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Structure–Induction Versus Structure–Toxicity Relationships for Polychlorinated Biphenyls and Related Aromatic Hydrocarbons

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A comparison of the structure-induction (involving rat and mouse *Ah* receptor binding) and structure–toxicity (*in vivo* guinea pig toxicity) relationships suggests that two receptors with structurally distinct binding properties may be involved. This is supported by demonstration of potentiated toxicity through a mechanism believed to involve the *Ah* receptor as a site of loss with respect to toxicity. Theoretical and working models are proposed for these separate receptors to aid in the search for other relevant binding proteins. The findings suggest that polychlorinated biphenyls that are relatively low in toxicity may have modulating properties on the action of highly toxic compounds with which they are normally found in the environment.

Introduction

Several members of the broad class of halogenated aromatic hydrocarbons including the polychlorinated biphenyls (PCBs), and halogenated dibenzodioxins and dibenzofurans produce a characteristic toxicity syndrome, manifestations of which are increased mortality, edema, hyperkeratosis, thymic involution and heptatotoxicity (*1,2*). The toxic compounds are also inducers of cytochrome P-448-mediated mixed-function oxidases (*1,3*). The toxicity and induction responses have both been proposed to involve initial binding of the hydrocarbon to the same cytosolic receptor, but the subsequent events are not understood. It is not known whether the toxicity and induction are causally related or whether they are coordinated but independent aspects of a pleiotropic response (*3*). In this work we attempt to help clarify this matter by examining the structure–activity relationships for both the induction and toxic responses. Studies of structure–activity relationships can help predict the potency of new compounds; distinguish those parts of the molecule that are essential for activity from those that modulate the activity; establish the size and shape of a receptor binding site; identify the type of binding between a compound and a receptor; suggest experiments to identify new regions of the binding site; differentiate between receptor-controlled potency and potency controlled by pharmacokinetics or transport; and identify receptor subtypes *in vivo* (*4*).

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Materials and Methods

Compounds

With the exception of 2,3,6,7-tetrabromobiphenylene (2,3,6,7-TBBene) the compounds used in this work were either obtained from commercial sources or synthesized (*5,6*) or isolated (*7*) from commercial mixtures according to published procedures. The 2,3,6,7-TBBene was obtained as a gift from Professor McOmie, University of Bristol, and it gave the expected mass spectral properties in our hands.

π-Complex Study

The nuclear magnetic response (NMR) spectra were recorded on a Nicolet high resolution FT-NMR spectrometer operating at 360 MHz equipped with Nicolet

1180E computer. Chemical shifts were measured relative to an internal tetramethylsilane (TMS) reference. All measurements were made at 21°C, the ambient temperature of the probe. The standard solution of 3-methylindole (skatole) in chloroform-*d* was prepared with the concentration (0.5 mM) just sufficient to observe an NMR signal (doublet) for the methyl protons. A known amount of acceptor (5–80 mM for 3-methycholanthrene and 10–80 mM for 2,3,7-trichlorodibenzo-*p*-dioxin) in chloroform-*d* was then added, bringing the total volume to 1 mL. The use of NMR in the study of π-complex formation has been previously (*8–9*) demonstrated.

Pharmacokinetic Studies

The excretion pattern of two persistent polychlorinated biphenyls (PCBs) in bile of guinea pigs was studied in the following way. One-month-old (approximately 350 g) male Hartley guinea pigs were anesthetized with 25 mg/kg sodium pentabarbital. A midline incision was made in the abdomen and the common bile duct cannulated with PE-50 tubing. The desired ^{14}C-PCB was injected into the left femoral vein at a volume of 0.5 mL/kg at a dose of 0.6 mg/kg. The PCBs were dissolved at a concentration of 1.2 mg/mL in Emulphor: ethanol:water (1:1:8) at a specific activity of 2 mCi/mmole. After injection of the ^{14}C-PCB, serial samples were collected for up to 6 hr after treatment, at which time the animals were sacrificed. Three animals were used for each PCB.

The bile samples were weighed and radioactivity determined by taking 100 μL samples into 7 mL Aquasol and analyzing in a Beckman 9800 liquid scintillation spectrometer. The resulting data were compiled and the cumulative percentage of the total dose calculated.

Samples of bile were also analyzed by TLC. A 500-μL portion of bile from the treated guinea pigs, taken from the 1.5-hr sample, was chromatographed on a 250-μL preabsorbent silica gel G plate in benzene:ethyl acetate (12:1) for 10 cm. The plate was scored in 1-cm sections, silica gel sections scraped off onto 20 mL Aquasol and radioactivity determined. Bile from untreated guinea pigs was spiked with ^{14}C-PCBs and co-run to determine the migration of the standard compounds in this system.

$LC_{50\text{-}30}$ Studies

Groups of six guinea pigs were exposed to these compounds either as mixtures in combined dosing experiments or as individual purified compounds. Relatively toxic compounds were administered in the microgram per kilogram body weight dose range, whereas the relatively nontoxic ones were administered in the milligram per kilogram body weight range. In combination dosing experiments, the less toxic compounds were administered first to all groups followed immediately by the more toxic compounds. Each guinea pig (Hartley strain, male, 3–4 weeks old, 200–250 g) was gavaged with 0.1 or 0.2 mL of the test compound in corn oil, depending on the compound's solubility, per 100 g body weight. Control animals were dosed similarly with corn oil alone. All guinea pigs were housed singly in suspended stainless steel wire cages and given food (ground NIH Feed A, Ziegler Bros., Gardner, PA) and water *ad libitum*.

Each animal was observed daily and weighed three times a week for the duration of the experiment (30 days). Animals dying during the study and those killed at the end were subjected to a postmortem examination, and target organs were fixed in buffered neutral 10% formalin for subsequent histopathologic examination. Target organs included the thymus, liver, spleen, adrenal, and testicle. Weights of target organs from survivors in the treated animals were recorded. Probit analysis (*10*) was used to evaluate the lethality data. Estimated $LD_{50\text{-}30}$ values [dose required to kill 50% of the animals 30 days postexposure (PE)] were not determined for all compounds tested mainly because of insufficient amounts of highly purified synthetic compounds, but $LD_{50\text{-}30}$ ranges were sufficient for comparison purposes to assess relative magnitudes of toxicity expressed as lethality.

For all lethal compounds or their combinations, a similar, clinocopathologic syndrome was observed. There was an almost immediate dose-related decrease in body weight gain or actual body weight loss in the treated groups as previously observed (*11*). Animals which survived began to regain weight within 7 to 10 days PE, while those which died showed progressive weight loss. Organ weight effects were also similar to those observed previously (*11*).

The lesions observed at necropsy and on histopathologic examination were also characteristic. This included marked thymic involution, adrenal cortical hemorrhage, testicular atrophy and hyperplasia of the transitional epithelium lining of the urinary tract from the renal pelvis to the urinary bladder. Changes in the adrenal gland and testicle were found mainly in animals that died. Thymic involution and transitional cell hyperplasia occurred at sublethal doses, and the severity of these lesions was dependent on the amount of compound administered.

Results

Several new experiments were performed to provide additional experimental support for arguments given in the discussion. Results of these experiments are described below.

Pharmacokinetics and Transport Considerations

3,4,3′,4′-Tetrachlorobiphenyl (34-TCB) and 2,4,5,2′,4′,5′,hexachlorobiphenyl (245-HCB) are both potent inducers of drug-metabolizing enzymes. 245-HCB is rel-

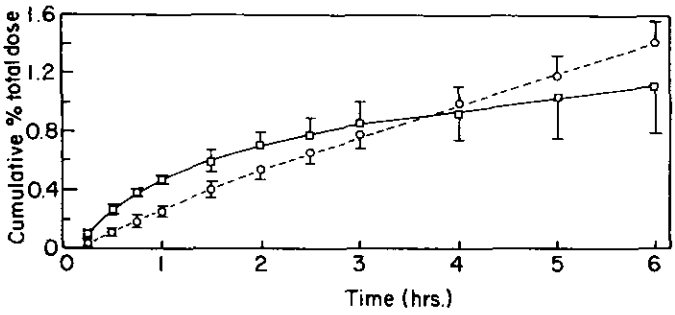


FIGURE 1. Biliary excretion of PCBs in guinea pigs: (□) 3,4-TCB; (○) 2,4,5-HCB.

atively nontoxic, while 34-TCB is one of the most toxic PCB isomers known. Since all the highly chlorinated PCBs have been shown to require metabolism for excretion that occurs almost completely via the biliary route, we decided to compare the excretion of these two PCBs in the bile in order to see if a difference in excretion in the guinea pig might account for the difference in toxicity in this highly sensitive species.

The excretion of ^{14}C-PCB-derived radioactivity in the bile of treated guinea pigs can be seen in Figure 1. With both isomers, less than 1% of the total dose is excreted within 4 hr of treatment. There was no detectable radioactivity in the treated bile that cochromatographed with either of the standard PCBs, suggesting that essentially all of the ^{14}C-PCB-derived radioactivity excreted in the bile is due to metabolites of the parent HCB. Thus, very low amounts of either 34-TCB or 245-HCB are excreted by guinea pigs after IV dosing, and what is eliminated is in the form of metabolites.

Structural Types and Comparisons

A relatively simple assay method (*12*) for the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) receptor in hepatic cytosol was used to measure 2,4,6-triiodophenol (2,4,6-TIP) binding to the *Ah* receptor. The assay is based on specific binding of the [^{125}I] iodovaleramide derivative of trichlorodibenzo-*p*-dioxin or [^{125}I] dioxin to cytosol-binding protein. Specific binding is taken as the difference between [^{125}I] dioxin bound in the absence of unlabeled TCDD and [^{125}I] dioxin bound in the presence of a large excess of unlabeled TCDD (or other competitors). Under these conditions 2,4,6-TIP (10^{-6} M) had a relative binding affinity of 92% (at 10 M^{-6} TCDD set equal to 100). Therefore, this single ring iodo compound is almost as effective as unlabeled TCDD in displacing the labeled dioxin.

Stacking versus Nonstacking Models

In order to investigate the hypothesis that charge-transfer complexes are involved in *Ah* receptor binding, an NMR method has been employed to study a series of π-molecular complexes between 3-methylindole acting as an electron donor and certain aromatic compounds, including 3-methylcholanthrene and 2,3,7-trichlorodibenzo-*p*-dioxin as an electron acceptor. These studies were severly limited by lack of a distinct NMR signal and low solubility for toxic acceptor compounds. However, for these two compounds, plots of the reciprocal of the observed shifts of donor protons versus the reciprocal of the concentration of acceptor molecules in chloroform-*d* gave straight lines [for 237-Tri CDD, $1/\Delta$ obs = 0.0957 + 52.9461 ($1/M_D$); for 3-MC, $1/\Delta$ obs = -0.0746 + 21.3495 ($1/M_D$)]. As demonstrated previously (*8,9*) these observed shifts are consistent with 1:1 molecular complexes often referred to as charge-transfer complexes and thus support the stacking-type interaction for both a halogenated and nonhalogenated compound which do bind the *Ah* receptor.

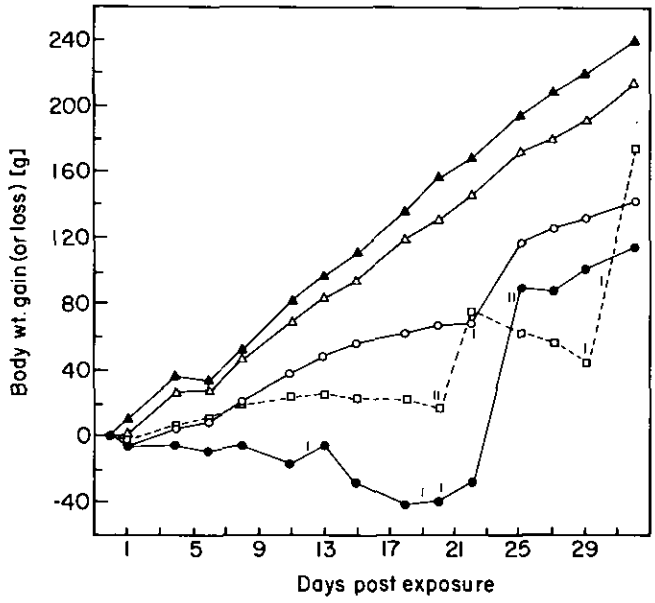


FIGURE 2. Mean (N = 6) body weight gain (or loss) versus days postexposure for pentabromonaphthalene (PBN) at 180 μg/kg and selected combinations: (Δ) control; (*f*) 2,4,5,2′,4′,5′-hexabromobiphenyl (HBB) at 50 mg/kg and 2,4,5,3′,4′,5′-HBB at 20 mg/kg; (●) 2,4,5,2′,4′,5′-HBB at 50 mg/kg, 2,4,5,3′,4′,5′-HBB at 20 mg/kg and PBN at 180 μg/kg; (○) PBN at 180 μg/kg; (□) 3-methylcholanthrene at 3 μg/kg and PBN at 180 μg/kg; superscript 1 denotes days on which one animal died.

Evidence for Two Receptors

The following experiment was designed to demonstrate synergism and the involvement of multiple receptor interactions. An approximate $LD_{50'30}$ dose of 1,2,4,6,7-pentabromonaphthalene (PBN) (180 μg/kg) was given in the presence and absence of polybrominated biphenyls (PBBs). Figure 2 and Table 1 show that the combination of nontoxic PBBs (2,4,5,2′,4′,5′- hexabromobiphenyl (HBB), a P-450 inducer and 2,4, 5,3′,4′,5′-HBB a mixed inducer) with PBN or of a relatively nontoxic polynuclear aromatic hydrocarbon, 3-methylcholanthrone (3-MC), with PBN resulted in an enhancement in toxicity both in terms of body weight gain (or loss) and lethality than when PBN or the nontoxic compounds are given alone. This potentiation of toxicity is even

Table 1. Weight gain on selected days for guinea pigs (X + SE) dosed with PBN, 2-HBBs or combination.[a]

Day	Weight gain, g[b]			
	Control	PBN	2-HBB	PBN + 2-HBB
1	10.2 ± 1.9	−5.7 ± 2.4	1.7 ± 2.3	−5.8 ± 1.7
5	26.3 ± 2.0	−0.8 ± 2.9	12.8 ± 6.6	−14.0 ± 4.4
8	51.0 ± 4.3	20.5 ± 3.7	46.0 ± 5.0	−4.7 ± 11.4
12	94.5 ± 6.4	47.0 ± 5.4	80.2 ± 5.9	−19.5 ± 24.3
13	97.5 ± 6.8	49.5 ± 5.2	83.5 ± 7.1	−1.6 ± 18.4[d]
15[c]	110.8 ± 8.2	56.3 ± 9.4	93.3 ± 7.4	−25.0 ± 23.7[d]
18[c]	134.8 ± 11.2	60.0 ± 17.2	118.3 ± 6.7	−39.6 ± 29.0[d]

[a]Each group started with six animals; initial weights were 253 ± 5 g (control), 252 ± 4 g (PBN), 248 ± 248 ± 6 g (2-HBBs) and 254 ± 5 g (PBN ± 2-HBBs).
[b]Relative to initial weight.
[c]Significant ($p < 0.05$) potentiation on these days by analysis of variance.
[d]Based on five animals; one animal died between days 12 and 13.

more dramtically pointed out by examining the changes in individual animal weights (Fig. 3). Although there was evidence of potentiation early as day 8, because of the high variability in the combination group (reflecting primarily a single animal who continually lost weight and finally died between days 12 and 13) this interaction is not statistically significant ($p < 0.05$ by analysis of variance) until day 15 (see Table 1). In separate experiments (data not shown), it was shown that neither PBB isomer when given alone with PBN produced a similar level of potentiation. The apparent requirement for both a P-450 and mixed inducing PBB in potentiation of toxicity in the guinea pig is being examined further.

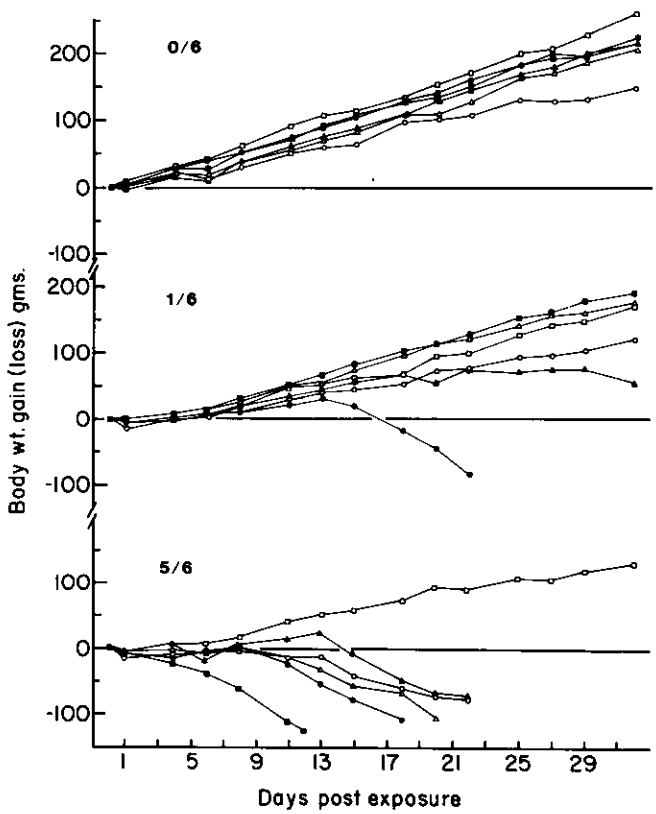


FIGURE 3. Individual body weight gain (or loss) versus days postexposure for 2,4,5,2′,4′,5′-hexabromobiphenyl (HBB) at 50 mg/kg and 2,4,5,3′,4′,5′-HBB at 20 mg/kg group (top), pentabromonaphthalene at 180 μg/kg group (middle) and the combination of the HBBs and PBN group (bottom). Number of deaths per group also shown.

Discussion

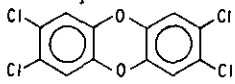
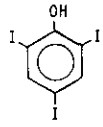
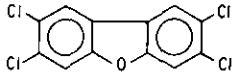
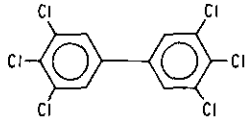
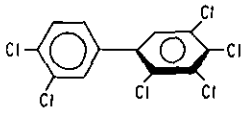
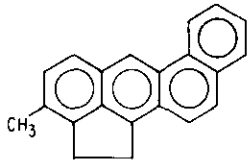
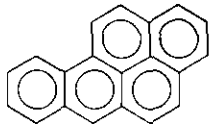
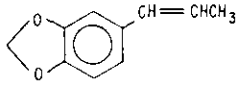
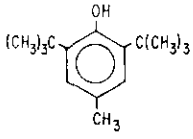
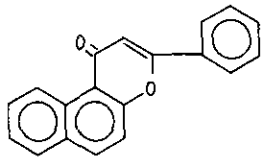
Pharmacokinetic and Transport Considerations

A cytosolic receptor, with a very high affinity for aromatic chemicals is believed to be the major product of the *Ah* regulatory genes. Attempts to characterize this receptor have been made with a variety of assays including charcoal-binding (*13*), isoelectric focusing following trypsin treatment (*14*), sucrose density gradient centrifugation following dextran–charcoal treatment (*15*), and DEAE–cellulose column chromatography (*16*). All of these assays are based on rapid separation of free from bound [^{3}H] TCDD. We have recently developed a method (*12*) based on [^{125}I] dioxin, which is simpler to perform and shows much less nonspecific interference.

The capacity of a number of aromatic hydrocarbons to displace dioxin from the cytosolic receptor has been determined as a measure of their binding affinities. An excellent correlation is seen between the capacity to displace the radioligand from the *Ah* receptor and the capacity to induce certain monooxygenase activities (*17*). Since several assay methods have been used to make these measurements, it is difficult to compare the results of one study in a quantitative sense with any other study. However, it is possible to compare these results qualitatively in order to try to gain insight into what might be common structural features of importance in receptor binding. Since the binding data on which the structure–induction considerations are based is from *in vitro* measurements, pharmacokinetic and distribution properties are not a major concern here, although one occasionally encounters solubility problems in performing the assays.

Using whole-animal toxicity data as the activity side of the structure–activity equation is not frequently done since any interpretation of relationships would ordinarily be complicated by differences in pharmacokinetic factors for the compounds under study. However,

Table 2. Structures of representative halogenated aromatic and nonaromatic compounds that bind the *Ah* receptor and approximate equilibrium dissociation constant K_d.

Structure	Compound	K_d, M
Halogenated aromatic compounds		
Structure I, 	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	10^{-8}
Structure II, 	2,4,6-Triiodophenol	10^{-8}
Structure III, 	2,3,7,8-Tetrachlorodibenzofuran	10^{-7}
Structure IV, 	3,4,5,3′,4′,5′-Hexachlorobiphenyl	10^{-7}
Structure V, 	2,3,4,5,3′,4′-Hexachlorobiphenyl	10^{-6}
Nonhalogenated aromatic compounds		
Structure VI, 	3-Methylcholanthrene	10^{-8}
Structure VII, 	Benzo(a)pyrene	10^{-8}
Structure VIII, 	Isosafrole	10^{-8}
Structure IX, 	Butylated hydroxytoluene	10^{-8}
Structure X, 	β-Naphthoflavone	10^{-7}

in the course of examining the lethality of various halogenated aromatic hydrocarbons in the guinea pig, we have examined using several animals species the differences in absorption (*18*), distribution (*19*), metabolism and excretion (*20*) properties of selected isomers and congeners including the biphenyls. In general, potency correlates with resistance to metabolism and persistence in the tissues, and differences in absorption and distribution are not remarkable. However, persistence in tissue alone is not sufficient for toxicity. While species differences can be demonstrated for these compounds, there is no evidence for significant differences in pharmacokinetic properties of these compounds in the guinea pigs. Our results (Fig. 1) comparing the excretion pattern of a toxic and nontoxic PCB in the bile are consistent with this argument. Since the guinea pig shows exquisite sensitivity to the toxic effects of these compounds (*11*), we felt that this animal model might afford useful structure–activity information for these classes of compounds.

Structural Types and Comparisons

It is seen that both halogenated and nonhalogenated aromatic compounds bind the *Ah* receptor, some with near equal affinities. An aromatic or extended conjugation system appears always to be required. Up until now, it has not been a practice to screen nonaromatic compounds for their affinity for the *Ah* receptor.

Table 2 shows a representative series of halogenated aromatic hydrocarbons that have been shown to bind the receptor in one or more assay systems. The compounds are shown roughly in order of decreasing affinity for the receptor. For purposes of comparisons, approximate K_d values are given as estimated from literature values (*24,17*) primarily reflecting ECs (competitor concentration which inhibits ^{3}H-TCDD or related ligand binding to the *Ah* receptor). This series illustrates that molecular size and shape are not the only determinants of binding since the single-ring iodo compounds binds effectively (92% relative to TCDD) while a basically nonplanar system (2,3,4,5,3′,4,4′-hexachlorobiphenyl) binds with a considerably decreased affinity, presumably because of additional steric interactions.

In the second portion of Table 2, the structures of several nonhalogenated aromatic hydrocarbons are shown in their rough order of binding to the receptor. A similar trend is seen here, in that single-ring compounds (such as butylated hydroxytoluene) again bind with good affinities and molecules with increasing conformational flexibility (as in β-naphthoflavone) show lowered binding affinities.

Therefore, for these representative halogenated and nonhalogenated compounds, the highest affinities were seen for the rigidly planar compounds (seven compounds) with as little as one ring (three compounds) and the lowest for the three compounds with nonrigid structures. Structural considerations such as these lead one to conclude that binding to the cytosolic receptor associated with the induction responses is facilitated by an aromatic ring system for which molecular size, halogen substitution and coplanarity of rings are not critical but may affect the strength of binding.

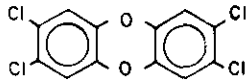
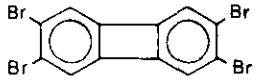
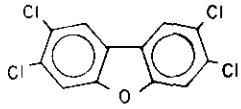
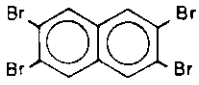
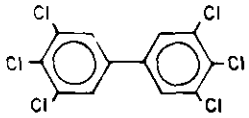
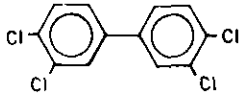
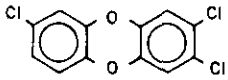
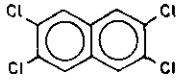
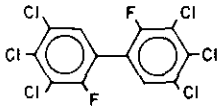
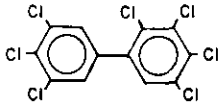
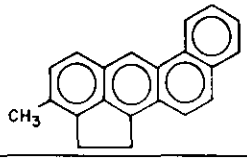
Differences in toxicity (as defined in the methods section for $LD_{50\text{-}30}$ studies) that cannot be explained on the basis of pharmacokinetic differences may be related to variations in some toxicologically relevant receptor. We have examined the structure–toxicity relationship (*21*) of a number of halogenated aromatic hydrocarbons in the guinea pig for comparison with the structure–induction relationships previously developed in the mouse (*3*). Here again, the primary interest is in detecting qualitative differences, but some quantitative considerations are also possible at this point.

Table 3 shows the structures of several highly toxic halogenated aromatic hydrocarbons in their approximate order of decreasing potency (estimated $LD_{50\text{-}30}$ dose given) in the guinea pig. The range of potency seen here is about 500 to 1. With the exception of the brominated naphthalene, all of these compounds in their planar or coplanar state are approximate isosteres (in relation to the dioxin structure). The increase in conformational flexibility found in the PCB structure does lower the toxicity as would be expected if specifically placed lateral halogens are important in receptor interaction. The presence of oxygen in the structure is not essential for high toxicity. We show for comparison several relatively nontoxic compounds. The presence of at least four lateral halogens in a planar or coplanar state is seen to be of major importance in toxicity in comparing the trichlorodioxin and *ortho*- substituted PCB structures with tetrachlorodioxin and the non-*ortho*-containing PCB structures. The importance of sufficient polarization about lateral halogens is seen in comparing the tetrachloronaphthalene with the relatively more polarized tetrabromonaphthalene. The relatively low toxicity (*22*) of 3-methylcholanthrene is interesting since this compound has been shown (*15*) to bind to the dioxin receptor with similar binding affinity to dioxin (TCDD). This approximately 10^4 difference in toxic potency cannot be explained entirely on the basis of differences in metabolism (*23,24*). In fact, this difference strongly suggests that the toxicity and induction responses are not causally related.

It is of interest to examine further the apparent lack of toxicity of *ortho*-substituted halogenated biphenyls, since some of these compounds have been shown to bind to the dioxin receptor and afford an induction response associated with cytochrome P-448. Table 4 shows the estimated $LD_{50\text{-}30}$ values for several halogenated biphenyl compounds in the guinea pig. With the exception of the 2,4,5,2′,4′,5′-hexachlorobiphenyl, all of these biphenyls afford an induction response associated with cytochrome P-448. The *ortho*-substituted isomers are in every case considerably less toxic than the non-*ortho*-containing compounds.

This is further demonstrated in comparing plots of mean body weight gain (or loss) in guinea pigs as a function of time after exposure (Fig. 4). Treatment with the two toxic PCBs (*5*), 3,4,3′,4′- and 3,4,5,3′,4′,5′-, results in a significant depression of weight gain at doses of 300 μg/kg. Some lethality (2/6) was seen for those treated with 3,4,5,3′,4′,5′-HCB. However, a 10-fold higher dose of 2,3,4,5,3′,4′,5′-heptachlorobiphenyl (*5*), the corresponding hepta isomer with one *ortho* chlorine added, showed a body weight gain essentially identical to the controls. The P-450 inducer, 2,4,5,2′, 4′,5′-HCB also had no effect over a 30-fold higher dose. Levels as high as 30,000 μg/kg of 2,4,5,3′,4′,5′-hexabromobiphenyl, a mixed inducing isomer (*7*), had slight effects on body weight gain but no lethality (data not shown). The difference in binding affinity of the *Ah* receptor for mixed inducing type and for P-448 inducing type PCBs is in the range of 10- to 100-fold so one might have expected to see some indication of toxicity. The lack of toxicity of the mixed inducers could be associated with a much smaller or negligible population of coplanar molecular conformers present which are isoteric with the TCDD structure.

Table 3. Structures of halogenated aromatic hydrocarbons of high and low toxicity in the guinea pig.

Structure	Compound	$LD_{50\text{-}30}$, μg/kg[a]
Compounds of high toxicity		
Structure I, 	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	2
Structure II, 	2,3,6,7-Tetrabromobiphenylene	< 10
Structure III, 	2,3,7,8-Tetrachlorodibenzofuran	7
Structure IV, 	2,3,6,7-Tetrabromonaphthalene	206
Structure V, 	3,4,5,3′,4′,5′-Hexachlorobiphenyl	500
Structure VI, 	3,4,3′,4′-Tetrachlorobiphenyl	< 1,000
Compounds of low toxicity		
Structure VII, 	2,3,7-Trichlorodibenzo-*p*-dioxin	> 29,000
Structure VIII, 	2,3,6,7-Tetrachloronaphthalene	>> 3,000
Structure IX, 	2,2′-Difluoro-3,4,5,3′,4′,5′-Hexachlorobiphenyl	>> 3,000
Structure X, 	2,3,4,5,3′,4′,5′-Heptachlorobiphenyl	> 3,000
Structure XI, 	3-Methylcholanthrene	> 10,000

[a]Estimated $LD_{50\text{-}30}$ in Hartley strain guinea pigs given a single oral dose and observed for 30 days.

Since the available population of coplanar conformers is the result of a dynamic equilibrium process, there have been no good ways to accurately estimate the relative populations although some attempt (*25*) has been made to associate the degree of coplanarity with the so-called "K-band" in their ultraviolet spectrum. We have approached this problem by estimating (*26*) through quantum chemical calculations the barrier to rotation about the pivot bond (estimation of barrier to rotation at 0° torsion angle in kcal/mole). Using the Gaussian-80 *ab initio* technique, all molecules that do not have *ortho* substitution have an energy minimum at 42° and barriers of approximately 3.6 kcal/mole at 0°. *Ortho* substitution results in an extremely high barrier at 0° and a shift of the energy minimum toward toward 90°. These calculations are consistent with the information available from X-ray crystallographic measurements on related compounds (*27*).

If one adjusts the energy barrier (3.6 kcal/mole value assumes no bond distortions) by 5 to 6% to allow for some bond distortions and uses this value (3.4 kcal/mole) in the Boltzman distribution equation to calculate a population of coplanar conformers, approximately 0.4% of the molecules could exist in the coplanar state. Small increases in this barrier to rotation have a remarkable effect on the population, e.g., raising the barrier to 5.0

kcal/mole results in only 0.03% coplanar conformers. It can be seen (Table 5) that by normalizing toxic potency of the non-*ortho*-containing PCBs by the percentage of coplanar conformers results in an adjusted potency approaching unity, i.e., equipotent to TCDD. If the population of coplanar conformers is indeed responsible for the observed acute toxicity for these PCBs, then one must speculate that the majority of the administered PCB is in a noncoplanar state and cannot reequilibrate to form additional coplanar conformers necessary for toxicity. If reequilibration were possible, the full concentration of PCB should be expressed as toxicity, but this apparently is not the case.

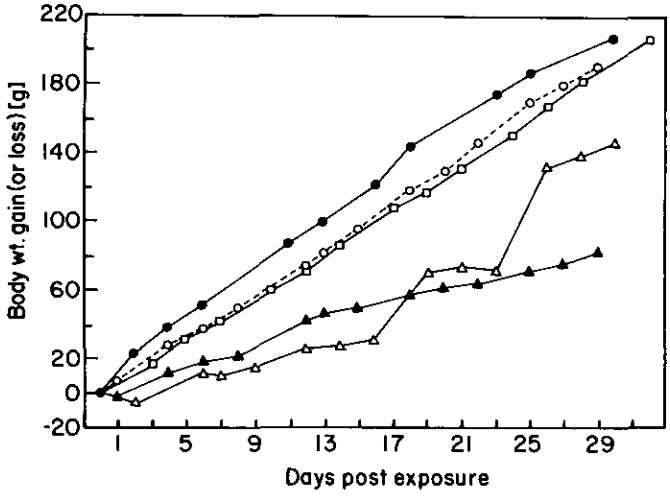


FIGURE 4. Mean (N = 6) body weight gain (or loss) versus days post exposure for selected PCBs: (*f*) 3,4,3′,4′ at 300 μg/kg; (Δ) 3,4,5′,3′,4′,5′ at 300 μg/kg; (□) 2,3,4,5,3′,4′,5′ at 3000 μg/kg; (●) 2,4,5,2′,4′,5′ at 10,000 μg/kg; (○) control.

Electronic Properties

There are circumstances under which polarizability may be the determining factor in governing the interaction of two molecules at long range. This is the attraction resulting from the instantaneous and simultaneous polarization of the two molecules and constitutes the dispersion energy (*28*). Of the intermolecular forces, the dispersion interaction alone depends on the square of the polarizability. Since polarizability is roughly proportional to the number of electrons, dispersion interactions can reasonably be expected to be dominant for large molecules. This appears to be the case for the PCBs and related compounds where permanent dipoles are weak and similar across the classes and where ionization energies are nearly identical throughout. We have recently (*29*) modified the Applequest-Carl-Fung method (*30*) so as to improve the computed polarizabilities of planar aromatic molecules, and we have devel-

Table 4. Effect of *ortho* substitution on toxicity of halogenated biphenyls in the guinea pig and approximate equilibrium dissociation constant K_d for *Ah* receptor response.

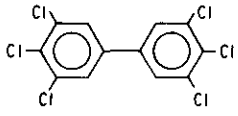
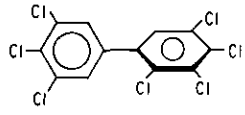
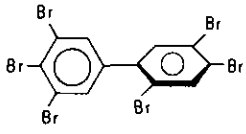
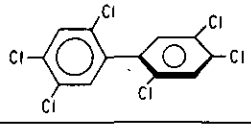
Structure		Compound	$LD_{30\text{-}50}$	K_d, M
Structure I,		3,4,5,3′,4′,5-Hexachlorobiphenyl	500 μg/kg	10^{-7}
Structure II,		2,3,4,5,3′,4′,5′-Heptachlorobiphenyl	> 3 mg/kg	10^{-6}
Structure III,		2,4,5,3′,4′,5′-Hexabromobiphenyl	> 30 mg/kg	10^{-6}
Structure IV,		2,4,5,2′,4′,5′-Hexachlorobiphenyl	> 10 mg/kg	10^{-5}

Table 5. Calculation of population of coplanar conformers in non-*ortho*-substituted biphenyl compounds and relevance to toxicity.

Compound	Potency relative to TCDD[a]	Population of effective coplanar conformers, %[b]	Adjusted potency[c]
3,4,5,3′,4′,5′	223	0.4	0.89
3,4,3′,4′	>> 166 < 552	0.2	>> 0.33 < 1.1

[a]Lethality in guinea pig on a molar basis.
[b]Obtained from Boltzman distribution equation [planar/nonplanar = $e^{-\Delta E/kT}$] using 3.4 kcal/mole for ΔE and 0.62 kcal/mole for kT and multiplying by 100% [k is Boltzman's constant and T is absolute temperature (310°K)].
[c]Multiplying observed molar potency by % of coplanar conformers.

Table 6. Comparison of polarizability components (Å^3) of toxic compounds.[a]

Compound	α_1		α_2		α_3		α	
	Calc	Exp	Calc	Exp	Calc	Exp	Calc	Exp
Toxic compounds								
3,3′,4,4′-TCB[b]	52.983	–	27.787	–	14.035	–	31.602	25.5
3,3′,4,4′,5,5′-HCB[b]	55.517	–	35.299	–	15.357	–	35.391	41.9
2,3,7,8-TCDD	58.271	–	28.591	–	14.325	–	33.729	–
Reference compounds								
1,2,3,4-TCDD	48.21	–	34.65	–	14.43	–	32.43	–
Benzene	12.347	12.27	12.347	12.27	6.508	6.65	10.401	10.40
Cl-Benzene	16.763	15.93	12.723	13.24	7.265	7.58	12.250	12.25
Biphenyl[b]	36.027	24.7	20.803	20.2	11.655	13.8	22.828	19.57

[a]Atom dipole interaction model for polarizability (*29*).
[b]Calculated at torsion angle of 0° (coplanar state).

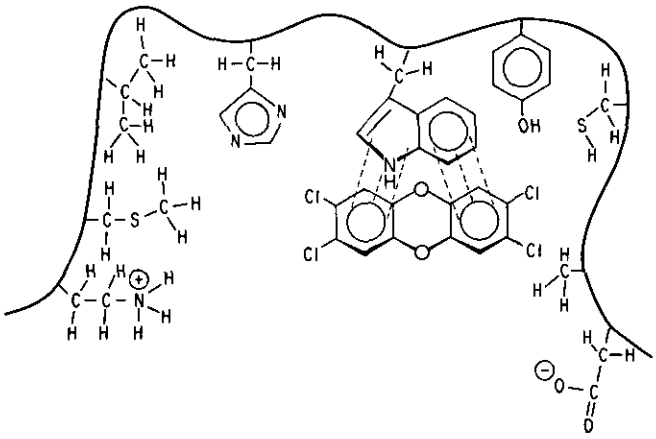


FIGURE 5. Illustration of *Ah* receptor stacking interaction model showing polarization through π-complexation.

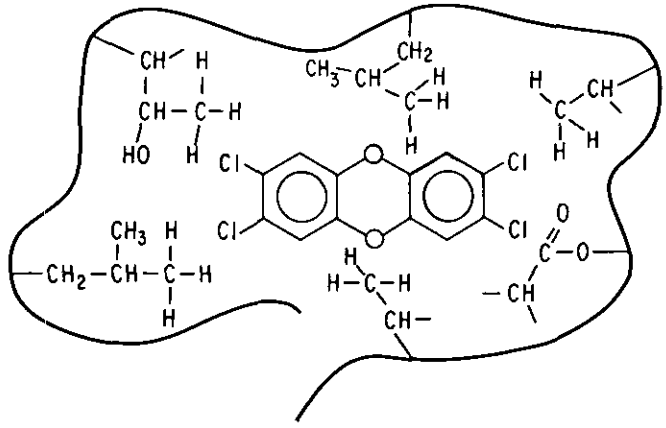


FIGURE 6. Illustration of a nonstacking lateral halogen polarization interaction model for putative "toxic" dioxin receptor showing "pocket" type interaction.

oped (*31*) a model based on such dispersion interactions to interpret the cytosol *Ah* receptor-PCB binding data obtained by others (*32*). The essential parameters in this model are the PCB polarizability and equilibrium separation of the receptor and the PCB. A quantitative structure–activity relationship has been derived in terms of these molecular parameters and should now be useful in predicting the potential of untested PCBs and possibly related compounds to bind to the *Ah* receptor and afford and inductive response.

The apparent requirement of planarity or coplanarity (as occurs in nonfused ring systems) of structure for high toxicity suggested that there might be an important and common electronic property associated with the placement of the lateral halogen atom. As discussed above, the polarizability properties of these molecules would appear to be important in such receptor interactions and could account for the importance of number and positions of halogens in the toxicity of these compounds. In our modification (*29*) of the Applequest-Carl-Fung method for computing polarizabilities of planar aromatic molecules, we were also able to compute the polarizability components (in x, y, and z coordinates) of the molecule. Preliminary comparisons of the computed components of three toxic structures have shown similarities in the pattern of polarizabilities especially with regard to the component in the direction of the lateral halogens (x-axis or α_1 in Table 6). It is the same strong polarization in the direction of the lateral halogens that perhaps contributes to the electropositive ring character important in binding to the *Ah* receptor.

Stacking versus Nonstacking Models

The diversity of structural types that bind the *Ah* receptor suggests a stacking type interaction of molecules as in charge-transfer type complexation (*33*) as investigated in our NMR study. It has been proposed (*3*) that a rectangle 3 × 10 Å with halogens in the four corners serves as a rough approximation, for the generalized structure–activity relationship involving the *Ah* receptor. It seems clear now that molecular size and shape are not controlling factors in binding the *Ah* receptor. A better model is based on molecular polarizability (dispersion) and equilibrium separation between receptor and effector molecule. Development of a theoretical model for the *Ah* receptor based on these molecular parameters provides additional support for this argument (*31*). This is a more universally applicable model (stacking or layering of molecules) since it can explain the exceptions found for the rectangular box model. An illustration of this model is shown in Figure 5. This illustration (and Fig. 6 also) is given only for descriptive purposes and is not meant to suggest that

anything is known about the specific microenvironment (nature of functional groups, etc.) of the receptor site.

In the structure–toxicity relationship planarity or coplanarity of structure and lateral halogenation are apparently important in the specific placement of enough halogens to effect a sufficient condition of polarization and its localization. This would be more consistent with a nonstacking (see Fig. 6) type receptor model. If both the toxicity and induction responses were mediated by the same receptor or different stacking type receptors, the two responses should have shown the same or very similar structure–activity relationships. Thus the structure–toxicity relationship appears to differ from the structure–induction relationship in that although both appear to involve polarizability properties of the molecule, the latter depends more upon molecular polarizability in a stacking type complex whereas the former may depend more on specific polarizability components involving the lateral halogens.

Evidence for Two Receptors

The proposal that the structure–induction and structure–toxicity relationships are different and may involve mechanistically different receptors is an interesting one and can be experimentally tested. From a theoretical basis, if one considers two compounds of the same type and with identical sites of action, a synergistic effect of the combination cannot be imagined (*34*). In this case, any competition for the receptor will usually decrease the frequency of the best interactions. With decreasing intrinsic activity of one of the components, the combined action will assume the form of a competitive antagonism. Now if one considers that there may be different sites of action for two compounds with the same type of activity, then it is possible to have a synergistic effect of the combination with respect to the primary activity because of a competition between an active compound and less active or inactive structurally related compound for the sites of loss (see below).

Figure 7 is a schematic illustration as to how this might work in the case of the relatively toxic brominated naphthalenes and the relatively nontoxic brominated biphenyls. In the absence of brominated biphenyls, a given level of brominated naphthalene is required to produce a certain level of action or toxic response. A portion of the administered compound is wasted on receptors (site of loss) not coupled to the toxic response. However, if a brominated biphenyl compound can be found which will preferentially occupy (in higher concentration) the sites of loss for the naphthalene, it should be possible to lower the toxic dose of the haphthalene required since more of it would now be available for interaction with the site of primary toxic action.

As a test for this hypothesis we examined the effects of selected polybrominated biphenyls on the toxicity of 1,2,4,6,7-pentabromonaphthalene in the guinea pig. The combination of nontoxic PBBs with PBN or of 3-MC was seen to potentiate toxicity both in terms of body weight gain (or loss) and lethality. If this demonstrated synergism is due to the *Ah* receptor functioning as a "site of loss" with respect to toxicity then a relatively nontoxic compound with much higher affinity for the *Ah* receptor should show this potentiation of PBN toxicity. As seen in Figure 2, combination of PBN with 3-MC resulted in a dramatic increase in the observed toxicity.

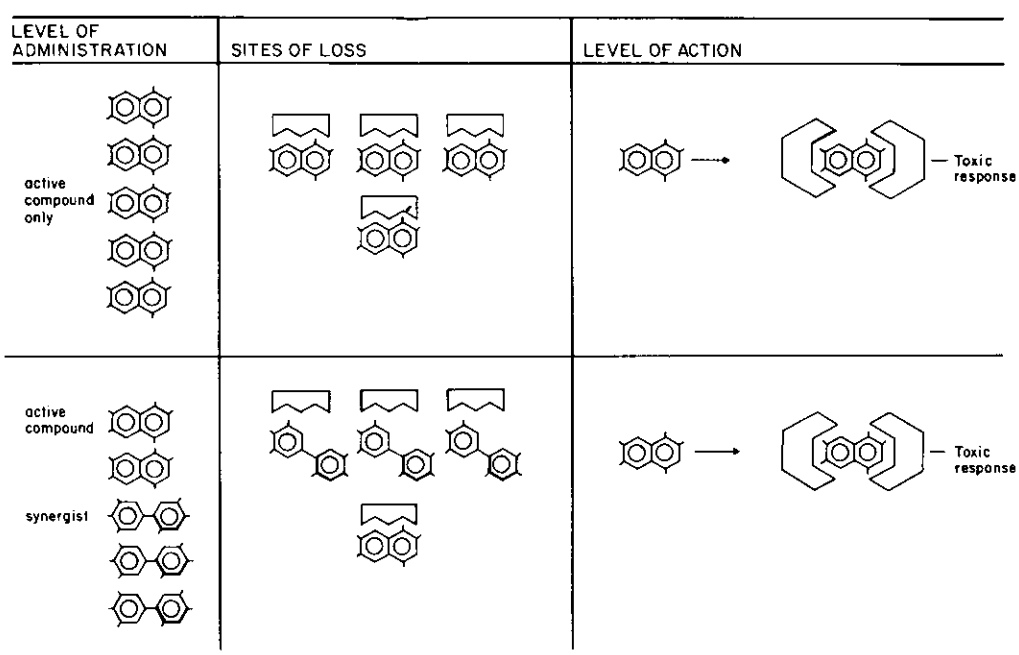


FIGURE 7. Possible molecular mechanism for potentiated toxicity of a planar halogenated aromatic hydrocarbon (represented by a substituted naphthalene) by a halogenated biphenyl.

Since this result was with only 3 μg/kg of 3-MC and the reported toxicity of 3-MC in guinea pigs is in the tens of milligrams per kilogram range (*24*), it is clearly not due to 3-MC toxicity nor to induction of arylhydrocarbon hydroxylase (AHH) activity (*35*).

As further evidence and an additional test for this mechanism, it has been shown (*36*) in preliminary work using the mouse teratogencity model (*37*) that the induction of cleft palates by TCDD can be significantly enhanced (> 10-fold) by addition of mixed-inducer PCB isomers that are relatively nontoxic but do bind to the *Ah* receptor (*32*). In contrast, a phenobarbital type inducing PCB isomer had no effect on the teratogenic potential of TCDD. These results are in agreement with those observed on body weight gain or lethality of PBN in guinea pigs.

It has recently been reported (*38*) that benzoxaprofen, a nonsteroidal antiinflammatory agent, decreased the toxicity of 34-TCB in the chick embryo and that it does so without altering the degree of mixed-function oxidase induction. This independent alteration of 34-TCB toxicity and induction further suggests that the two phenomona are not casually related and can also be interpreted in terms of two separate receptors.

Conclusion

Comparison of the structure–induction and structure–toxicity relationships suggests that they are not identical but may both depend upon polarizability properties of molecules. Structural considerations and experimental models for receptor interactions further suggest that interaction with the *Ah* receptor may involve a stacking interaction while binding to the putative "toxic" receptor may not be stacking in nature. The existence of this second receptor for toxicity is postulated to account for the differences in the structure–activity relationships.

The proposal for multiple receptor involvement is tested experimentally by demonstrating the potential for synergism between selected combinations of chemicals for which the *Ah* receptor can serve as a site of loss with respect to the primary toxic action. Synergism has been observed both in guinea pig toxicity and mouse teratogenicity animal models.

These results also suggest that induction by some compounds may be an indirect response due to potentiation of the activity of some endogenous ligand(s) for these receptors. Thus, the structure–induction relationship may not always reflect true induction potential which could explain the apparent lack of a casual relationship between the induction and toxic responses. But it would still be necessary to invoke multiple receptors to account for the differences in the structure–activity relationships.

The work suggests that a more toxicologically relevant binding protein should be sought consistent with the requirements of coplanarity and halogenation. The environmental concern about PCBs and PBBs should include an analysis of their ability to modulate the toxic action of planar or coplanar halogenated hydrocarbons with which they are frequently found in the environment.

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