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COMPETITIVE BINDING TO THE CYTOSOLIC 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN RECEPTOR

EFFECTS OF STRUCTURE ON THE AFFINITIES OF SUBSTITUTED HALOGENATED BIPHENYLS—A QSAR ANALYSIS

STELVIO BANDIERA,* THOMAS W. SAWYER,† MARY ANNE CAMPBELL,* TOSHIO FUJITA ‡ and STEPHEN SAFE†§

*The Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario N1G 2W1 Canada; †Department of Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4466, U.S.A.; and ‡Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan 606

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Abstract—The proposed mechanism of action of the toxic halogenated aromatics, typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), involves the initial binding to a high-affinity, low-capacity, cytosolic receptor protein. Previous studies have shown that several 4′-halo-2,3,4,5-tetrachlorobiphenyls bind to the TCDD receptor and that a lateral substituent on both phenyl rings is required for activity. Using an extended series of eighteen 4′-substituted-2,3,4,5-tetrachlorobiphenyls as probes, the effects of a variable lateral substituent on receptor binding affinity and the induction of aryl hydrocarbon hydroxylase (AHH) *in vivo* and in rat hepatoma H-4-II E cells have been determined. For most substituents, there was an excellent correlation between the rank-order potency for receptor binding and the rank-order potency for AHH induction. Based on *in vitro* binding affinities (EC_{50} values) of the 4′-substituted tetrachlorobiphenyls, a multiparameter regression equation was formulated correlating the binding constants to physicochemical substituent parameters. For thirteen compounds out of the present series, multiple regression analysis of the binding data led to the following equation: $\log(1/EC_{50}) = 1.53\sigma + 1.47\pi + 1.09HB + 4.08$, $r = 0.978$. The results suggest that halogen substitution on both phenyl rings is not a requirement for binding and that hydrophobic (π) and electronic (σ) substituent constants and a variable for hydrogen bond (HB) formation are significant parameters describing relative binding avidities of this series of substituted biphenyls for the TCDD receptor.

Halogenated aromatic hydrocarbons such as the polychlorinated biphenyls (PCBs),‖ dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) possess a number of common physicochemical, biologic and toxic properties. For example, (1) within each class there exists a multiplicity of isomers and congeners (PCBs, 209; PCDFs, 135; and PCDDs, 75); (2) the biologic and toxic potencies of individual PCBs, PCDDs and PCDFs are remarkably dependent on structure with the most active compounds similar in structure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic individual halogenated aromatic compound [1–5]; (3) TCDD and approximate isostereomers elicit a number of common toxic responses including a wasting syndrome, porphyria, thymic atrophy, chloracne and hepatotoxicity [4–8]; and (4) TCDD and related compounds induce cytochrome "P-448"¶-dependent monooxygenases including aryl hydrocarbon hydroxylase (AHH) [1, 3, 5, 9–13].

Studies with chlorinated dibenzo-*p*-dioxins have shown that the potency of individual congeners to induce AHH activity correlates very closely to their toxic potency (i.e. LD_{50}) [1, 2]. An initial and obligatory step in enzyme induction is believed to involve the reversible binding of the inducer to a cytoplasmic receptor protein [1, 2, 14, 15]. The receptor has a very high affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylchlolanthrene, and other polycyclic aromatic hydrocarbons [1, 14, 15]. Moreover, there appears to be an excellent correlation between the potencies of individual PCDD congeners to induce AHH activity and their affinities for receptor binding [1, 2, 14]. These structure–activity relationships and related studies with genetically inbred mice [2] support the hypothesis that the receptor:halogenated aryl hydrocarbon interaction initiates the sequence of events leading to the expression of a typical pleiotypic response produced by TCDD and congeners.

§ Address all correspondence to S. Safe.

‖ Abbreviations: AHH, aryl hydrocarbon hydroxylase, DMSO, dimethyl sulfoxide; EC_{50}, effective concentration of test compound necessary to give 50% of maximal response (for receptor binding experiments, the effective concentration of competitor necessary to reduce specific binding of [^{3}H]TCDD to 50% of the maximal value obtained in the absence of competitor); EROD, ethoxyresorufin *O*-deethylase; GLC gas–liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MC, 3-methylcholanthrene; NMR, nuclear magnetic resonance; PB, phenobarbitone; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TLC, thin-layer chromatography.

¶ In this paper, we refer to the form(s) of cytochrome induced by MC collectively as "cytochrome P-448". The term cytochrome P-450 refers to all forms of microsomal cytochrome P-450.

Comparable studies with pure, synthetic PCB isomers and congeners have shown that both receptor binding affinities and AHH inducing activities of the individual PCBs were also dependent on structure [10–12, 16]. The most active compounds, 3,3′,4,4′-tetra-, 3,3′,4,4′,5-penta- and 3,3′,4,4′,5,5′-hexachlorobiphenyl, are approximate isostereomers of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [10–13]. Several mono-*ortho* chloro substituted derivatives of these laterally-substituted PCBs were less active as inducers of AHH and exhibited less affinity for the cytosolic receptor [12, 16, 17]. Furthermore, for all PCB isomers and congeners tested, the relative order for binding correlated well with the rank-order for induction of AHH activity in cell culture [17, 18].

The natures of the receptor binding site(s) and of the ligand–receptor interactions are not well understood. From studies on structure–activity relationships of various classes of halogenated aromatic compounds, Poland and co-workers [1, 2] suggested that the basic requirement for compounds binding to the receptor was a molecular structure which conformed to a planar rectangle 3 × 10 Å with halogen atoms in the four corners. In terms of molecular orbital parameters, the electron acceptor properties of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were assumed to be important [19, 20]. Moreover, based on calculations with a few polychlorinated and polybrominated biphenyls, it was proposed that the coplanarity of halogenated aromatics facilitated steric fit to the receptor whereas the ability to bind effectively was dependent upon the net polarizability of the ligand molecule [21, 22].

Quantitative analysis of structure-activity relationships (QSAR) has been developed recently for correlating the variation in biological activity of a series of congeners [23–26]. This approach can be employed to study the intermolecular interactions between drugs and their receptor sites or, more specifically, between PCB isomers and the hepatic TCDD-receptor. In general, drug–receptor interactions involve such forces as hydrophobic bonding, hydrogen bonding, van der Waals' energy, electrostatic energy, valence-bond energy and repulsion or strain energies of the bonds [26]. The strength of the interaction depends upon the sum of various energies which, for a series of substituted congeners, can be approximated by the use of free-energy-related physicochemical substituent parameters [23–27]. For members of a congeneric series, a statistically significant correlation between biological activity and substituent constants can be developed by multiple regression analysis [23, 24, 26, 27].

An assessment of the effects of substituents on the binding affinities of halogenated aryl hydrocarbons requires a ligand with at least two important characteristics: (1) the polychlorinated ligand should both bind to the receptor protein and elicit the pleiotypic responses typical of TCDD and related isostereomers; and (2) the substitution pattern of the ligand should permit the introduction of diverse functional groups at a critical lateral position.

Previous studies have shown that 2,3,4,4′,5-pentachlorobiphenyl binds to the cytosolic TCDD-receptor from rat liver [16, 28] and induces cytochrome "P-448" dependent monooxygenases in rat hepatoma H-4-II E cells in culture [18, 28]. Moreover, this PCB congener induced AHH and caused thymic involution in the responsive C57BL/6J mice but did not elicit these effects in the nonresponsive DBA/2J mice [29]. Since 2,3,4,4′,5-pentachlorobiphenyl produced biologic and toxic responses comparable to the toxic halogenated aromatic hydrocarbons and since the structure contains a single lateral substituent in the *para* (i.e. 4′) position on one of the phenyl rings, this substitution pattern can be used to probe for the effects of different functional groups on the activity of 4′-substituted tetrachlorobiphenyls.

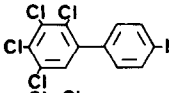
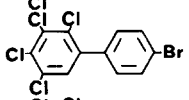
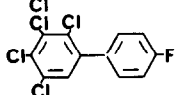
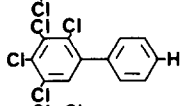
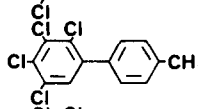
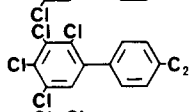
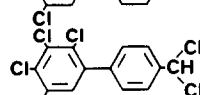
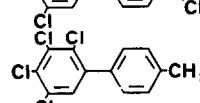
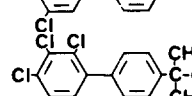
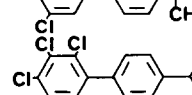
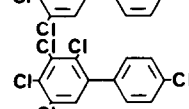
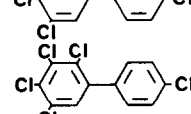
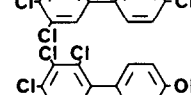
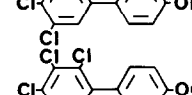
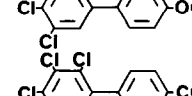
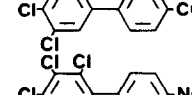
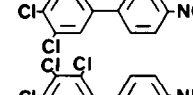
Previously, we reported that for a series of 4′-halo-2,3,4,5-tetrachlorobiphenyls there was an excellent correlation between the relative binding affinities of the 4′-iodo, 4′-bromo, 4′-chloro and 4′-fluoro analogs for the receptor and their potencies as AHH inducers both *in vivo* and *in vitro* [28]. The present study reports the effects of structure on the binding affinities of eighteen 4′-substituted-2,3,4,5-tetrachlorobiphenyls for the cytosolic receptor protein. A multiparameter regression equation correlating the binding constants to substituent parameters is determined. Moreover, the relative binding affinities for the individual compounds are compared to their relative potencies as *in vitro* inducers of AHH activity, part of the receptor-mediated pleiotypic response.

MATERIALS AND METHODS

Synthesis and purification of para-*substituted* 2,3,4,5-*tetrachlorobiphenyls.* The 4′-substituted-2,3,4,5-tetrachlorobiphenyls were synthesized by the Cadogan coupling of the *para*-substituted anilines (15–20 mmoles) with excess 1,2,3,4-tetrachlorobenzene (100–150 mmoles) at 125° for 18 hr (summarized in Table 1). The synthesis and purification of 2,3,4,4′,5-pentachlorobiphenyl was reported previously [12, 29]. The chlorinated benzene was removed, and the crude product was adsorbed on 20–30 g silica gel which was added as an upper component to a layered Florisil/silica gel or alumina/silica gel column. The nonpolar coupling products (4′-alkyl and 4′-halo derivatives) were eluted with petroleum spirit (b.p. 40–60°) and the more polar methoxy, cyano, nitro, acetyl and acetylamino products were eluted with petroleum spirit:diethyl ether mixtures as required. The crude coupling products were purified by TLC, and the residue was crystallized from methanol as described [12, 17]. Purities were determined by gas chromatography using a Hewlett–Packard model 5710 chromatograph equipped with a ^{63}Ni electron capture detector and an 0.6 cm × 1.2 m glass column packed with 3% OV 101 Ultrabonded Carbowax 20 m (80–100 mesh, RFR Corp., Hope, RI). Structures of purified substituted tetrachlorobiphenyls were confirmed by their 200 MHz NMR and mass spectra (determined on a VG Micromass 7070 mass spectrometer). The impurities present in the PCB isomers represent chlorinated biphenyl analogues of similar structure.

Chemicals. 2,3,7,8-Tetrachloro[^{3}H]dibenzo-*p*-dioxin (sp. act. 50–52 Ci/mmole) was a gift from Dr.

Table 1. Synthesis and properties of 4'-substituted-2,3,4,5-tetrachlorobiphenyls

Structure	Name	Synthetic precursors	GLC purity
	4'-Iodo-2,3,4,5-Tetrachlorobiphenyl	4-Iodoaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-Bromo-2,3,4,5-Tetrachlorobiphenyl	4-Bromoaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-Fluoro-2,3,4,5-Tetrachlorobiphenyl	4-Fluoroaniline[b] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-Hydro-2,3,4,5-Tetrachlorobiphenyl	Aniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-Methyl-2,3,4,5-Tetrachlorobiphenyl	*p*-Toluidine[c] 1,2,3,4-Tetrachlorobenzene[a]	>98%
	4'-Ethyl-2,3,4,5-Tetrachlorobiphenyl	4-Ethylaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>97%
	4'-Isopropyl-2,3,4,5-Tetrachlorobiphenyl	4-Isopropylaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-*n*-Butyl-2,3,4,5-Tetrachlorobiphenyl	4-*n*-Butylaniline[a] 1,2,3,4-Tetrachlorobenzene	>98%
	4'-*t*-Butyl-2,3,4,5-Tetrachlorobiphenyl	4-*t*-Butylaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>98%
	4'-Phenyl-2,3,4,5-Tetrachlorobiphenyl	4-Aminobiphenyl[a] 1,2,3,4-Tetrachlorobenzene[a]	>97%
	4'-Trifluoromethyl-2,3,4,5-Tetrachlorobiphenyl	4-Aminobenzotrifluoride[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-Cyano-2,3,4,5-Tetrachlorobiphenyl	4-Cyanoaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>96%
	4'-Hydroxy-2,3,4,5-Tetrachlorobiphenyl	Demethylation of methoxy derivative	>97%
	4'-Methoxy-2,3,4,5-Tetrachlorobiphenyl	*p*-Anisidine[a] 1,2,3,4-Tetrachlorobenzene	>98%
	4'-Acetyl-2,3,4,5-Tetrachlorobiphenyl	4-Acetylaniline[d] 1,2,3,4-Tetrachlorobenzene[a]	>97%
	4'-Nitro-2,3,4,5-Tetrachlorobiphenyl	4-Nitroaniline[a] 1,2,3,4-Tetrachlorobenzene[a]	>99%
	4'-N-Acetylamino-2,3,4,5-Tetrachlorobiphenyl	4-Acetylaminoaniline[d] 1,2,3,4-Tetrachlorobenzene[a]	>97%

Purchased from [a]Aldrich Chemical Co., Milwaukee, WI, [b]Pfaltz and Bauer Inc., Stanford, CT, [c]British Drug House, Carle Place, NY, [d]Chemical Procurement Laboratories Inc., College Point, NY.

A. B. Okey, Division of Clinical Pharmacology, Hospital for Sick Children (Toronto, Ontario). The radiolabeled TCDD used in the present study contained less than 20% of the tritiated trichloro- and pentachlorodibenzo-*p*-dioxin congeners, as determined by the manufacturer and confirmed in our laboratory by gas–liquid chromatography. The contaminants have been reported to be considerably less active as inducers of AHH activity and as competitors for specific cytosolic binding sites [12, 14]. In the cytosol receptor binding assay, a 20% tritiated impurity of 2,3,7-trichlorodibenzo-*p*-dioxin would represent less than 3% of the specific binding obtained with tritiated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, because of the lower affinity of the former congener for the receptor. Moreover, equivalent receptor binding results were obtained in a subsequent study using radiolabeled TCDD of greater than 95% purity.

[G^{3}H]Benzo[*a*]pyrene (40 Ci/mmole) was purchased from the Amersham Corp. (Oakville, Ontario).

Dextran (average M_r = 82,000), Hepes, NADP$^+$, NADH, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase (Baker's yeast), benzo[*a*]pyrene, rhodamine B, bovine serum albumin and ethylisocyanide (EIC) were purchased from the Sigma Chemical Co. (St. Louis, MO). Dithiothreitol was obtained from the Eastman Kodak Co. (Rochester, NY); 4-dimethylaminoantipyrine (DMAP) from the Aldrich Chemical Co. (Milwaukee, WI); 3-methylcholanthrene (MC) from Pfaltz & Bauer, Inc. (Stamford, CT); decolorizing charcoal (Norit A) from BDH Chemicals (Toronto, Ontario); and sucrose (SDS grade) from Beckman Instruments (Fullerton, CA). Dimethyl sulfoxide (DMSO), glycerol (A.C.S. grade), and EDTA were from the Fisher Scientific Co. (Toronto, Ontario). Sodium phenobarbitone (PB) was supplied by the Ontario Veterinary College (Guelph, Ontario). Ethoxyresorufin was synthesized according to the method of Burke and Mayer [30].

Animals. One-month-old male Wistar rats (average weight 100 g), were purchased from Woodlyn Laboratories, Ltd. (Guelph, Ontario). They were housed in wire cages, allowed free access to Purina Rat Chow No. 5002 and water, and were maintained on a diurnal cycle of 13 hr of light/11 hr of darkness.

Isolation of microsomes and biochemical assays. The *para*-substituted 2,3,4,5-tetrachlorobiphenyls were dissolved in corn oil and injected intraperitoneally on days 1 and 3 at a dose level of 150 μmoles/kg per injection. Rats injected with PB, MC and corn oil served as controls [12, 17]. Microsomes (105,000 *g* pellet) were prepared from perfused rat livers by differential centrifugation as described [17]. Enzymic activities including 4-dimethylaminoantipyrine *N*-demthylase, benzo[*a*]pyrene hydrosylase and ethoxyresorufin *O*-deethylase, as well as spectral assays including the concentration of cytochrome b_5 and the carbon monoxide (CO)- and ethylisocyanide (EIC)-difference spectra, were determined as previously reported [12, 17]. The animals were killed by cervical dislocation on day 6.

Growth and treatment of cells and enzymatic assays. Rat hepatoma H-4-II E cells (supplied by Dr. J. Bradlaw) were grown as a continuous cell line in α-minimum essential medium without ribonucleosides, deoxyribonucleosides and sodium bicarbonate, but with L-glutamine as described [18, 31]. After reaching confluency the cultures were trypsinized and seeded in 100 × 20 mm Petri dishes at 10^6 cells/plate in 8 ml of medium. The cells were allowed to attach for 24 hr, and then the spent medium was removed and replaced with fresh medium. The test chemical in DMSO was added to the cell culture system so that the final concentration of DMSO in the culture medium was 0.5%. After incubation of the chemical for 72 hr, the medium was removed and the cultures were washed thoroughly with phosphate-buffered saline (PBS), pH 7.4. The cells were then harvested by scraping with a Tris–sucrose (0.05 to 0.2 M) buffer, pH 8.0. The cellular protein pellet was recovered after centrifugation for 5 min at 10,000 *g*, resuspended in the Tris–sucrose buffer, and the suspension made up to a concentration of 1.0 mg protein/ml. Ethoxyresorufin *O*-deethylase and aryl hydrocarbon hydroxylase assays were determined as described [18].

Cytosol receptor binding. Hepatic cytosol [105,000 *g* supernatant prepared in HEDG buffer: Hepes 25 mM, EDTA 1.5 mM, dithiothreitol 1 mM, glycerol (10%, v/v), pH 7.6] was prepared from non-fasted, immature male Wistar rats as previously reported [16].

The procedure used to quantitate the cytosol receptor was sucrose density gradient analysis following dextran–charcoal treatment. This assay has been shown to be more reliable in separating a class of high-affinity, low-capacity sites from non-saturable binding than DEAE-cellulose column chromatography or dextran–charcoal adsorption methods [15].

Samples for sucrose density gradient analysis were prepared by incubating 1 ml of cytosol (5–6 mg protein) with 10 nM [^{3}H]TCDD for 1 hr at 0–2°. [^{3}H]TCDD was added in 10 μl of dimethyl sulfoxide/ml of cytosol. In competitor experiments, an equal amount of dimethyl sulfoxide containing the desired concentration of competitor was added. A control containing [^{3}H]TCDD plus an equal volume of solvent (20 μl), but no competitor, was also prepared. After incubation, the unbound and loosely bound [^{3}H]TCDD and/or competitor were removed by agitating the cytosol with a dextran–charcoal pellet (10 mg charcoal/mg dextran, pelleted from HEDG buffer). Following a 15-min incubation at 0–2°, the dextran–charcoal was removed by centrifugation at 4000 *g* for 15 min. Aliquots of cytosol were taken both before and after dextran–charcoal treatment for determination of "total" and "bound" radioactivity. Three hundred microliters of the charcoal-treated cytosol was layered onto 5–20% sucrose gradients prepared in HEDG buffer. Gradients were centrifuged in a Beckman SW 50.1 rotor at 48,000 rev/min for 16 hr (g_{av} = 216,000) at 2°. After centrifugation, forty fractions of 0.12 ml each were collected per gradient into 7 ml plastic miniscintillation vials. Radioactivity in each fraction was determined by liquid scintillation counting and corrected for counting efficiency.

Multiple linear regression analysis. The relationships between various substituent parameters and receptor binding constants were examined by means of regression analysis. The calculations were performed with a FACOM M200 computer at the Data Processing Center of Kyoto University (Kyoto, Japan).

To each substituent group at the 4′-position of 2,3,4,5-tetrachlorobiphenyl, a hydrophobic parameter (π), an electronic parameter (σ), and a hydrogen-bonding accepting parameter (HB) were assigned.

The hydrogen bonding parameter, HB, is an indicator variable which takes a value of 1 for substituents which are hydrogen acceptors but a value of 0 for nonhydrogen bonders (see Ref. 32).

For the electronic effect of 4′-substituents, σ_{para} (Hammett constant) was use. The σ_p values were obtained from the literature [33].

The hydrophobic parameter, π, defined by equation 1,

$$\pi = \log P_X - \log P_H \quad (1)$$

(where P_X and P_H are the partition coefficients of 4′-substituted-2,3,4,5-tetrachlorobiphenyl and 4′-unsubstituted-2,3,4,5-tetrachlorobiphenyl, respectively, in the *n*-octanol/water system) was estimated by means of a recently developed method (see Ref. 34). According to this method, π values of the hydrogen-bondable 4′-substituents were estimated using equation 2,

$$\pi_{X/PhC_6HCl_4} = 0.94\pi_{X/PhH} + 0.19\rho_X \quad (2)$$

where the suffix, X/PhC_6HCl_4, indicates π values from the 4′-substituted-2,3,4,5,-tetrachlorobiphenyls and the suffix, X/PhH, refers to π values from monosubstituted benzenes. As equation 2 illustrates, hydrophobic substituent parameters for this series of 4′-substituted tetrachlorobiphenyls were determined not only by the π values of monosubstituted benzenes ($0.94\pi_{X/PhH}$) but also by an additional factor ($0.19\rho_X$). The coefficient of the $\pi_{X/Ph}$ term, 0.94, is derived empirically from a number of disubstituted benzene series. The fact that its value is somewhat less than 1 means that the "intrinsic" hydrophobicity of substituents in disubstituted benzenes is lightly, but significantly, lower than in monosubstituted benzenes [34]. The secor.d term, $0.19\rho_X$, describes the solubility modifying effect of the electron-withdrawing 2,3,4,5-tetrachlorobiphenyl on the hydrogen bonding interactions of each 4′-substituent between the octanol and water phases. For substituents incapable of hydrogen bonding such as alkyl and halogen, the ρ value is 0 [34]. The electron-withdrawing effect of the 2,3,4,5-tetrachlorobiphenyl group is expressible by the σ_p value which was estimated as σ_p $(C_6Cl_5) \times 4/5 = 0.19$ [33]. The $\pi_{X/PhH}$ values were taken from the literature [33].

RESULTS

Enzyme induction in rat hepatoma cells in culture. Figure 1 summarizes the effects of fifteen of the eighteen 4′-substituted-2,3,4,5-tetrachlorobiphenyls as inducers of AHH and EROD enzyme activities in the rat hepatoma H-4-II E cell cultures. Increasing concentrations of the test compounds led to dose-related increases in both enzyme activities which was usually followed by a decrease at higher concentrations of the inducer (see Ref. 18). From plots of dose–response curves in H-4-II E cells, EC_{50} values for induction of AHH and EROD were determined for each inducer by inverse linear regression. The EC_{50} values for the fifteen *para*-substituted tetrachlorobiphenyls, presented in Fig. 1, are shown in rank-order of competitive potency for receptor binding.

Compounds which were among the best inducers of both AHH and EROD activities include 4′-trifluoromethyl-, 4′-iodo- and 4′-bromo-2,3,4,5-tetrachlorobiphenyl, in that order. The 4′-ethyl-, 4′-acetyl- and 4′-isopropyl-substituted derivatives were similar in potency to 2,3,4,4′,5-pentachlorobiphenyl as inducers of both enzyme activities, but they were at least an order of magnitude weaker than the three best inducers. Six of the remaining substituted tetrachlorobiphenyls were, by comparison, relatively weak inducers of AHH and EROD. Moreover, pretreatment of H-4-II E cells with 4′-hydroxy- and 4′-hydro-2,3,4,5-tetrachlorobiphenyl did not produce any appreciable induction of either enzyme activity.

Enzyme induction in vivo. The effects of pretreatment with PB, MC and PB plus MC (coadministered), as well as with 4′-iodo-, 4′-bromo-, 4′-chloro- and 4′-fluoro-2,3,4,5-tetrachlorobiphenyl on the enzymic and spectral characteristics of the hepatic microsomal enzymes in the immature male Wistar rat were summarized and discussed in a previous report [28].

Administration of 4′-isopropyl-, 4′-*t*-butyl-, 4′-trifluoromethyl-, 4′-cyano-, 4′-nitro- and 4′-*N*-acetylamino-2,3,4,5-tetrachlorobiphenyl to rats resulted in significantly increased activities of microsomal benzo[*a*]pyrene hydroxylase and EROD, as well as increased levels of cytochrome b_5 and cytochrome P-450. however, only 4′-cyano-, 4′-nitro- and 4′-*N*-acetylamino-2,3,4,5-tetracholorbiphenyl produced a significant induction of microsomal DMAP *N*-demethylase. Two other compounds, 4′-phenyl- and 4′-acetyl-2,3,4,5-tetrachlorobiphenyl, were relatively poor inducers of benzo[*a*]pyrene hydroxylase. The 4′-phenyl derivative, however, produced some induction of microsomal DMAP *N*-demethylase and ethoxyresorufin *O*-deethylase enzyme activities. At concentrations of 150 μmoles/kg, the six remaining compounds (namely 4′-hydro-, 4′-methyl-, 4′-ethyl-, 4′-*n*-butyl-, 4′-hydroxy- and 4′-methoxy-2,3,4,5-tetrachlorobiphenyl) produced no significant induction of the microsomal enzyme activities tested and did not increase cytochrome b_5 or cytochrome P-450 levels.

Competitive binding of para-*substituted tetrachlorobiphenyls.* Incubation of rat hepatic cytosol with 10 nM 2,3,7,8-tetrachloro[^{3}H]dibenzo-*p*-dioxin ([^{3}H]TCDD) for 1 hr at 0–2° resulted in the formation of a binding complex which was detectable as a peak of radioactivity following sucrose density gradient centrifugation and subsequent fractionation. The peak sedimented at approximately Fraction 25 under our experimental conditions (see Ref. 16). In the

presence of 10 μM nonradiolabeled TCDD or 10 μM 3-methylcholanthrene, this peak was completely abolished. The amount of [^{3}H]TCDD specifically bound to hepatic cytosol, either in the absence or presence of competitors, was measured from the area under the peak.

To study the effect of different substituent parameters on the binding site of the receptor, the abilities of eighteen *para*-substituted 2,3,4,5-tetrachlorobiphenyls to compete against [^{3}H]TCDD for the receptor were tested. The results of this competitive binding between 10 nM [^{3}H]TCDD and various concentrations of the substituted biphenyls are shown in Fig. 2. From interpolation of such full dose–response curves, EC_{50} values were derived for each of the biphenyls. Although the equilibrium dissociation constant, K_d, is routinely employed as a measure of the affinity of binding to the receptor, K_d values measured by Scatchard plot analyses were difficult to quantitate accurately (see Ref. 15). For this reason, EC_{50} values were chosen as a measure of the relative affinities of the substituted PCBs for the receptor. The rank-order competitive potency of the eighteen compounds against [^{3}H]TCDD is shown in Fig. 3.

As expected, compounds that were good inducers of cytochrome "P-448" dependent monooxygenases were the most potent competitors for the hepatic

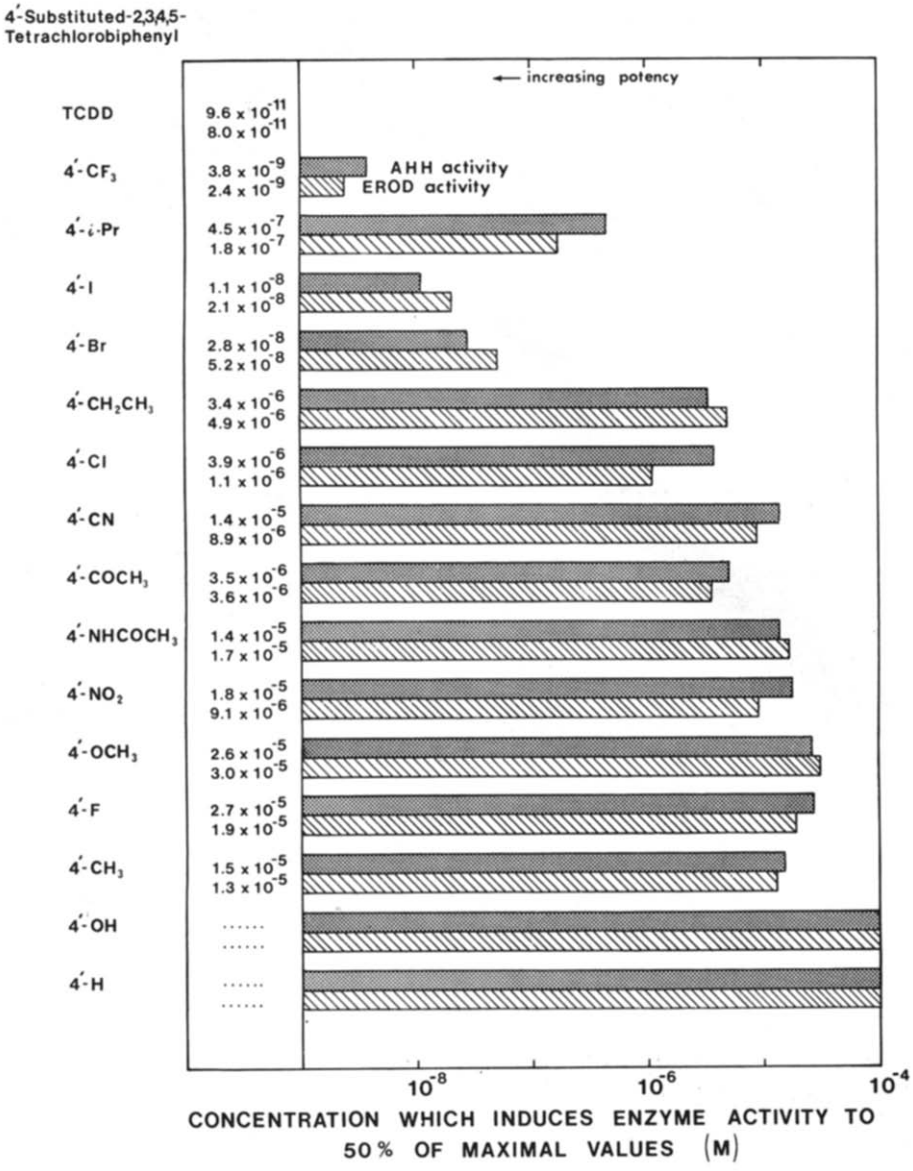


Fig. 1. Comparative EC_{50} values of the 4′-substituted-2,3,4,5-tetrachlorobiphenyls for induction of aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (EROD) in rat hepatoma H-4-II E cells. H-4-II E cells were incubated with various concentrations of 4′-substituted tetrachlorobiphenyls for 72 hr and then harvested. Enzyme activities were assayed fluorimetrically. The EC_{50} values were obtained from plots of specific activity (pmoles per mg protein per min) versus molar concentration of the 4′-substituted tetrachlorobiphenyls. Enzyme activities at each biphenyl concentration were determined in triplicate.

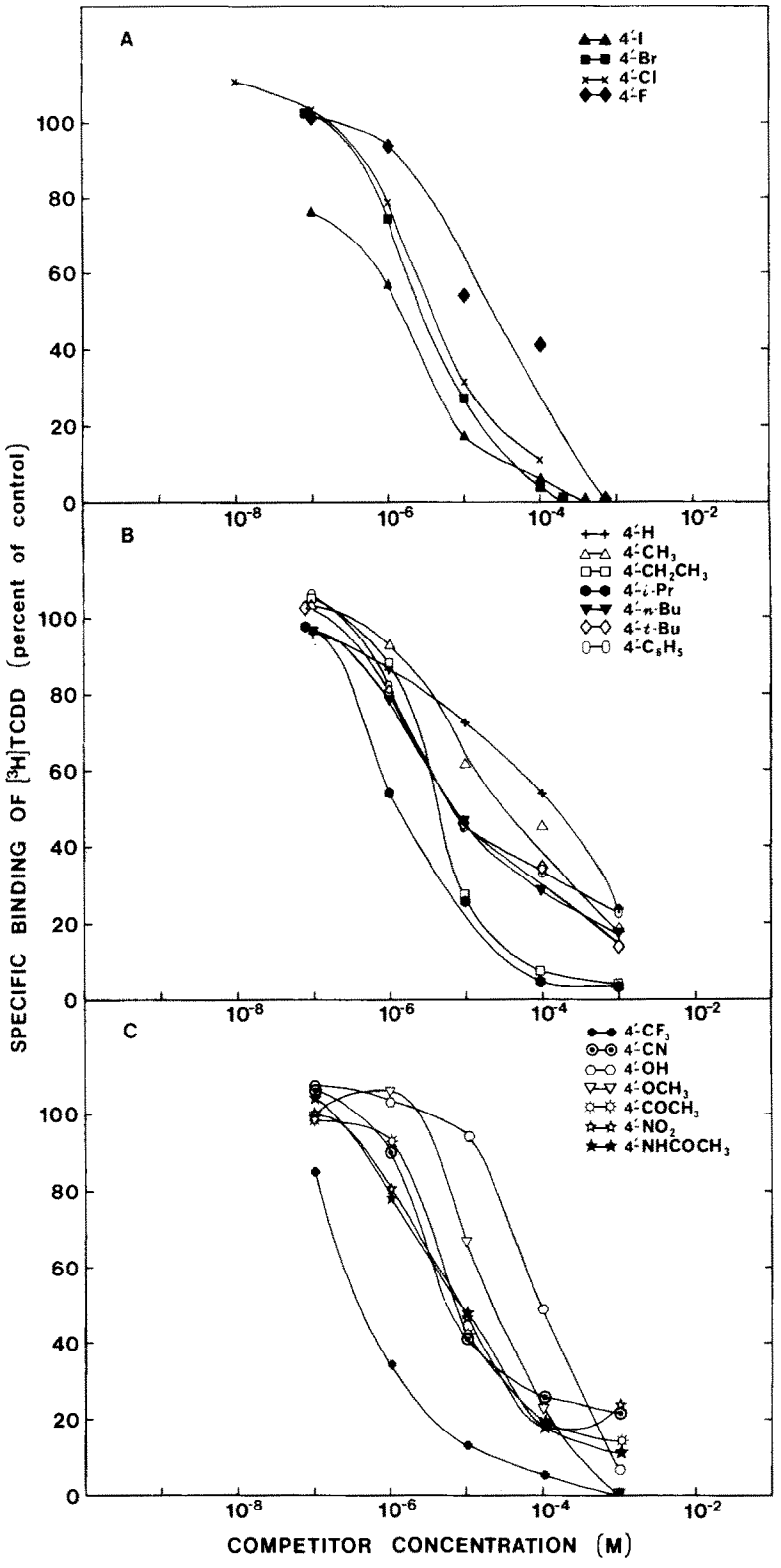


Fig. 2. Comparative potency of 4'-substituted-2,3,4,5-tetrachlorobiphenyls as competitors with [^{3}H]TCDD for specific binding to rat cytosolic receptor. (A) Competition by 4'-halo-2,3,4,5-tetrachlorobiphenyls. Hepatic cytosol (5–6 mg protein/ml) was incubated with 10 nM [^{3}H]TCDD in the presence of various concentrations (given on the abscissa) of the 4'-substituted tetrachlorobiphenyls. Cytosol incubated with 10 nM [^{3}H]TCDD in the absence of competitor served as control. (B) Competition of 4'-alkyl- and 4'-aryl-2,3,4,5-tetrachlorobiphenyls. (C) Competition by remaining 4'-substituted-2,3,4,5-tetrachlorobiphenyls. For 4'-nitro- and 4'-chloro-2,3,4,5-tetrachlorobiphenyl, the dose–response curves shown represent the mean of three determinations at each competitor concentration.

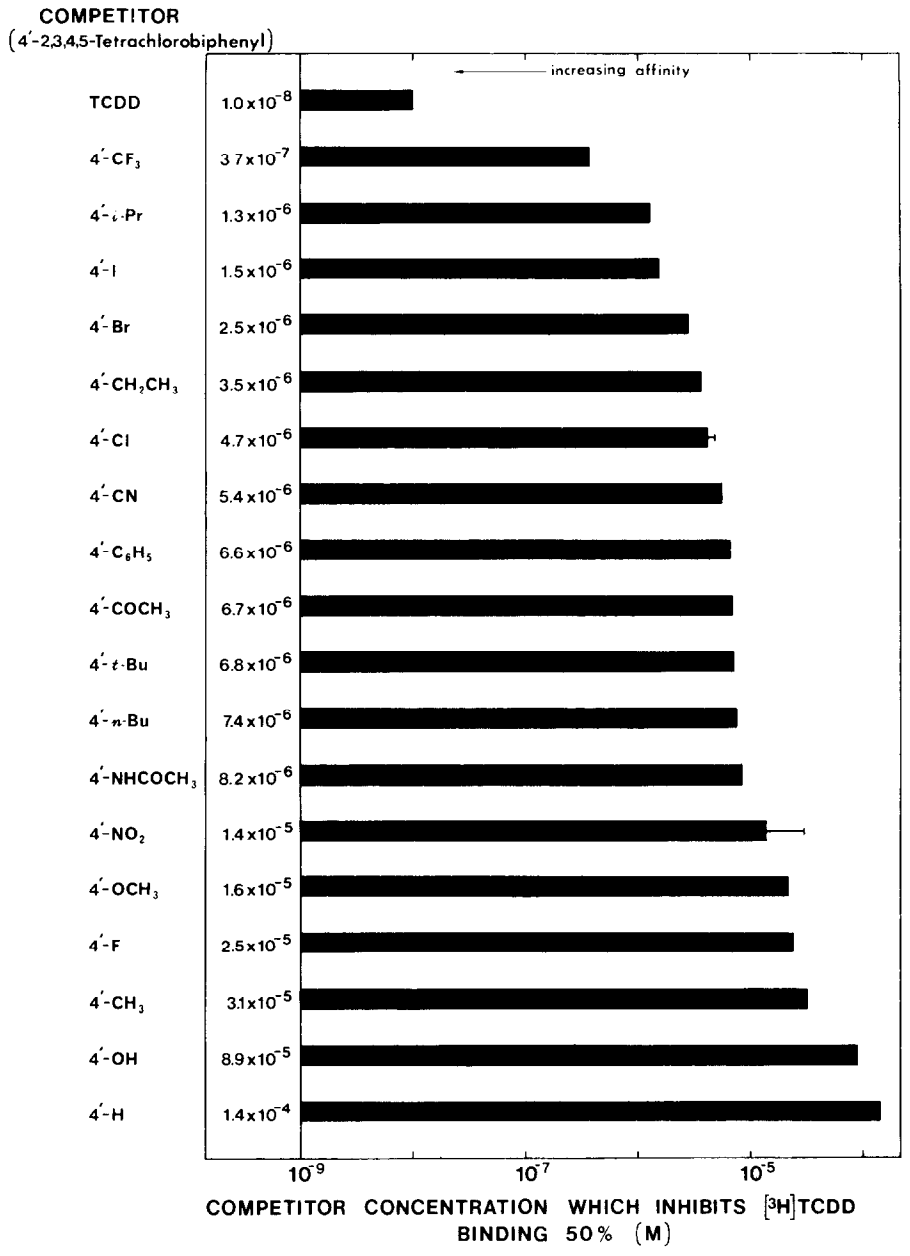


Fig. 3. Comparitive EC_{50} values of the 4'-substituted-2,3,4,5-tetrachlorobiphenyls for specific binding to hepatic cytosolic TCDD receptor. All values are expressed as molar concentrations. For 4'-nitro- and 4'-chloro-2,3,4,5-tetrachlorobiphenyl, the EC_{50} values represent the mean of three determinations. Standard deviation is indicated by the error bars.

cytosol receptor. However, 4'-isopropyl-2,3,4,5-tetrachlorobiphenyl, which was a good ligand for the [^{3}H]TCDD binding site(s) with a binding affinity approximately equivalent to that of 4'-iodo- and 4'-bromo-2,3,4,5-tetrachlorobiphenyl, was a weaker inducer of both AHH and EROD activities *in vitro*. There were five compounds with greater affinity for the receptor than 2,3,4,5'-pentachlorobiphenyl, also a potent mixed-type inducer of cytochrome P-450. It is noteworthy that two of these five substituted biphenyls, namely the ethyl- and isopropyl-*para*-substituted tetrachlorobiphenyls contained a non-halogen substituent on the second phenyl ring. Of the five, 4'-trifluoromethyl-2,3,4,5-tetrachlorobiphenyl had the greatest affinity for the receptor with an EC_{50} value approximately ten times less than that of the pentachlorobiphenyl.

Correlation of receptor binding with substituent constants. To determine the ideal requirements of a ligand for the receptor binding sites in rat hepatic cytosol, we attempted to develop a correlation of receptor binding with substituent constants. For fifteen compounds out of the present series of *para*-substituted biphenyls, electronic, hydrophobic and hydrogen-bonding substituent constants were examined with respect to the EC_{50} values calculated from

Table 2. Substituent parameters and analysis of binding constants

4'-Substituent	$\pi_{X/PhH}$*	ρ_X†	$\pi_{X/Ph}C_6HCl_4$	σ	HB	log(1/EC50) Obs	log(1/EC50) Calc‡	Δ
H	0	0	0	0	0	3.85	4.20	−0.35
OH	−0.67	0.94	−0.46	−0.37	1	4.05	4.20	−0.15
CH_3	0.56	0	0.54	−0.17	0	4.51	4.67	−0.16
F	0.14	0	0.13	0.06	0	4.60	4.45	0.15
OCH_3	−0.02	0.27	0.03	−0.27	1	4.80	4.98	−0.18
$COCH_3$	−0.55	0.16	−0.50	0.50	1	5.17	5.36	−0.19
CN	−0.57	0	−0.55	0.66	1	5.27	5.52	−0.25
Cl	0.71	0	0.68	0.23	0	5.33	5.41	−0.08
CH_2CH_3	1.02	0	0.98	−0.15	0	5.46	5.27	0.19
Br	0.86	0	0.83	0.23	0	5.60	5.61	−0.01
I	1.12	0	1.08	0.18	0	5.82	5.86	−0.04
$CH(Ch_3)_2$	1.53	0	1.47	−0.15	0	5.89	5.92	−0.03
CF_3	0.88	0	0.85	0.54	0	6.43	6.06	0.37
NO_2	−0.28	−0.14	−0.30	0.78	0§	4.85	4.89	−0.04
$NHCOCH_3$	−0.97	0.91	−0.76	0	1	5.09	4.32	0.77
C_6H_5	1.96	0	1.88	−0.01	0	5.18‖	6.65	−1.47
$C(CH_3)_3$	1.98	0	1.90	−0.20	0	5.17‖	6.41	−1.24
$(CH_2)_3CH_3$	2.08	0	1.99	−0.16	0	5.13‖	6.59	−1.46

* Taken from Ref. 33.
† Taken from Ref. 34.
‡ Calculated by equation 3.
§ See text.
‖ Not included in equation 3.

competition binding assays (shown in Fig. 3). Multiple regression analysis of the data led to equation 3,

$$\log(1/EC_{50}) = \underset{(0.57)}{1.39\sigma} + \underset{(0.43)}{1.31\pi} + \underset{(0.61)}{1.12HB} + \underset{(0.39)}{4.20}$$

$$N = 15 \quad S.D. = 0.31 \quad r = 0.916 \qquad (3)$$

$$F = 19.20 \ (\alpha < 1\%)$$

in which hydrophobic π, electronic σ, and a variable HB for hydrogen bond formation are significant parameters determining the variations in the binding constants. The number of compounds is given by N, S.D. is the standard deviation, r is the correlation coefficient and F is the value of the F-ratio. The figures in parentheses are the 95% confidence intervals. The results of the analysis are shown in Table 2.

The 4'-*N*-acetylamino derivative is the compound the log(1/EC50) of which is most poorly predicted by equation 3. For the 4'-nitro compound, the HB value in equation 3 was arbitrarily taken as 0. However, the nitro group is capable of acting as a hydrogen acceptor with an HB value of 1. When this value was used in equation 3, its affinity was predicted to be about ten times higher than the observed value. The hydrogen bonding interaction may not be insignificant for this compound although the mechanism is not clear. Additionally, the the binding of 4'-nitro-2,3,4,5-tetrachlorobiphenyl to the receptor may be related to the planarity of the nitro group with the biphenyl ring system. A nitro group on a benzene ring can lie either in plane or out of plane with the phenyl ring. The preferred orientation is probably planar, since conformational energy is required to rotate an uncrowded NO_2 out of the ring plane [20]. However, compounds with out-of-plane nitro groups demonstrate greater affinity for the receptor (unpublished results, [20]).

Deleting these two outliers, the analysis was repeated to yield equation 4,

$$\log(1/EC_{50}) = \underset{(0.37)}{1.53\sigma} + \underset{(0.30)}{1.47\pi} + \underset{(0.41)}{1.09HB} + \underset{(0.26)}{4.08}$$

$$N = 13 \quad S.D. = 0.18 \quad r = 0.978$$

$$F = 65.90 \ (\alpha < 0.01\%) \qquad (4)$$

Although the quality of the correlation is much improved, the inferences drawn from the correlation are not changed significantly.

The 4'-*n*-butyl, 4'-*t*-butyl and 4'-phenyl compounds were not included in these correlations. Their log(1/EC50) values were much lower than those predicted by equations 3 and 4. The van der Waals' volume of these three substituents is among the highest, being 41.8 cm^3/mole for the *n*-butyl and *t*-butyl groups and 45.8 cm^3/mole for phenyl. The value for other substituents used here is less than 35 cm^3/mole (i.e. 34.12 cm^3/mole for isopropyl and 33.23 cm^3/mole for *N*-acetylamino, Ref. 35). Although the use of various types of steric parameters did not produce meaningful correlations for the present series of compounds, there seems to be a certain limiting size in the receptor site to accommodate the biphenyl derivatives.

DISCUSSION

Inspection of the results of competitive binding experiments (Fig. 3 and Table 2) shows that some substituent groups had the effect of enhancing the

binding of 2,3,4,5-tetrachlorobiphenyl relative to that of 4′-chloro-2,3,4,5-tetrachlorobiphenyl (2,3,4,4′,5-pentachlorobiphenyl), while others diminished it. The relative competitive potency of the 4′-substituted-2,3,4,5-tetrachlorobiphenyls followed the order $CF_3 > CH(CH_3)_2 > I > Br > CH_2CH_3 > Cl$ for the most effective ligands. In fact, 4′-CF_3-2,3,4,5-tetrachlorobiphenyl competed with [^{3}H]TCDD for the specific binding sites almost as effectively as 3,3′,4,4′,5-pentachlorobiphenyl, the most potent competitor of the PCBs previously tested [16]. Our finding that both the ethyl- and isopropyl-substituted-tetrachlorobiphenyls also demonstrated a relatively high affinity for the receptor suggests that halogen substitution in both phenyl rings is not a requirement for binding, in contrast to earlier hypotheses [21, 22].

To determine which substituent parameters are important, the competitive binding data (presented in Fig. 3) were subjected to multi-parameter regression analysis. It was found that the binding constants were best correlated by the equation (given in the Results):

$$\log(1/EC_{50}) = 1.39\sigma + 1.31\pi + 1.12HB + 4.20$$

This correlation means that increasing both substituent electron withdrawing activity and hydrophobicity increased binding avidity of the ligands for the receptor. Hydrogen bond accepting substituents also favor this binding. The coefficient of the HB term, 1.12, means that the molarity of the H-donor in the receptor region is 12- to 13-fold that of the octanol phase which is used as a model of the hydrophobic receptor site [32].

We can now speculate on the factors affecting ligand–receptor binding. It would appear that steric factors do not play a significant role in interactions at the binding site as far as the compounds included in the correlation are concerned. However, steric effects may be important with other ligands having a 4′-substitutent the volume of which is bulkier than 35 cm^3/mole or with ligands which may contain two vicinal bulky groups on a biphenyl, or possibly a dibenzo-*p*-dioxin, backbone. This may be particularly applicable to compounds which contain sterically crowded nitro groups in the lateral positions. Furthermore, it seems probable that absolute planarity for chlorobiphenyls is not required for effective binding to the receptor, since the substituted biphenyls in the present study all contain an *ortho* chlorine atom which is expected to hinder planar conformations. In fact, 4′-trifluoromethyl-2,3,4,5-tetrachlorobiphenyl, which is predicted to be significantly nonplanar, demonstrates a competitive affinity for the receptor almost equivalent to that for 3,3′,4,4′-tetra- and 3,3′,4,4′,5-pentachlorobiphenyl.

Factors which do appear important for effective interaction at the binding site include hydrophobic, hydrogen-bonding, and electronic forces. The free energy-related electronic parameter in the present correlation is the normal Hammett constant which includes both field and inductive effects [36] and correlates, to some extent, with the group dipole moment [37]. Of course, this includes polarizability effects as predicted by earlier studies [21, 22]. That hydrophobicity and especially hydrogen-bond formation are also important is not surprising since both of these forces are believed to be involved in the binding of estrogen and progesterone to their respective receptor proteins [38, 39]. Thus, the [^{3}H]TCDD binding site(s) can be postulated to be a hydrophobic pocket of a limited size containing one or more slightly polarizable groups.

The correlation between the relative binding affinities of 4′-substituted-2,3,4,5-tetrachlorobiphenyls and the expression of a proposed receptor-mediated pleiotypic response, namely the induction of AHH activity in rat hepatoma cells in culture, has also been evaluated. With the exception of 4′-isopropyl-2,3,4,5-tetrachlorobiphenyl, the rank-order potency of the 4′-substituted-2,3,4,5-tetrachlorobiphenyls as competitors with [^{3}H]TCDD for the receptor binding sites (determined as EC_{50} values) correlated well with their rank-order potency as *in vitro* inducers of benzo[*a*]pyrene hydroxylase and ethoxyresorufin *O*-deethylase activities, both of which are cytochrome "P-448" dependent monooxygenases. In addition, most of the compounds which induced AHH activity *in vitro* also increased the activity of microsomal benzo[*a*]pyrene hydroxylase *in vivo*, at the concentrations tested. There was one obvious exception; 4′-ethyl-2,3,4,5-tetrachlorobiphenyl did not induce either enzyme activities or cytochrome levels over control values *in vivo* but did induce both AHH and EROD *in vitro*. Moreover, this compound demonstrated a relatively high affinity for the [^{3}H]TCDD binding site. Tissue residue levels (not shown) indicate that the anomalous result obtained for 4′-ethyl-2,3,4,5-tetrachlorobiphenyl with respect to hepatic microsomal enzyme induction may be explained on the basis of insufficient retention of this compound in liver.

It should be noted that, although all compounds tested competed with [^{3}H]TCDD for binding to the receptor, two 4′-substituted-2,3,4,5-tetrachlorobiphenyls (4′-hydroxy- and 4′-hydro) did not induce either AHH activity or EROD activity in cell culture or *in vivo*. Three possible explanations can be proposed to account for this discrepancy: (1) binding of these "noninducers" to the receptor at high concentrations represents a nonspecific interaction (as reported previously with other PCB congeners, Ref. 16); (2) these compounds are weak competitive antagonists or partial agonists; and (3) induction of enzyme activities may be a reflection of the persistence of these compounds so that rapid metabolism to less active derivatives would be expected, particularly *in vivo*, for the more polar compounds such as the 4′-hydroxy- and 4′-methoxy analogues.

Since the ability to bind effectively to the cytoplasmic receptor, with concomitant induction of cytochrome P-448 dependent monoxygenase activities, is either enhanced or reduced by various substituent groups on the tetrachlorobiphenyl backbone, the variation in biological activity must be dependent on the properties inherent in the substituents. Multiple linear regression analysis has proved to be useful for describing the relative importance of various physicochemical determinants for binding to the cytosolic TCDD receptor in the present study. This type of analysis can also be used to predict the comparative binding affinities and even the induction

potencies of other laterally-substituted biphenyls, dibenzofurans and dibenzo-*p*-dioxins.

Currently, more research with synthetic model ligands is underway to delineate the nature of the receptor–substrate interactions and confirm the relationship between receptor binding affinities and the expression of a pleiotypic response by halogenated aryl hydrocarbons.

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