed to XAR-5 film (Eastman Kodak Co.) with an intensifying screen (Cronex; Dupont, Wilmington, DE) at −70° to develop the autoradiograph (21).

Results

Binding of [^{125}I]Cl_3D*p*D to subcellular fractions of liver homogenate. Liver homogenates from control (solvent-treated) and TCDD-treated C57BL/6J mice were incubated with radioligand (10^{-10} M) and subjected to subcellular fractionation (Table 1). In homogenate from control mice ~25% of the radiolabel was found in the 100,000 × *g* pellet, 42% was associated with the cytosol fraction, and remainder was distributed in the crude nuclear and mitochondrial pellets. In contrast, in liver homogenate from TCDD-treated mice 65% of the added [^{125}I]Cl_3D*p*D was associated with the microsomal fraction, with the remainder about equally distributed between the 600 × *g* pellet, the 10,000 × *g* pellet, and the 100,000 × *g* supernatant fraction. Incubation of liver homogenate from TCDD-treated animals with a 100-fold greater concentration of radioligand (10^{-8} M) produced a similar fractionation pattern, indicating the pool of sites in the microsomal pellet is not saturable by this concentration of ligand. [^{125}I]Cl_3D*p*D (1 × 10^{-10} mol/kg) was administered to mice pretreated with the solvent (control) or TCDD, the animals were killed 24 hr later, and their liver homogenates were subjected to the same fractionation scheme. As expected, the total radioactivity in TCDD-treated liver was higher (~9-fold) than in control liver. The microsomal fraction of the liver homogenate from control animals contained 48% of the radioactivity, whereas that from TCDD-treated animals contained 68% of the radiolabel. From these studies we conclude that the major ligand-binding species in liver homogenate that is increased by TCDD administration is located in the microsomal fraction.

Biochemical characterization. In Table 2 are shown the effects of various *in vitro* treatments on the total ligand binding of liver homogenate from TCDD-treated mice. Heating the homogenate at 60° produces a rapid loss in binding capacity, with a maximal effect by 10 min. The residual binding capacity in heat-inactivated homogenate from TCDD-treated mice is considered nonspecific binding and is roughly equivalent to the binding capacity of homogenate from control mice (data not shown). The binding capacity of this liver homogenate is diminished by mercurials and *p*-dioxane and by proteolysis with

TABLE 1

Localization of [^{125}I]Cl_3DpD in liver cell fractions

C57BL/6J male mice were treated with TCDD (3×10^{-7} mol/kg) in *p*-dioxane or the vehicle alone intraperitoneally and were (a) killed 72 hr later for *in vitro* incubation with [^{125}I]Cl_3DpD or (b) treated with [^{125}I]Cl_3DpD (1×10^{-10} mol/kg) after 48 hr and killed 24 hr later. For each treatment group, the livers from three mice were pooled, weighed, homogenized in 9 volumes (v/w) of MEN buffer containing 0.25 M sucrose, and filtered through 100-μm nylon mesh. For the sample incubated *in vitro*, 10^{-10} or 10^{-8} M radioligand (10^{-10} M [^{125}I]Cl_3DpD plus 100-fold excess unlabeled ligand) was added to the homogenate and incubated for 45 min at 20°. For each homogenate, the radioactivity was determined for a 0.5-ml aliquot and expressed for the entire 5-ml sample in the first line of the table (dpm of homogenate = 100%). Five milliliters of each homogenate were subjected to sequential centrifugation, 600 × g for 10 min, 10,000 × g for 10 min, and 100,000 × g for 1 hr. Each pellet was resuspended in buffer and resedimented. The radioactivity in each fraction was determined and expressed as the percent of total dpm in the homogenate. The sum of dpm in all fractions was equal to 93 to 98% of that in the homogenate.

Cell Fraction	[^{125}I]Cl_3DpD Distribution				
	In vitro incubation			*In vivo* administration	
	Control (10^{-10} M ligand)	TCDD-treated (10^{-10} M ligand)	TCDD-treated (10^{-8} M ligand)	Control	TCDD-pretreated
			dpm		
Homogenate	238,200[a]	235,000	238,000	11,400	95,100
			% of homogenate		
600 × *g* pellet	15.4	9.0	8.9	17.3	13.6
10,000 × *g* pellet	10.8	8.7	9.1	11.7	12.4
100,000 × *g* pellet	24.7	65.3	67.7	48.3	67.7
100,000 × *g* supernatant	42.4	10.5	11.1	19.0	4.3

[a] dpm in 5 ml of homogenate = 100%.

TABLE 2

Effect of various agents on [^{125}I]Cl_3DpD binding

Liver homogenate from TCDD-treated mice, at 2 mg wet weight/ml in MEN buffer, was subjected to the indicated treatments and then diluted with MEN to 0.5 mg/ml and binding was determined under standard conditions (0.25 mg of homogenate, 10 mg/ml BSA, and 0.3 pmol of radioligand/ml for 45 min at 20°). Following the addition of $HgCl_2$, *p*-hydroxymercuribenzoate, trypsin, and *p*-dioxane, the homogenate was incubated for 30 min at 20° and then overnight at 4° before dilution. The values for radioactivity were rounded to the nearest 100 dpm.

Treatment	Total Bound Ligand	
	dpm	*% of initial binding*
Homogenate (control)	15,800	100
60°		
2 min	12,400	78.5
5 min	5,300	33.5
10 min	3,700	23.4
15 min	3,700	23.4
$HgCl_2$		
200 μM	13,000	82.3
500 μM	10,500	66.5
1 mM	3,500	22.2
2 mM	3,100	19.6
p-Hydroxymercuribenzoate		
200 μM	15,200	96.2
500 μM	11,700	74.1
1 mM	7,400	46.8
2 mM	4,300	27.2
Homogenate (control)	17,500	100
p-Dioxane, 50 μl/ml	4,100	23.4
Trypsin, 100 μg/ml	2,600	14.9

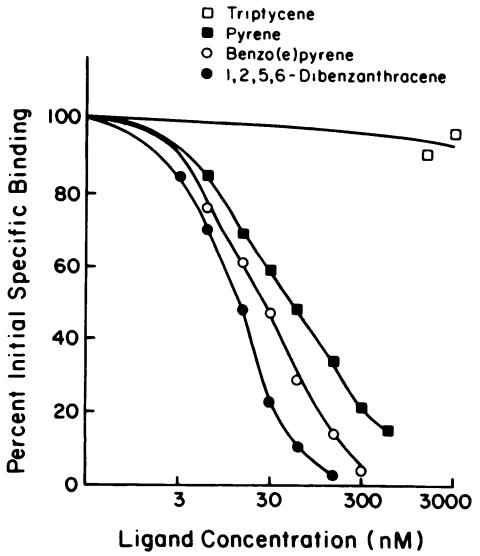

Fig. 1. Competitive binding of polycyclic aromatic hydrocarbons. Competitive binding of polycyclic aromatic hydrocarbon congeners was determined in the standard assay that contained 0.3 pmol of [^{125}I]Cl_3DpD, 10 mg of BSA, and 0.25 mg of liver homogenate from TCDD-treated mice, in a 1-ml volume (see Experimental Procedures). In the absence of competitor, total binding of [^{125}I]Cl_3DpD was 17,337 dpm, nonspecific binding was 4,128 dpm, and initial specific binding was 13,209 dpm (100%).

trypsin. These observations suggest that the specific binding moiety is protein in nature.

Competitive binding. We examined the capacity of a variety of compounds to compete with [^{125}I]Cl_3DpD for specific binding to liver homogenate from TCDD-treated mice. In Fig. 1 are seen typical competitive binding curves, and in Table 3 is presented the summary of these experiments. A number of halogenated dibenzo-*p*-dioxins, halogenated biphenyls, and polycyclic aromatic hydrocarbons, compounds that are all aromatic and planar (or can assume a planar configuration), compete for binding. Nonplanar compounds were inactive, e.g., triptycene, *ortho*-chloro-substituted biphenyls, and several nonaromatic or cage-like compounds.

The overall structure-activity relationship for binding to liver homogenate, at first glance, is similar to that for binding to the Ah receptor; however, on closer examination, the differences are obvious. For instance, benzo(*e*)pyrene, 3,3′,5,5′-tetrachlorobiphenyl, and 1-chloro-, 2,3-dichloro-, 2,7-dichloro-, and 1,2,3,4-tetrachlorodibenzo-*p*-dioxin bind to the TCDD-stimulated liver homogenate species but do not compete for the Ah receptor (22, 23). The relatively high concentration of unlabeled 2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin (EC_{50} = 90 nM) and other compounds necessary to decrease by one half the binding of [^{125}I]Cl_3DpD (0.3 nM) is noteworthy and most likely is attributable to the large pool of binding sites (see below).

Affinity and pool size of binding moiety in liver homogenate. A saturation binding isotherm was generated by incubating liver homogenate from TCDD-treated mice with

TABLE 3

Competitive binding of compounds with [^{125}I]Cl_3D*p*D for the TCDD-induced liver homogenate binding species

The capacity of various compounds to compete with [^{125}I]Cl_3D*p*D (0.3 nM) for specific binding to liver homogenate from TCDD-treated C57BL/6J mice was determined (as detailed in Experimental Procedures). The table is a composite of many experiments. *Inactive* indicates that there was no significant reduction in radioligand binding by a competing ligand concentration of 3000 nM.

Compound	EC_{50}
	nM
Dibenzo-*p*-dioxins	
1-Chloro-	300
2,3-Dichloro-	120
2,7-Dichloro-	105
2,3-Dichloro-7-hydroxy-	300
1,2,3,4-Tetrachloro-	120
2,3,7,8-Tetrachloro-	30
2-Iodo-7,8-dibromo-	105
2-Iodo-3,7,8-trichloro-	90
Dibenzofuran	60
2,3,7,8-Tetrachloro-	
Biphenyls	
2,2′,3,3′Tetrachloro-	Inactive
3,3′,4,4′-Tetrachloro-	30
3,3′,5,5′-Tetrachloro-	750
2,2′,3,3′,4,4′-Hexachloro-	Inactive
2,2′,4,4′,6,6′-Hexachloro-	Inactive
2,2′,3,3′,4,4′,5,5′,6,6′-Dodecachloro	Inactive
3,3′,5,5′-Tetrachloro-4,4′-diol	Inactive
Polycyclic aromatic hydrocarbons	
1,2,5,6-Dibenzoanthracene	14
Benzo(*a*)pyrene	15
Benzo(*e*)pyrene	27
Triphenylene	57
Pyrene	60
Triptycene	Inactive
β-Naphthoflavone	30
α-Naphthoflavone	150
Miscellaneous	
α-Hexachlorocyclohexane	Inactive
Mirex	Inactive
Kepone	Inactive
Dieldrin	Inactive
Hemin	Inactive
17β-Estradiol	Inactive
Pregnenolone-16α-carbonitrile	Inactive
3,3′,4,4′-Tetrachlorobiphenylether	Inactive
2,6-Dichlorobenzonitrile	Inactive
4,4′-Benzophenone	Inactive

various concentrations of radioligand (Fig. 2A). Nonspecific binding was determined as that observed with heat-inactivated homogenate. Specific binding (total binding minus nonspecific binding) was determined and plotted according to the method of Scatchard (24) (Fig. 2B). In a series of five experiments, the average apparent equilibrium dissociation constant, K_D, was 56.0 ± 15.5 nM, the pool size, B_{max}, was 22.0 ± 5.2 nmol/g of liver (5.51 pmol/0.25 mg of liver homogenate), and the correlation coefficient of the regression line, r was 0.960 ± 0.017. The estimates of K_D and B_{max} must be viewed as rough approximations, because the binding assay is dependent on the addition of a large pool of competing low affinity sites, i.e., BSA.

Identification of the [^{125}I]Cl_3D*p*D-binding species. It is useful to summarize the distinctive properties of the [^{125}I]Cl_3D*p*D-binding moiety. There is a small amount of specific binding in the liver of control mice (i.e., total ligand binding minus binding to heat-inactivated homogenate). The administration of TCDD or other agonists for the Ah receptor greatly increases specific ligand binding in liver but not in other tissues examined. The [^{125}I]Cl_3D*p*D-binding species is a hepatic microsomal protein. There is a large amount of this binding species in liver of TCDD-treated mice (B_{max} = 20 nmol/g of liver). This moiety has a well defined structure-binding relationship, with all ligands being aromatic and planar.

TCDD (and other agonists for the Ah receptor) induce two major proteins in the microsomal fraction of liver, cytochromes P_1-450 and P_3-450.[1] Cytochrome P_1-450 is inducible in liver and other tissues, whereas cytochrome P_3-450 is inducible only in liver (26). Thus, cytochrome P_3-450 is a likely candidate for the [^{125}I]Cl_3D*p*D-binding species.

To test this hypothesis, we covalently labeled the microsomal fraction with an ^{125}I-photoaffinity ligand (an analogue of [^{125}I]Cl_3D*p*D), resolved the microsomal proteins by gel electrophoresis, and searched for the coincidence of a protein(s) that was immunochemically stained with antibody to cytochrome P_3-450 and was radiolabeled by the photoaffinity ligand. The validity of the experiment resides in the specificity of the two reagents. [^{125}I]N_3Br_2D*p*D is a selective photoaffinity label for the Ah receptor (17). Given the general similarity of the structure-binding relationship for the Ah receptor and the TCDD-induced microsomal binding species, this compound holds promise as a selective label for the latter. We had available a polyclonal rat antiserum to rat cytochrome P-450c that cross-reacts with mouse cytochromes P_1-450 and P_3-450.[2]

We first compared the reversible binding of [^{125}I]Cl_3D*p*D and [^{125}I]N_3Br_2D*p*D to the microsomal fraction of various tissues (Table 4). Total binding of [^{125}I]Cl_3D*p*D to liver microsomes from control mice is relatively low, and TCDD treatment of mice greatly enhances total binding (TCDD-treated/control liver microsomal binding = 11-fold). In contrast, [^{125}I]N_3Br_2D*p*D total binding to the hepatic microsomal fraction from control animals is always higher than that of [^{125}I]Cl_3D*p*D, and the ratio of binding in TCDD-treated/control liver microsomes is lower, ~3-fold. The binding of [^{125}I]N_3Br_2D*p*D is qualitatively similar to that of [^{125}I]Cl_3D*p*D but is less selective.

Aliquots of the same microsomal preparations used in the experiments shown in Table 4 were incubated with [^{125}I]N_3Br_2D*p*D and covalently labeled by photolysis and the components were resolved by denaturing gel electrophoresis and transferred to nitrocellulose. Shown in Fig. 3A is the immunochemical stain of the Western blot, using polyclonal antiserum that stains both cytochrome P_1-450 (the slower migrating band) and cytochrome P_3-450 (the faster migrating band). In liver microsomes from control mice, there is a detectable quantity of cytochrome P_3-450. TCDD administration induces both proteins in liver microsomes, but only cytochrome P_1-450 is induced in kidney and lung. In Fig. 3B, an autoradiograph of the nitrocellulose blot, it can be seen that the photoaffinity ligand labels both proteins in liver microsomes from TCDD-induced animals, but cytochrome P_3-450 is more extensively labeled.

[1] Cytochromes P_1-450 (M_r = 58,900) and P_3-450 (M_r = 58,200) are the major cytochromes in C57BL/6 mouse liver induced by Ah agonists, and their cloned sequences exhibit overall nucleotide and amino acid homology of 68 and 73%, respectively (27). Cytochrome P_1-450 has the highest turnover number for aryl hydrocarbon hydroxylase activity and cytochrome P_3-450 has the highest turnover number for acetanilide 4-hydroxylase activity. The orthologous form of cytochrome P_1-450 in rat is P-450c, and in rabbit LM_6; the orthologous form of cytochrome P_3-450 in rat is P-450d, and in rabbit LM_4 (28).

[2] Rabbit polyclonal antiserum to rat cytochrome P-450c recognizes common epitopes on rat P-450c and P-450d and on orthologous mouse P_1-450 and P_3-450 (28, 29). The two murine proteins have amino acid sequences that are 93% similar to those of their respective rat homologs (28).

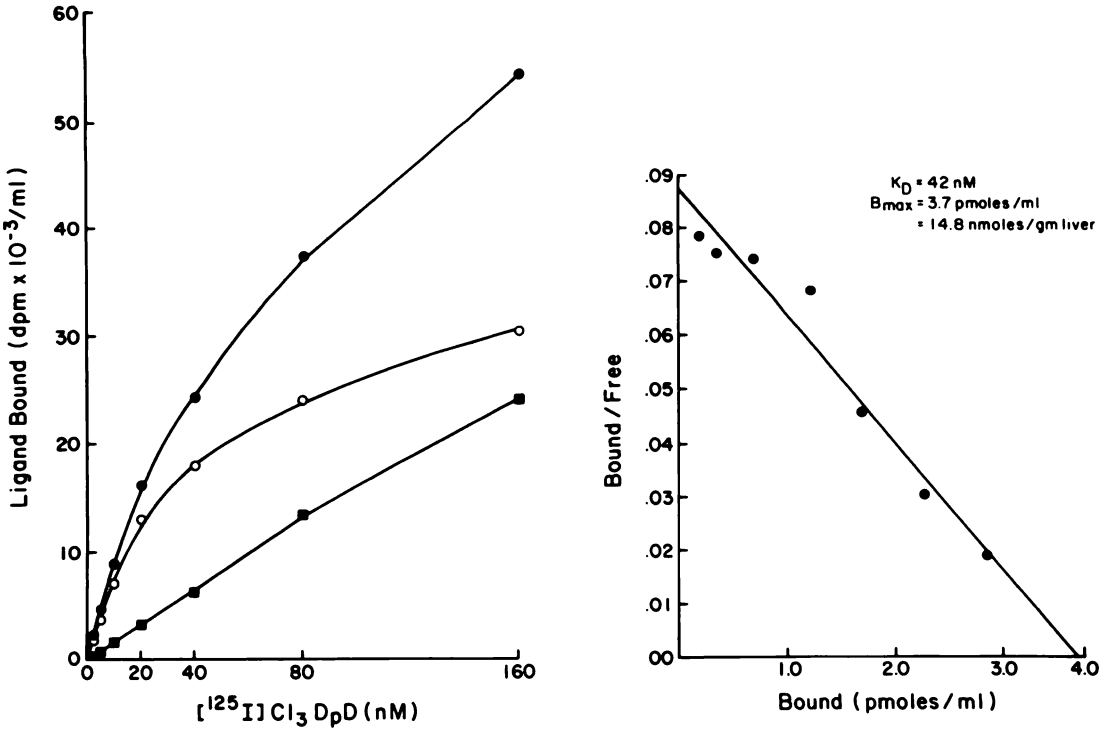

Fig. 2. A, Saturation binding curve. [^{125}I]Cl_3DpD was diluted with unlabeled compound 1:24, so the specific activity was 10,670 dpm/pmol. Varying concentrations of [^{125}I]Cl_3DpD were incubated with 0.25 mg of liver homogenate from TCDD-treated mice in MEN buffer that contained 10 mg/ml BSA, in a 1-ml volume, and binding was determined (see Experimental Procedures). Nonspecific binding was determined as that occurring to homogenate heated at 60° for 10 min. Specific binding was determined as the difference between total and nonspecific binding. ●, Total; ■, nonspecific; and ○, specific binding. B, Scatchard plot. The data in A were replotted according to the method of Scatchard *et al.* (24). The linear regression line and parameters were determined by computer program. Linear regression coefficient r = 0.975 and Hill coefficient = 0.990.

There is also faint labeling of cytochrome P_1-450 in the kidney and lung microsomes from TCDD-treated animals. From these studies we conclude that a major component of the TCDD-induced hepatic microsomal binding species is cytochrome P_3-450. Because the binding of the photoaffinity ligand is less selective than that of [^{125}I]Cl_3DpD (Table 4), it is difficult to evaluate whether the photoaffinity labeling of cytochrome P_1-450 implies that this isozyme has an *in vivo* role in ligand binding.

Discussion

In this report, we have characterized the TCDD-induced hepatic binding species, its subcellular localization, binding specificity, affinity, and pool size, and we have provided evidence that this binding species or a major component of this species is cytochrome P_3-450.

Evidence that the hepatic microsomal binding species is cytochrome P_3-450. In the accompanying report (13), we have found that TCDD administration to C57BL/6J mice enhances the hepatic uptake of [^{125}I]Cl_3DpD *in vivo* and the binding of this radioligand to liver homogenate *in vitro*. We have assumed that stimulation of a single moiety, the [^{125}I]Cl_3DpD-binding species, is responsible for both phenotypes, because enhancement of the *in vivo* and *in vitro* effects show similar dose-response curves, structure-activity relationships, and genetic segregation. Although a small amount of [^{125}I]Cl_3DpD specific binding is found in liver homogenate from control animals, an appreciable pool of specific binding site (B_{max} = 20 nmol/g of liver) is seen only after administration of agonists for the Ah receptor. The TCDD-induced [^{125}I]Cl_3DpD-binding species is in the microsomal fraction of liver, and no stimulation of binding is seen in other tissues examined.

This distinctive profile prompted the conjecture that the [^{125}I]Cl_3DpD-binding species might be cytochrome P_3-450. Demonstration that in liver microsomes from TCDD-treated mice the major species covalently labeled by the ^{125}I-photoaffinity ligand is cytochrome P_3-450, as identified by immunochemical staining on a Western blot, provides support for this hypothesis. However, cytochrome P_1-450 is also labeled to a considerable extent in liver microsomes, and to a small extent in kidney and lung microsomes, from TCDD-pretreated mice. It should be noted that the reversible binding of the photoaffinity ligand [^{125}I]N_3Br_2DpD is less specific (more hydrophobic) than the binding of [^{125}I]Cl_3DpD (Table 4). The photoaffinity ligand faintly labels several bands (M_r 45,000 to 55,000) in liver microsomes from control mice (Fig. 3, *lane 1*) and in liver microsomes from TCPOBOP- and 2,2′,4,4′,5,5′-hexabromobiphenyl-treated mice (data not shown), although administration of these later agents produces no increase in hepatic uptake of [^{125}I]Cl_3DpD *in vivo* (13). Thus, we feel that the labeling of cytochrome P_1-450 and these other bands are *in vitro* artifacts attributable to the hydrophobicity of the photoaffinity ligand and that the induction of cytochrome P_3-450 is largely responsible for the *in vivo* uptake and *in vitro* binding of [^{125}I]Cl_3DpD and [^{3}H]TCDD (12).

In rats, administration of agonists for the Ah receptor induces cytochrome P-450c and P-450d (orthologues of the murine cytochromes P_1-450 and P_3-450, respectively) in liver micro-

TABLE 4

Reversible binding of [^{125}I]Cl_3DpD and [^{125}I]N_3Br_2DpD to the microsomal fraction of tissues from variously pretreated mice

C57BL/6J male mice (10 weeks old) were treated with p-dioxane (0.3 ml/kg) or TCDD (1×10^{-7} mol/kg in p-dioxane), intraperitoneally. Forty-eight hours later animals were killed, tissues from four animals were pooled, and the microsomal fraction was prepared from liver, kidney, brain, and lung. In a second experiment, C57BL/6J male mice (10 weeks old) were treated with dimethyl sulfoxide (4 ml/kg), 3,3′,4,4′,5,5′-hexabromobiphenyl (2 mg/kg), 2,2′,4,4′,5,5′-hexabromobiphenyl (50 mg/kg), or TCPOBOP (3 mg/kg) with dimethyl sulfoxide as the vehicle, intraperitoneally, and killed 72 hr later and microsomes were prepared from pooled livers of two like-treated animals. C57BL/6J (Ah^d/Ah^d) male mice (7 weeks old) were treated with 3,3′,4,4′,5,5′-hexabromobiphenyl (20 mg/kg in dimethyl sulfoxide) and killed 72 hr later and microsomes were prepared from the livers of two like-treated animals. The microsomal fractions were stored in 0.25 M sucrose at −80° until use. The microsomal fractions equivalent to 20 μg of protein were incubated with MEN buffer containing 10 mg BSA and 0.2 pmol of [^{125}I]Cl_3DpD (1.38×10^5 dpm) or 0.3 pmol of [^{125}I]N_3Br_2DpD (1.45×10^5 dpm), in a 1.0-ml volume for 45 min at 20°, c and the bound radioactivity was determined as outlined in Experimental Procedures. Radioactivity was rounded to nearest 100 dpm.

Mice	Tissue	Treatment	Total Bound Ligand	
			[^{125}I]Cl_3DpD	[^{125}I]N_3Br_2DpD
			dpm/20 μg of microsomal protein	
C57BL/6J	Liver	Control	1,800	6,100
	Liver	TCDD	19,700	18,300
C57BL/6J	Kidney	Control	1,200	4,900
	Kidney	TCDD	2,400	7,200
C57BL/6J	Brain	Control	1,300	5,600
	Brain	TCDD	2,600	5,900
C57BL/6J	Lung	Control	2,200	8,900
	Lung	TCDD	2,800	9,600
C57BL/6J	Liver	Control	4,400	6,200
C57BL/6J	Liver	3,3′,4,4′,5,5′-Hexabromobiphenyl	12,100	12,600
C57BL/6J	Liver	2,2′,4,4′,5,5′-Hexabromobiphenyl	6,200	6,600
C57BL/6J	Liver	TCPOBOP	3,000	8,200
C57BL/6J (Ah^d/Ah^d)	Liver	3,3′,4,4′,5,5′-Hexabromobiphenyl	2,000	5,800

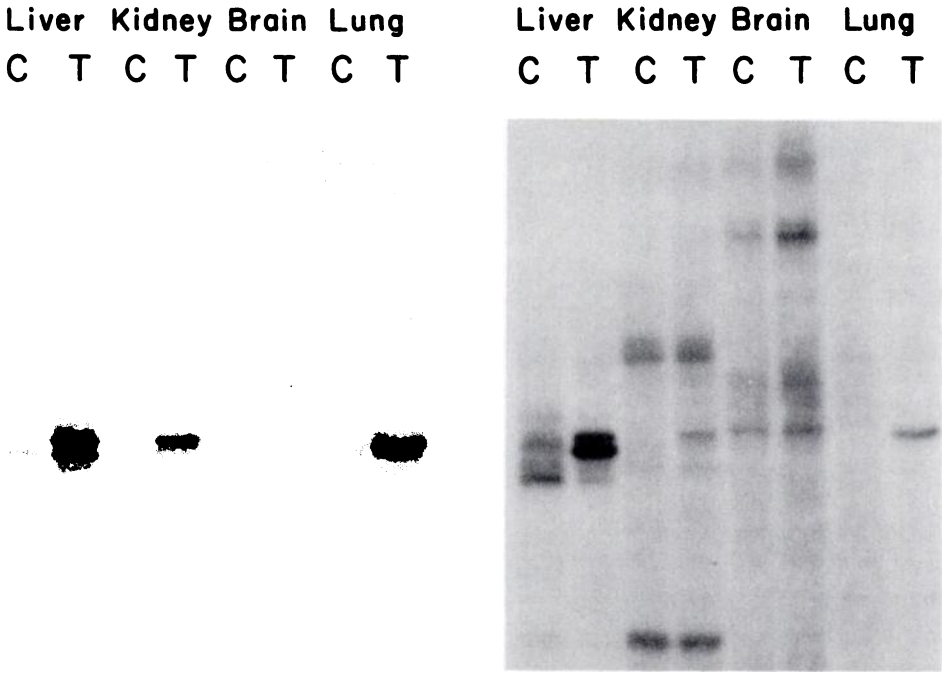

Fig. 3. Photoaffinity labeling of the microsomal fraction and immunochemical staining of the resolved proteins. The microsomal preparations described in Table 4 were incubated, at a concentration of 100 μg of protein/ml in MEN plus 10 mg/ml BSA and [^{125}I]N_3Br_2DpD, 0.3 pmol/ml (1.45×10^6 dpm/ml), in a 10-ml volume at 20° for 45 min, followed by irradiation with Westinghouse FS-20 UV lights (80 W; 4 cm; 20 sec) and the subsequent addition of 2-mercaptoethanol to a concentration of 10 mM (17). The incubation mixture was filtered through a GF/A filter and washed three times with 1.5 ml of MEN buffer containing 10 mM 2-mercaptoethanol and 1 mg of BSA/ml and three times with the same buffer without BSA. The radioactivity in each filter was quantified by γ-scintillation counting and the filter was then shaken for 30 min at 20° with 1 ml of electrophoresis sample buffer, containing 2% lithium dodecyl sulfate, to elute the protein. Elution of the filter-bound radiolabel was 67.8 ± 2.6% (mean ± standard deviation; eight experiments). Solubilized radioactivity was subjected to denaturing electrophoresis on polyacyrlamide slab gels and blotted to nitrocellulose. The nitrocellulose blot was first stained with polyclonal rabbit antiserum to rat cytochrome P-450c and visualized with peroxidase stain and then used to develop an autoradiograph (see Experimental Procedures). A, Immunochemical stain; B, autoradiograph. *C*, Microsomes from control animals; *T*, microsomes from TCDD-treated animals.

somes. Voorman and Aust (30) recently reported that in rats treated with 3,3′,4,4′,5,5′-hexabromobiphenyl (a) this compound remained associated with hepatic microsomal cytochrome P-450d throughout purification of this isozyme, (b) this compound was reversibly bound to P-450d in a nearly 1:1 ratio, and (c) by immunoprecipitation of the solubilized cytochromes, 3,3′,4,4′,5,5′-hexabromobiphenyl was associated only with cytochrome P-450d and not P-450c. They further observed that following the administration of other agonists for the Ah receptor, including TCDD, the major fraction of these compounds in liver microsomes immunoprecipitated with cytochrome P-450d.

The administration of isosafrole (not an agonist for the Ah receptor) selectively induces cytochrome P_3-450 (and not P_1-450) (19, 31). However dietary administration of this compound did not stimulate hepatic uptake of [^{125}I]Cl_3DpD or the *in vitro* binding of this ligand to liver homogenate (data not shown). This is most likely attributable to the known high affinity binding of the isosafrole metabolite to cytochrome P_3-450 (31). Thus, we might infer that [^{125}I]Cl_3DpD binds to cytochrome P_3-450 at or near the enzyme active site. In support of this, Voorman and Aust (30) found that, in an *in vitro* reconstituted system with cytochrome P-450d, the addition of 3-methylcholanthrene or 3,3′,4,4′,5,5′-hexabromobiphenyl produces a concentration-dependent inhibition of P-450d-mediated enzyme activity.

Comparison of the binding by the TCDD-induced microsomal species and the Ah receptor. There is considerable similarity between the structure-binding relationships of these two species; both bind planar aromatic compounds. Based on the limited data in Table 3, binding by the TCDD-induced microsomal species is more inclusive and less stereospecific than binding to the Ah receptor. All Ah receptor agonists tested bind to the microsomal species. The similarity of the structure-binding relationships of these two moieties is interesting and perhaps more than fortuitous. The induction of cytochrome P-450 isozymes (and their monooxygenase activity) by foreign lipophilic chemicals is often viewed as an adaptive response, to enhance the rate of metabolism of these compounds and their elimination from the body. Thus, one might speculate that it makes teleological sense that the recognition site for a group of these compounds (the Ah receptor) has binding specificity similar to that of the catalytic site in the induced isozyme (cytochrome P_3-450) that metabolizes these compounds.

It is interesting to contrast the binding parameters for the TCDD-induced microsomal binding species and the Ah receptor. We estimated that the former has an apparent binding affinity for [^{125}I]Cl_3DpD, K_D, of 5.6×10^{-8} M and a pool size, B_{max}, of 2.2×10^{-6} mol/g of liver. In C57BL/6J mice, the hepatic Ah receptor has an affinity for TCDD, K_D, of 4×10^{-12} M, and B_{max} is approximately 1.2×10^{-11} mol/g of liver (120 fmol/mg of cytosolic protein) (25). Thus, the induced microsomal binding species has an affinity 10^4 times less than the Ah receptor but a pool size that is approximately 2×10^3 greater. Our data suggest that, even for a trace dose of a very high affinity ligand for the Ah receptor (i.e., [^{125}I]Cl_3DpD), the large pool size of the induced microsomal binding species is more important in determining hepatic localization than the high affinity of the Ah receptor.

Finally, let us consider the *in vivo* consequences of this phenomenon. The administration of an agonist for the Ah receptor produces a dose-related induction of hepatic microsomal cytochromes P_1-450 and P_3-450, and this generally increases the metabolism of the inducing compound [e.g., polycyclic aromatic hydrocarbons and TCDD (32)]. Induction of hepatic cytochrome P_3-450 results in an increase in hepatic uptake of the agonist, an effect that will be most apparent with potent, slowly metabolized agonists (e.g., TCDD) where the hepatic concentration of the compound is less than the B_{max} of the induced microsomal binding species, i.e., cytochrome P_3-450. Thus, agonists for the Ah receptor may affect their pharmacokinetics in two ways, dose-related enhancement of the hepatic localization of the compound and stimulation of the rate of metabolism of the agonist. For TCDD (and probably most halogenated aromatic hydrocarbon congeners), metabolism is the rate-limiting step in elimination. Thus, the rate of elimination (i.e., whole body half-life) of TCDD should be a function of the dose of agonist, an effect that has not been observed (3). Examination of this corollary will be the subject of a subsequent communication.[3]

References

1. Poland A., and J. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–554 (1982).
2. Allen, J. R., J. P. Van Miller, and D. H. Norback. Tissue distribution, excretion and biological effects of [^{14}C]tetrachlorodibenzo-*p*-dioxin in rats. *Food Cosmet. Toxicol.* **13**:501–505 (1975).
3. Rose, J. Q., J. C. Ramsey, T. H. Wentzler, R. A. Hummel, and P. J. Gehring. The fate of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin following single and repeated oral doses to the rat. *Toxicol. Appl. Pharmacol.* **36**:209–226 (1976).
4. Gasiewicz, T. A., L. E. Geiger, G. Rucci, and R. A. Neal. Distribution, excretion and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in C57BL/6J, DBA/2J and B6D2F_1/J mice. *Drug Metab. Dispos.* **11**:397–403 (1983).
5. Olson, J. R., T. A. Gasiewicz, and R. A. Neal. Tissue distribution, excretion and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the golden syrian hamster. *Toxicol. Appl. Pharmacol.* **56**:78–85 (1980).
6. Gasiewicz, T. A., and R. A. Neal. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin tissue distribution, excretion, and effects on clinical chemical parameters in guinea pigs. *Toxicol. Appl. Pharmacol.* **51**:329–340 (1979).
7. Olson, J. R. Metabolism and disposition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in guinea pigs. *Toxicol. Appl. Pharmacol.* **85**:263–273 (1986).
8. Bowman, R. E., S. L. Schantz, N. C. A. Weerasinghe, J. Meehan, J. Pan, M. L. Gross, K. M. Boehm, J. P. Van Miller, and P. G. Welling. Clearance of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from body fat of rhesus monkeys following chronic exposure. *Toxicologist* **7**:158 (1987).
9. Poiger, H., and C. Schlatter. Pharmacokinetics of 2,3,7,8-TCDD in man. *Chemosphere* **15**:1489–1494 (1986).
10. Pirkle, J. L., W. H. Wolff, D. G. Patterson, Jr., L. L. Needham, J. E. Michael, J. C. Miner, and M. R. Peterson. Estimates of the half-life of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in ranch hand veterans, in *Seventh International Symposium on Chlorinated Dioxins and Related Compounds*, Abstract HT-08. (1987).
11. Neal, R. A., J. R. Olson, T. A. Gasiewicz, and L. E. Geiger. The toxicokinetics of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mammalian systems. *Drug Metab. Rev.* **13**:355–385 (1982).
12. Teitelbaum, P. J. The hepatic uptake of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the mouse. Ph.D. thesis, University of Rochester, 1979.
13. Poland, A., P. Teitelbaum, E. Glover, and A. Kende. Stimulation of *in vivo* hepatic binding of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin by the administration of agonists for the Ah receptor. *Mol. Pharmacol.* **36**:121–127 (1989).
14. Wilson, N. M., M. Christou, C. R. Turner, S. A. Wrighton, and C. R. Jefcoate. Binding and metabolism of benzo(*a*)pyrene and 7,12-dimethylbenz(*a*)anthracene by seven purified forms of cytochrome P-450. *Carcinogenesis (Lond.)* **5**:1475–1483 (1984).
15. Thomas, P. E., A. Y. H. Lu, D. E. Ryan, S. B. West, J. Kawalek, and W. Levin. Multiple forms of rat liver microsome P-450: immunochemical evidence with antibody against cytochrome P-448. *J. Biol. Chem.* **251**:1385–1391 (1976).
16. Hjelm, H., K. Hjelm, and J. Sjoquist. Protein A from *Staphylococcus aureus*: its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS Lett.* **28**:73–78 (1972).
17. Poland, A., E. Glover, F. H. Ebetino, and A. S. Kende. Photoaffinity labeling of the Ah receptor. *J. Biol. Chem.* **261**:6352–6365 (1986).
18. Laemmli, U. K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
19. Towbin, H., T. Staehelin, J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
20. Glass, W. F., R. C. Briggs, and L. S. Hnilica. Identification of tissue-specific nuclear antigens transferred to nitrocellulose from polyacrylamide gels. *Science* **221**:70–72 (1981).
21. Laskey, R. A., and A. D. Mills. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. *FEBS Lett.* **82**:314–316 (1977).
22. Poland, A., E. Glover, and A. Kende. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. *J. Biol. Chem.* **251**:4936–4946 (1976).
23. Poland, A., and E. Glover. Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmacol.* **13**:924–938 (1977).
24. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).

[3] A. Poland, P. Geiger, and E. Glover. Manuscript in preparation.

25. Bradfield, C. A., Kende, A. S., and A. Poland. Kinetic and equilibrium studies of Ah receptor-ligand binding: use of [^{125}I]-2-iodo-7,8-dibromodibenzo-*p*-dioxin. *Mol. Pharmacol.* **34**:229–237 (1988).
26. Tuteja, N., F. J. Gonzalez, and D. W. Nebert. Developmental and tissue-specific differential regulation of the mouse dioxin-inducible P_1-450 and P_3-450 genes. *Dev. Biol.* **112**:177–184 (1985).
27. Kimura, S., F. J. Gonzalez, and D. W. Nebert. The murine *Ah* locus: comparison of the complete cytochrome P_1-450 and P_3-450 cDNA nucleotide and amino acid sequences. *J. Biol. Chem.* **259**:10705–10713 (1984).
28. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P-450 gene superfamily: recommend nomenclature. *DNA* **6**:1–11 (1987).
29. Thomas, P. E., J. Reidy, L. M. Reik, D. E. Ryan, D. R. Koop, and W. Levin. Use of monoclonal antibody probes against rat hepatic cytochromes P-450c and P-450d to detect immunochemically related isozymes in liver microsomes from different species. *Arch. Biochem. Biophys.* **235**:239–253 (1984).
30. Voorman, R., and S. D. Aust. Specific binding of polyhalogenated aromatic hycrocarbon inducers of cytochrome P-450d to the cytochrome and inhibition of its estradiol-2-hydroxylase activity. *Toxicol. Appl. Pharmacol.* **90**:69–78 (1987).
31. Ryan, D. E., P. E. Thomas, and W. Levin. Hepatic microsomal cytochrome P-450 from rats treated with isosafrole. *J. Biol. Chem.* **255**: 7941–7955 (1980).
32. Wroblewski, V. J., and J. R. Olson. Effect of monooxygenase inducers and inhibitors on the hepatic metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat and hamster. *Drug Metab. Dispos.* **16**:43–51 (1988).

Send reprint requests to: Alan Poland, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706.