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# Assessing the Role of *ortho*-Substitution on Polychlorinated Biphenyl Binding to Transthyretin, a Thyroxine Transport Protein[1]

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***ortho*-Substituted polychlorinated biphenyls (PCBs) make up a large part of the PCB residue found in the environment and human tissues. Our laboratory as well as others have demonstrated that *ortho*-substituted congeners exhibit important biological activities by aryl hydrocarbon (Ah) receptor-independent mechanisms, including changes in second messenger systems necessary for normal cell function and growth. Previous structure-activity relationship (SAR) studies on second messengers and transthyretin (TTR; prealbumin) binding focused little attention on the *ortho*-substituted PCBs. Disruption of thyroid hormone (TH) transport is one potentially important mechanism by which PCBs can alter TH homeostasis. A more systematic study of PCB binding to TTR, a major TH transport protein, was undertaken, in which the role of *ortho*-substitution was more thoroughly investigated. Results from this study indicated that the *ortho*-only substituted series showed significant binding activity and the relative affinities were 2,2′,6 > 2,2′ = 2,6 ≫ 2 = 2,2′,6,6′. As anticipated on the basis of steric considerations, bromine was shown to be more active as an *ortho*-substituent where the relative affinity of 2,2′-Br was equivalent to 2,2′,6-Cl. The congener patterns (di-*meta*-substitution in one or both rings) most closely resembling the diiodophenolic ring of thyroxine ($T_4$) showed the highest binding activity. Multiple *ortho*-substituents were shown to decrease binding activity in such patterns. Congener patterns (single *meta*-substitution in one or both rings) more closely resembling the monoiodophenolic ring of $T_3$ showed significantly lower binding activity, consistent with the relatively low binding activity of $T_3$ and smaller size of chlorine compared to iodine. The addition of *ortho*-substitution to such patterns gave variable results depending on the substituent relationship (adjacency or nonadjacency) to the pattern. Some patterns such as 2,2′,4,4′,5,5′ showed good binding activity and represent common congeners in the commercial Aroclor mixtures and in the environment. The binding potencies of *ortho*-PCBs to TTR may represent a signature SAR that predicts specific biologic/toxic effects. In this regard, the binding potencies were consistent with measured biological activities of these PCBs, including effects on cell dopamine content, $Ca^{2+}$ homeostasis, and protein kinase C translocation in neuronal cells and brain homogenate preparations.** © 2000 Academic Press

*Key Words:* **polychlorinated biphenyls; competitive transthyretin binding; prealbumin binding; structure-activity relationship; thyroid hormones; molecular modeling; neurotoxicity**

Polychlorinated biphenyls and related chemicals have been reported to decrease circulating thyroid hormones (TH) during development (Morse *et al.*, 1993; Ness *et al.*, 1993; Goldey *et al.*, 1995) and in adult animals (Bastomsky and Murphy, 1976; ATSDR, 1989; Porterfield, 1994; Kodavanti *et al.*, 1998a). Developmental exposure to PCBs also causes behavioral alterations such as decreases in motor activity, hearing loss (Goldey *et al.*, 1995), and cognitive deficits in rats (Schantz *et al.*,1995). In humans, developmental exposure of infants to PCBs resulted in decreased neurological optimality scores in children, delayed psychomotor development, and cognitive deficits (Pluim *et al.*, 1992; Rogan and Gladen, 1992; Koopman-Esseboom *et al.*, 1994a; Jacobson and Jacobson, 1996). In addition, *in vitro* structure-activity relationship (SAR) studies indicate that *ortho*-substituted PCBs, which are noncoplanar in nature, decreased neuronal dopamine content and perturbed intracellular second messenger systems that are essential for the normal functioning and growth of neurons (Seegal, 1996; Kodavanti and Tilson, 1997; Tilson and Kodavanti, 1998). *ortho*-Substituted congeners have been reported to accumulate preferentially in brain during PCB exposure (Seegal *et al.*, 1990; Ness *et al.*, 1994; Kodavanti *et al.*, 1998b) and some of the neurotoxic effects (Pluim *et al.*, 1992; Koopman-Esseboom

[1] The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and is approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use. These findings in part were presented at the 18th Symposium of Halogenated Environmental Organic Pollutants (Dioxin '98), August 17–21, 1998, in Stockholm, Sweden, and have been published in *Organohalogen Compounds* 1998, **37,** 101–104.

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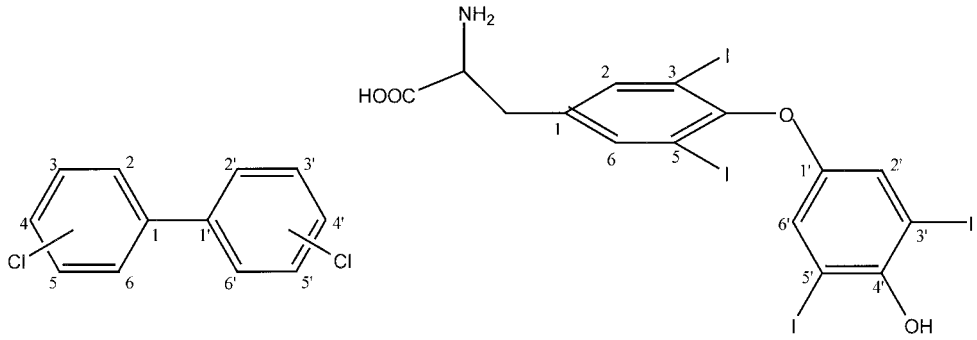


**FIG. 1.** The numbering system and common structural properties of thyroxine ($T_4$) and polychlorinated biphenyl (PCB) molecules.

*et al.*, 1994b; Goldey and Crofton, 1998) could be mediated by changes in TH by *ortho*-substituted PCBs.

The molecular events leading to PCB perturbations in TH homeostasis are not fully understood. The possible mechanisms include (1) direct interaction with the thyroid hormone receptor either as an agonist or antagonist (Gordon *et al.*, 1973); (2) increased $T_4$ elimination through the induction of hepatic uridine diphosphate (UDP) glucuronysyltransferase (Beetstra *et al.*, 1991); and (3) alterations of serum transport of TH (Rickenbacher *et al.*, 1986; Lans *et al.*, 1994). Recently, Cheek *et al.* (1999) investigated these mechanisms and concluded that disruption of TH transport is one of the key mechanisms by which PCBs alter TH homeostasis, although PCBs and their hydroxy metabolites have been shown to interact with a $T_4$-specific nuclear receptor (McKinney *et al.*, 1987; Cheek *et al.*, 1999) as well as induce hepatic UDP glucuronidation (Beetstra *et al.*, 1991; Barter and Klaassen, 1992, 1994). In nonmammalian vertebrates, the major transport protein is prealbumin (transthyretin [TTR]), while some mammals including humans have a second binding protein, thyroid-binding globulin (Larsson *et al.*, 1985). However, TTR is a highly conserved TH binding and transport protein in all vertebrate species including humans (Larsson *et al.*, 1985). TTR may also serve as a model for other $T_4$-specific binding proteins such as the deiodinase responsible for conversion of $T_4$ to $T_3$ in tissues (Rickenbacher *et al.*, 1989) and $T_4$-specific nuclear proteins, some of which may function as transcription factors (Abdukarimov, 1983; McKinney *et al.*, 1987; Wagner *et al.*, 1995). In addition, TTR has other multifunctional properties, which justify its separate study, including transport of $T_4$, retinol binding protein, and vitamin A (Blake and Oatley, 1977), and its apparent important role in maintaining biochemical thyroid status in tissues and cells (Ramaker and Wood, 1990). It has also been suggested that TTR contains a DNA interaction domain (Blake and Oatley, 1977). Although there is good reason to believe that TTR and related $T_4$-specific binding proteins might be involved in the action of PCBs in biological systems, direct evidence to establish such a linkage is lacking.

Studies in our laboratory (McKinney *et al.*, 1985; Rickenbacher *et al.*, 1986) as well as others (Lans *et al.*, 1993, 1994) have previously demonstrated that some PCBs and their hydroxylated derivatives (potential metabolites) bind strongly to transthyretin and can potentially compete with $T_4$ in biological systems. PCBs and $T_4$ have several common structural features (Fig. 1). Our previous structure-activity relationship (SAR) studies indicated that lateral chlorine substitution on PCBs is important for the binding. Strong binders were four to eight times better than $T_4$ and *ortho*-substitution in the presence of lateral-substitution did not appear to appreciably lower binding. Furthermore, it was observed that a single lateral chlorine along with *ortho*-substitutions (2,4,6-pattern) could lead to significant binding activity (McKinney *et al.*, 1985; Rickenbacher *et al.*, 1986). In our previous work, several biphenyl thyroid hormone analogs were shown to bind strongly to TTR, supporting the importance of the linear biphenyl system in interacting with the specific binding domain of the protein. Consistent with the suggestion of others (Somack *et al.*, 1982), our previous molecular modeling studies (Rickenbacher *et al.*, 1986; McKinney *et al.*, 1987) indicated that *para* hydroxylation was not necessarily required for binding and that a chlorine atom could reasonably replace the hydroxyl group. Molecular modeling and energy calculations have also been used in our previous work (Pedersen *et al.*, 1986) to assess the binding mode and torsional angle (about PCB pivot bond) requirements for PCB binding to TTR.

Previous studies (McKinney *et al.*, 1985; Rickenbacher *et al.*,1986; Lans *et al.*, 1993, 1994) on PCB binding to TTR have not adequately addressed the role of *ortho*-substitution relative to *meta*- or *para*-substitution and the relative importance of hydroxyl group substitution compared to chlorine substitution (particularly in the *para* positions). Besides, the steric constraints imposed by the binding site and other possible differences in the binding assay conditions used in different laboratories have not been adequately addressed. Therefore, the objectives of the present study are: (1) to optimize the conditions of the binding assay, which included the choice of buffer (imadazole-acetate vs Tris–HCl) and temperature (4°C vs

room temperature); (2) to undertake a systematic evaluation of the competitive binding behavior of *ortho*-only PCBs to TTR and compare them with *ortho*-only polybrominated biphenyls (PBBs; mono-*ortho* and di-*ortho*) to obtain more stereochemical information about the binding site; (3) to evaluate the binding of lateral-only PCBs for effects of varying the *meta* and *para* substituent pattern; and (4) to determine the effects of introducing *ortho* halogens into lateral-only PCBs by this modified competitive binding assay.

## MATERIALS AND METHODS

### *Chemicals*

All PCB and PBB congeners (purity > 99%) were purchased from Accu Standard (New Haven, CT), with the exception of PCBs 162 (2,3,3′,4′,5,5′) and 156 (2,3,3′,4,4′,5), which were previously synthesized and fully characterized (Goldstein *et al.*, 1981). L-Thyroxine ($T_4$), 3,5,3-triiodothyronine ($T_3$), and Sephadex G-25, particle size 50–150 $\mu$m, were purchased from Sigma (St. Louis, MO). Sephadex was deaerated by boiling for at least 1 h in Tris buffer (pH 8.0) and gradually cooled to 4°C. For gel filtration, minicolumns (2 ml bed volume in graduated glass pipets) were used.

[$^{125}$I]L-$T_4$ with a specific activity of 1250 $\mu$Ci/$\mu$g and radiochemical purity of >99% was purchased from New England Nuclear (NEN). Human prealbumin (transthyretin [TTR], 95%) was purchased from Calbiochem (La Jolla, CA).

### *Preparation of Stock PCBs*

Stock solutions of PCB congeners were prepared by dissolving them in dimethyl sulfoxide (DMSO). A 1-$\mu$l (0.2% of total incubation volume) aliquot of stock solution (different concentrations) was added to the incubation mixture to yield the desired final concentrations. DMSO at this concentration did not affect [$^{125}$I]$T_4$ binding to TTR.

### *Competitive [$^{125}$I]$T_4$ Binding Assay*

A modification of the gel filtration binding assay described by Somack *et al.* (1982) was used to measure the ability of various PCBs to compete with [$^{125}$I]L-$T_4$ for the high-affinity TTR binding site. The assay mixture was comprised of 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1 mM NaCl and 1 mM EDTA, 10 nM TTR, 20 nM L-$T_4$ (including 0.66 nM of [$^{125}$I]L-$T_4$, 150,000 cpm), and competitors (cold $T_4$ or PCBs) with increasing concentrations (1 to 1000 nM). The final volume of the assay mixture was 0.5 ml. After incubation at 25°C for 1 h, the mixtures were quickly cooled to 4°C, and a 0.4-ml portion was filtered at 4°C on Sephadex G-25 minicolumns. With an additional volume of 1.2 ml Tris buffer (pH 8.0), the protein-bound [$^{125}$I]L-$T_4$ and the competitor were removed from the column (total 1.6-ml fraction). Slight nitrogen pressure was applied to achieve an elution time of this fraction of 40–60 s, minimizing the dissociation of the complex. Radioactivity was counted by Packard Cobra autogamma counter (Packard Instruments, Meriden, CT). Nonspecific binding was determined in the presence of 1000 nM cold L-$T_4$ and this value was subtracted from the total binding to get the specific binding. Competitive binding curves were made by plotting specific TTR-bound [$^{125}$I]L-$T_4$ (percentage of control) against added nM competitor (L-$T_4$, PCBs, or PBBs) concentration. To optimize the conditions for the competitive [$^{125}$I]L-$T_4$ binding, [$^{125}$I]L-$T_4$ binding in 0.1 M imidazole-acetate (IA) buffer (pH 7.4) containing 1 mM EDTA was compared to binding in Tris buffer at room temperature (RT). Also, using Tris buffer, an incubation temperature of 4°C for 24 h was compared with RT for 1 h. The control-specific binding in Tris buffer at RT was 2.81 ± 0.36 pmol [$^{125}$I]L-$T_4$ /5 pmol TTR/1 h incubation ($n$ = 10).

### *Equilibrium [$^{125}$I]$T_4$ Binding Assay*

Equilibrium binding analyses were performed by evaluating competitive binding data of prototypical congeners from each class. The competitive binding assays were conducted as previously described. The concentrations used for these prototypic PCBs ranged from 1 to 1000 nM. The binding potencies of 2,2′,6 (*ortho*-only series), 3,3′,5,5′ (lateral-only series), and 2,2′,4,4′,5,5′ (*ortho*-lateral series) PCBs were compared with L-$T_4$ and L-$T_3$ in these analyses. The ratio of free to bound [$^{125}$I]L-$T_4$ was calculated for each analog at added concentrations and linear regression curves were plotted for the free/bound against the concentration of L-$T_4$, L-$T_3$, and PCBs.

### *Molecular Modeling*

The molecular modeling and calculation of torsional angles were performed using the PC-based modeling program HyperChem (Release 5.1 Pro for Windows copyright 1997, Hypercube Inc., Gainesville, FL). Gas-phase geometry minimization was accomplished with the molecular mechanics force field MM+. The Polak–Ribiere conjugate gradient was used with a terminating gradient of 0.01 kcal/mol.

### *Statistical Analyses of Binding Data*

The binding data using the two different buffers at RT and the data using Tris buffer at two different temperatures for different PCBs (2,2′,4,4′-, 2,2′,5,5′-, 2,2′,4,4′,5,5′- or 3,3′,4,4′,5,5′-) and L-$T_4$ were analyzed by separate two-way ANOVA for each compound with buffer or temperature as one factor and concentration as the other. The competitive binding data (three experiments done in duplicates) with different series of PCBs (*ortho*-, lateral-, *ortho*-lateral-chlorines, or *ortho*-bromo series) were analyzed by separate two-way ANOVAs with PCB as one factor and concentration as the other. Post hoc comparisons were made using Dunnett's $t$ test. The level of significance was set at $p < 0.05$. IC50 (concentration that inhibits the control activity by 50%) values were calculated from the regression line fit to the linear portion of the curve using GraphPad Instat Software. The binding potencies of each analog relative to that of thyroxine were calculated by the ratio of unlabeled $T_4$ concentration at 50% of total binding (IC50, L-$T_4$) vs competitor concentration at 50% of total binding (IC50, competitor) [IC50 (L-$T_4$)/IC50 (competitor)].

## RESULTS

### *Optimizing Conditions for the Competitive [$^{125}$I]$T_4$ Binding Assay with Prealbumin (TTR)*

The results in Fig. 2 summarize the competitive binding of [$^{125}$I]L-$T_4$ by selected PCBs along with cold L-$T_4$ in two buffer systems (imidazole acetate [IA] and Tris–HCl buffer) at room temperature (RT). The two-way ANOVA indicated a significant interaction of the effects in two buffers tested for each of the PCBs; however, there was no significant interaction for L-$T_4$ indicating that the effects of PCBs, but not L-$T_4$, were different in these two buffers. The post hoc comparisons for each PCB indicated a significant effect of concentration starting at 3–30 nM. The decreases in [$^{125}$I]L-$T_4$ binding to TTR by PCBs were greater in Tris buffer compared to IA buffer (Fig. 2). Likewise, a significant difference was observed for assays in Tris buffer performed at RT vs 4°C (Fig. 3). The two-way ANOVA indicated a significant interaction of the effects in two temperatures tested for each of the PCBs, indicating that the effects of these PCBs were greater at RT for 1 h when compared to 4°C for 24 h. The post hoc comparisons for each PCB indicated a significant effect of concentration start-

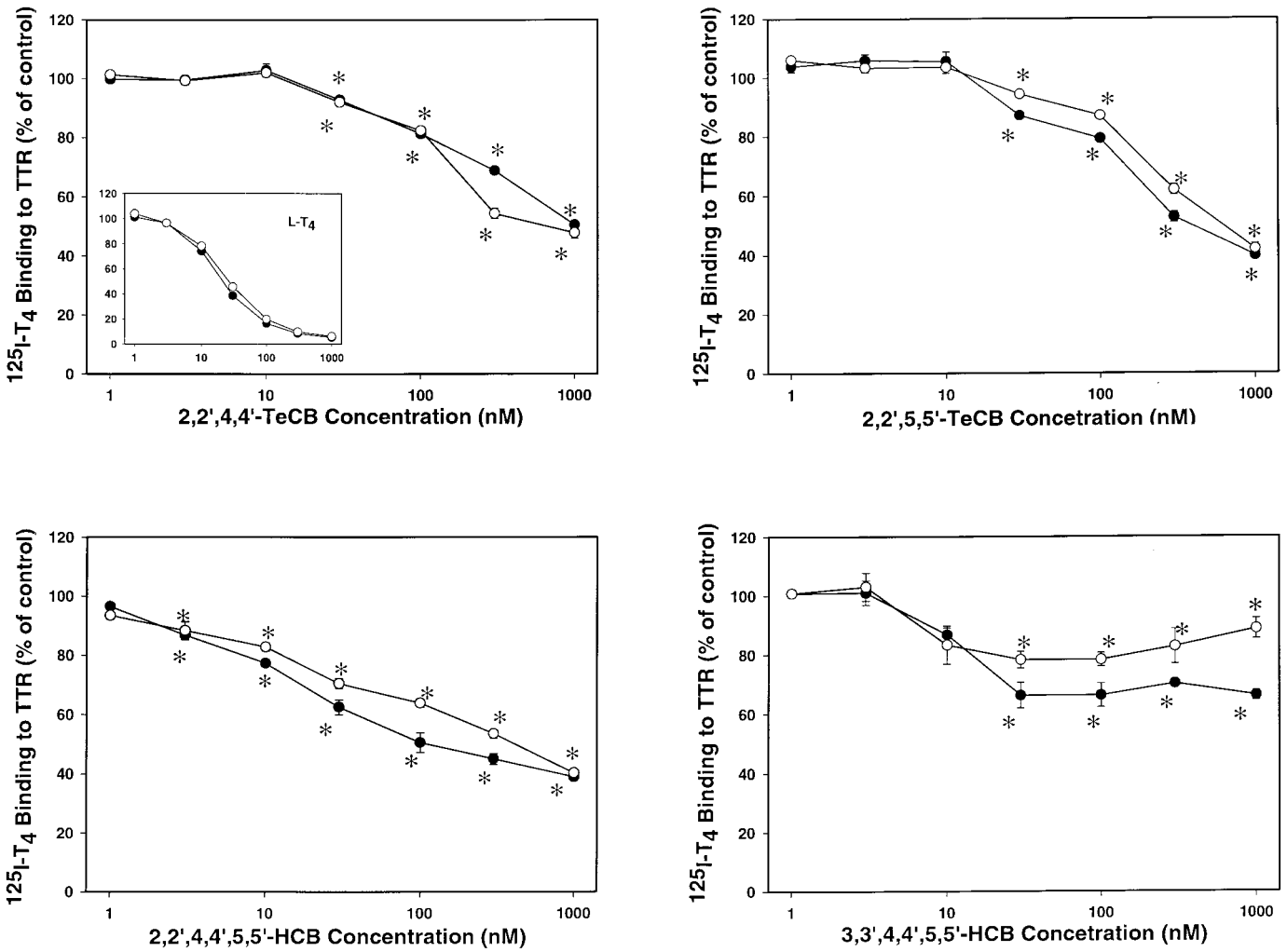


**FIG. 2.** Competitive binding of selected PCBs with [$^{125}$I]L-$T_4$ to transthyretin in Tris buffer (pH 8.0; filled circle) and imidazole-acetate (IA) buffer (pH 7.4; open circle). The insert shows competitive binding L-$T_4$ to transthyretin in these two buffers. The incubation was at 25°C (room temperature [RT]) for 1 h. The binding was represented as percentage of control (2.81 ± 0.36 pmol [$^{125}$I]L-$T_4$ /5 pmol TTR/1 h incubation; $n = 10$). Data points are mean values of three experiments done in duplicate. Asterisks indicate that these values are significantly different from the respective controls at $p < 0.05$.

ing at 10–30 nM. These preliminary results indicate that Tris buffer and incubation at RT are better conditions for this assay and all further competitive binding experiments were conducted under these conditions. Under these conditions, the IC50 value for L-$T_4$ on the TTR binding is 49 nM (Table 1), which is in agreement (15–62 nM) with previous reports from our lab (McKinney *et al.*, 1985; Rickenbacher *et al.*, 1986; Lans *et al.*, 1993) as well as others (Cheek *et al.*, 1999). The solvents (DMSO, methanol, or isopropanol), at the concentrations (0.2%) used in this assay, did not alter [$^{125}$I]L-$T_4$ binding to TTR.

### *Equilibrium Binding Assay with Prototypical PCB Congeners*

Results for prototypical PCBs indicate that all three classes (*ortho*-only, lateral-only, *ortho*-lateral) represented bind competitively to TTR. Intersection of the regression lines at zero of free/bound [$^{125}$I]L-$T_4$ vs competitor concentration (pmol) demonstrated the competitive nature of the displacement of [$^{125}$I]L-$T_4$ from TTR by different classes of PCBs (Fig. 4). The slopes of the regression lines clearly indicate the affinity of PCBs to TTR (steeper slope = greater affinity).

### *Competitive [$^{125}$I]$T_4$ Binding to TTR with ortho-Only PCBs*

The results from competitive binding studies with the *ortho*-only series are presented in Fig. 5. The two-way ANOVA indicated a significant interaction of concentration and the tested *ortho*-only PCBs. The effect of *ortho*-only PCBs on specific [$^{125}$I]$T_4$ binding increased with added *ortho*-chlorine substitutions but was completely abolished upon full *ortho*-substitution, as seen for 2,2′,6,6′-tetrachlorobiphenyl. In the *ortho*-only series, mono-*ortho* PCB (2-Cl) was inactive, with increased effect on [$^{125}$I]$T_4$ binding seen for the di-*ortho* PCBs

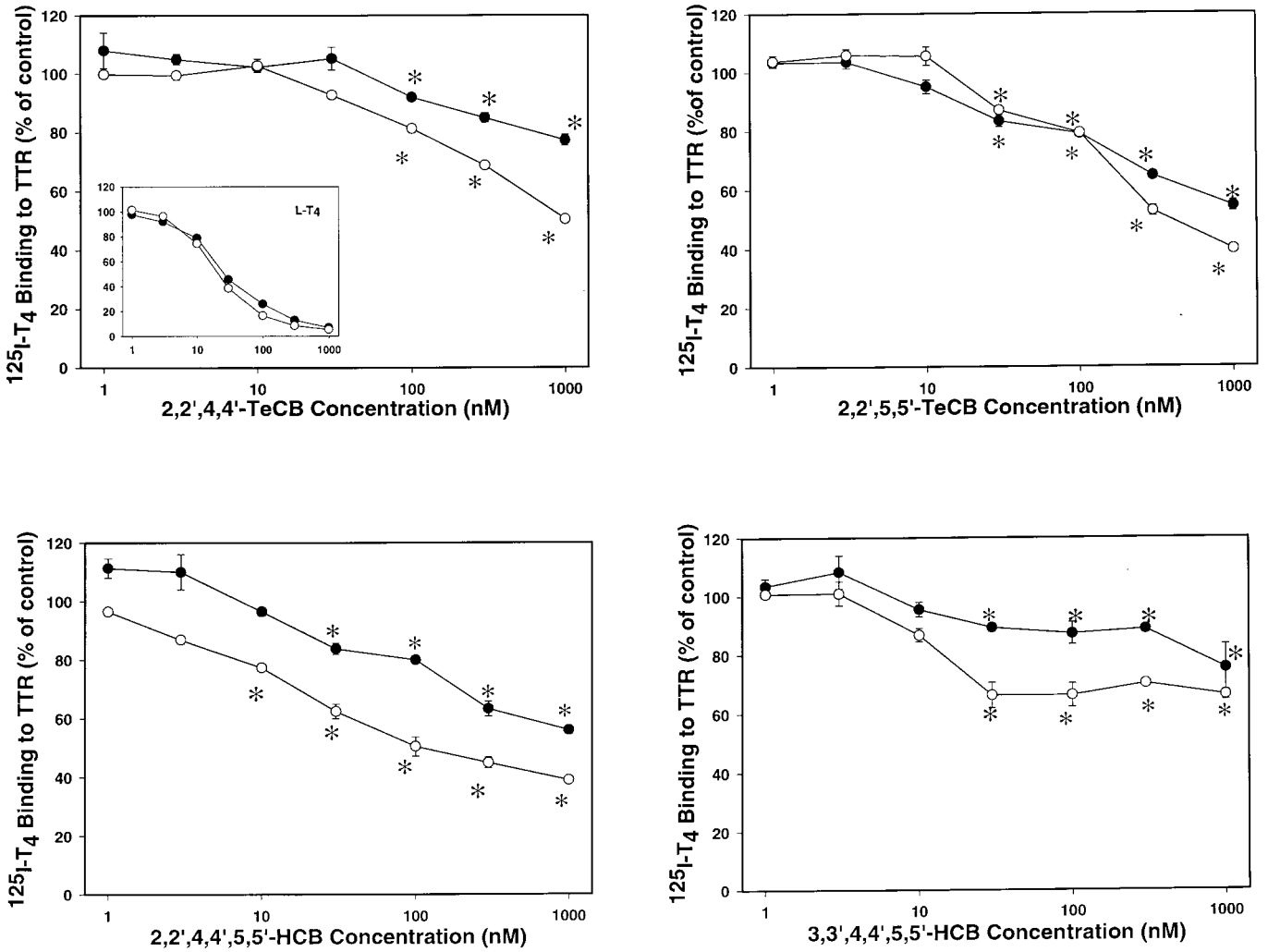


**FIG. 3.** Competitive binding of selected PCBs with [$^{125}$I]L-$T_4$ to transthyretin at 4°C for 24 h (filled circle) and room temperature (RT, 25°C) for 1 h (open circle) in Tris buffer. The insert shows competitive binding L-$T_4$ to transthyretin at these two temperatures. All other details are as mentioned in the legend to Fig. 2. Data points are mean values of three experiments done in duplicate. Asterisks indicate that these values are significantly different from the respective controls at $p < 0.05$.

(2,2′- and 2,6-$Cl_2$), reaching a maximum with the tri-*ortho* PCB (2,2′,6-$Cl_3$), and then becoming inactive again with the fully *ortho*-substituted PCB (2,2′,6,6′-$Cl_4$) (Fig. 5A). The unique binding behavior of the *ortho*-only PCBs suggests that steric interactions may be important, and this was evaluated by molecular modeling (Fig. 6). As expected, the torsional angle for the *ortho*-only series was seen to increase from about 39 degrees for the 2-Cl up to about 82 degrees for the 2,2′,6,6′-$Cl_4$ compound (Fig. 6). This has the effect of lowering the rotational degrees of freedom about the pivot bond and changing the overall shape in the longest dimension from one that is more ellipsoid (cigar) shaped to one that is more cylindrical shaped. Such properties may affect access of the molecule to the binding site of the protein as well as the fit to the binding site. When 2,2′-$Cl_2$ was overlayed (Fig. 7B) with the phenolic ring system of $T_4$, there was close spatial correspondence of the phenyl rings with the iodine atoms when the pivot bond axis was oriented perpendicularly to the axis passing through the ether oxygen and the 4-hydroxy group. This secondary binding mode appears to be limited to certain *ortho*-only-substituted biphenyls and can be compared with the primary binding mode (Fig. 7A) for lateral-substituted PCBs, in which the lateral chlorines show close spatial correspondence with the phenolic ring iodines. As anticipated on the basis of steric considerations, bromine was shown to be more active as an *ortho*-substituent. The two-way ANOVA indicated a significant interaction of concentration for the tested *ortho*-only polybrominated biphenyls. The 2-bromo biphenyl was active with increasing activity seen on di-*ortho* substitution (2,2′- and 2,6-dibromobiphenyls) (Fig. 5B). Two *ortho*-bromines on the

**TABLE 1**

**$IC_{50}$ Values and Relative Potencies of L-Thyroxine, L-T3, Different Classes of Polychlorinated Biphenyls (PCBs), and Polybrominated Biphenyls (PBBs)**

| IUPAC No. | Competitor[a] | $IC_{50}$ (nM) | Relative potency | % Control at 1000 nM |
|---|---|---|---|---|
| | L-Thyroxine[b] | 49 | 1 | 15 |
| | L-$T_3$[b] | 614 | 0.08 | 28 |
| 1 | 2-CB[c] | NA[d] | <0.01 | 88 |
| 4 | 2,2′-DCB | >1000 | <0.04 | 66 |
| 10 | 2,6-DCB | >1000 | <0.04 | 66 |
| 19 | 2,2′,6-TCB | 820 | 0.06 | 45 |
| 54 | 2,2′,6,6′-TeCB | NA | <0.01 | 98 |
| | 2-Br biphenyl[c] | >1000 | <0.04 | 68 |
| | 2,2′-$Br_2$ biphenyl | 914 | 0.05 | 50 |
| | 2,6-$Br_2$ biphenyl | 657 | 0.08 | 36 |
| 11 | 3,3′-DCB | >1000 | <0.04 | 50 |
| 12 | 3,4-DCB | >1000 | <0.04 | 56 |
| 14 | 3,5-DCB | 84 | 0.59 | 22 |
| 15 | 4,4′-DCB | NA | <0.01 | 91 |
| 35 | 3,3′,4-TCB | 854 | 0.06 | 50 |
| 37 | 3,4,4′-TCB | NA | <0.01 | 74 |
| 38 | 3,4,5-TCB | 26 | 1.9 | 15 |
| 39 | 3,4′,5-TCB | 289 | 0.17 | 41 |
| 77 | 3,3′,4,4′-TeCB | NA | <0.01 | 60 |
| 80 | 3,3′,5,5′-TeCB | 7 | 7.05 | 16 |
| 126 | 3,3′,4,4′,5-PeCB | >1000 | <0.04 | 92 |
| 127 | 3,3′,4,5,5′-PeCB | 6 | 8.23 | 14 |
| 169 | 3,3′,4,4′,5,5′-HCB | 43 | 1.15 | 73 |
| 28 | 2,4,4′-TCB | 950 | 0.05 | 47 |
| 33 | 2′,3,4-TCB | 796 | 0.06 | 50 |
| 34 | 2′,3,5-TCB | 199 | 0.25 | 25 |
| 43 | 2,2′,3,5-TeCB | 884 | 0.06 | 50 |
| 47 | 2,2′,4,4′-TeCB | 918 | 0.05 | 45 |
| 48 | 2,2′,4,5-TeCB | 651 | 0.08 | 36 |
| 52 | 2,2′,5,5′-TeCB | 699 | 0.07 | 40 |
| 85 | 2,2′,3,4,4′-PeCB | 96 | 0.51 | 37 |
| 94 | 2,2′,3,5,6′-PeCB | >1000 | <0.04 | 68 |
| 95 | 2,2′,3,5′,6-PeCB | 97 | 0.51 | 44 |
| 99 | 2,2′,4,4′,5-PeCB | 244 | 0.2 | 30 |
| 100 | 2,2′,4,4′,6-PeCB | 256 | 0.19 | 33 |
| 101 | 2,2′,4,5,5′-PeCB | 243 | 0.2 | 41 |
| 102 | 2,2′,4,5,6′-PeCB | >1000 | <0.04 | 51 |
| 104 | 2,2′,4,6,6′-PeCB | >1000 | <0.04 | 65 |
| 105 | 2,3,3′,4,4′-PeCB | >1000 | <0.04 | 92 |
| 110 | 2,3,3′,4′,6-PeCB | 19 | 2.6 | 23 |
| 111 | 2,3,3′,5,5′-PeCB | 18 | 2.74 | 15 |
| 118 | 2,3′,4,4′,5-PeCB | >1000 | <0.04 | 70 |
| 128 | 2,2′,3,3′,4,4′-HCB | NA | <0.01 | 90 |
| 133 | 2,2′,3,3′,5,5′-HCB | >1000 | <0.04 | 62 |
| 136 | 2,2′,3,3′,6,6′-HCB | >1000 | <0.04 | 63 |
| 138 | 2,2′,3,4,4′,5′-HCB | 28 | 1.76 | 34 |
| 153 | 2,2′,4,4′,5,5′-HCB | 90 | 0.55 | 40 |
| 155 | 2,2′,4,4′,6,6′-HCB | >1000 | <0.04 | 63 |
| 156 | 2,3,3′,4,4′,5-HCB | >1000 | <0.04 | 69 |
| 162 | 2,3,3′,4′,5,5′-HCB | 21 | 2.35 | 35 |
| 180 | 2,2′,3,4,4′,5,5′-HeCB | 690 | 0.07 | 48 |

[a] Abbreviations: CB, chlorobiphenyl; DCB, dichlorobiphenyl; TCB, trichlorobiphenyl; TeCB, tetrachlorobiphenyl PeCB, pentachlorobiphenyl; HCB, hexachlorobiphenyl; HeCB, heptachlorobiphenyl.

[b] L-$T_4$ and analogs were purchased from Sigma (St. Louis, MO).

[c] All the PCBs and PBBs were purchased from Accu Standard (New Haven, CT).

[d] NA, not active.

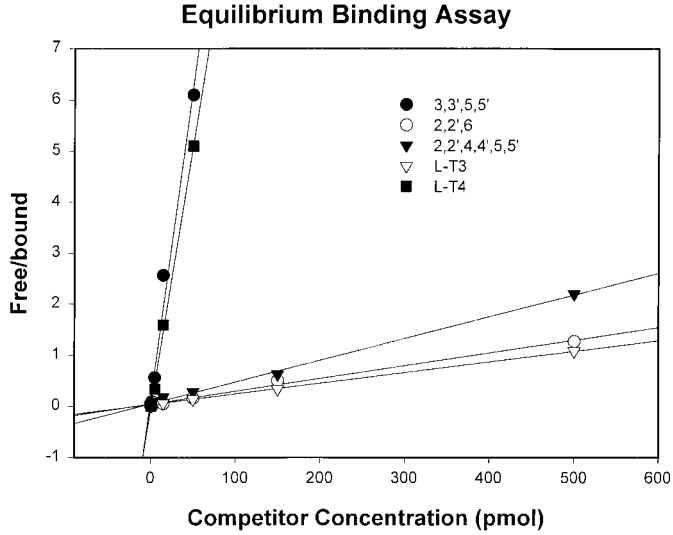


**FIG. 4.** Equilibrium competition binding assay for $T_4$, $T_3$, and prototypic congeners representing different classes of PCBs. These and further assays were conducted in Tris buffer at RT. Unlabeled L-$T_3$; L-$T_4$; 2,2′,6; 3,3′,5,5′; and 2,2′,4,4′,5,5′ were tested for their ability to displace [$^{125}$I]L-$T_4$ from high affinity binding sites as described under Materials and Methods. The steeper the slope, as seen with L-$T_4$ and 3,3′,5,5′-, the greater the affinity to TTR, whereas the shallow slope, as seen with L-T3, 2,2′,6-, and 2,2′,4,4′,5,5′-, indicates relatively lower affinity to TTR.

biphenyl molecules were approximately equivalent to three *ortho*-chlorines in terms of their effects on [$^{125}$I]$T_4$ binding (Fig. 5; Table 1).

### *Competitive [$^{125}$I]$T_4$ Binding to TTR with Lateral-Only PCBs*

For the sake of clarity, the lateral-only PCBs were divided into lightly (≤3 chlorines) and heavily (≥4 chlorines) chlorinated groups. For both the groups, the two-way ANOVA indicated a significant interaction of concentration and the appropriate PCBs. For lateral-only lightly chlorinated PCBs (Fig. 8A; Table 1), competitive binding was observed in the order of 3,4,5 > 3,5 > 3,4′,5 > 3,3′,4 > 3,3′ > 3,4,4′/4,4′ indicating the significance of *meta* and *para* substitutions on PCB congeners. For lateral-only heavily chlorinated PCBs (Fig. 8B; Table 1), competitive binding was observed in the order of 3,3′,4,5,5′ > 3,3′,5,5′ > 3,3′,4,4′,5 > 3,3′,4,4′. Combined evaluation of lateral-only series (Figs. 8A and 8B), clearly indicated that having both *para* (4,4′) positions substituted contributes to decreased binding activity,while having all *meta* (3,3′,5,5′) positions occupied contributes to increased activity. The lateral-only PCB series as a group showed greater binding affinity when compared to *ortho*-only PCBs (Table 1). These results are consistent with previous molecular modeling studies (Pedersen *et al.*, 1986; Rickenbacher *et al.*, 1986; McKinney *et al.*, 1987) that emphasized the importance of *meta, para* substitutions in PCB binding to TTR.

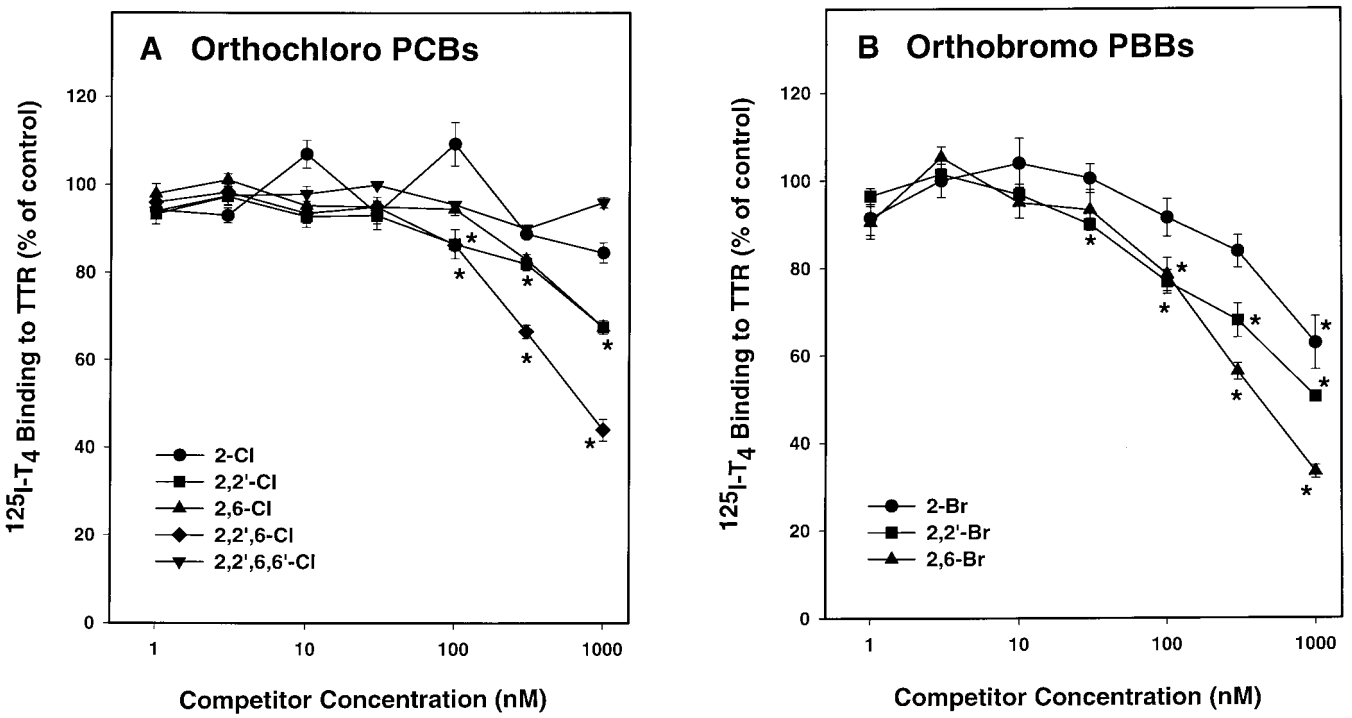


**FIG. 5.** Competitive binding of *ortho*-only chloro-biphenyls (A) and *ortho*-only bromo-biphenyls (B), as evaluated by TTR-$T_4$ competitive binding assay. Data points are mean values of three experiments done in duplicate. All other details are as mentioned in the legend to Fig. 2. Asterisks indicate that these values are significantly different from the respective controls at $p < 0.05$.

*Competitive [$^{125}$I]$T_4$ Binding to TTR with ortho-Lateral PCBs*

To understand the relative importance of *ortho*-substitution on lateral PCBs, three categories of PCBs were selected. They are *ortho*-substitution on *meta* PCBs (Fig. 9A), *ortho*-substitution on *para* PCBs (Fig. 9B), and *ortho*-substitution on *meta-para* PCBs (Fig. 9C). For all three categories, the two-way ANOVA indicated a significant inter-

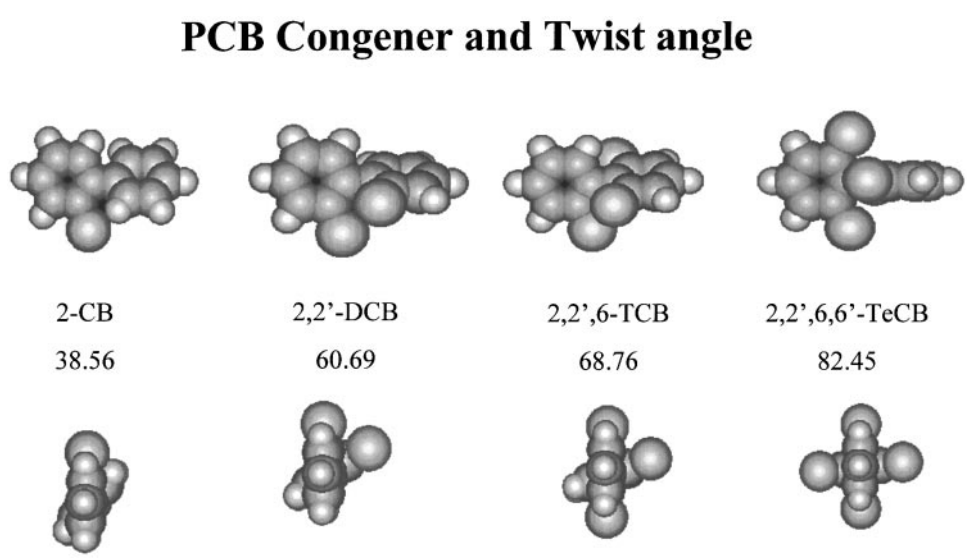


**FIG. 6.** Effect of *ortho*-chloro substitution on stereochemistry showing the twist angle for *ortho*-only PCBs. As the number of *ortho* chlorine substitutions increases, there is a tendency toward lower rotational degrees of freedom about the pivot bond and increased torsional angle (39 to 82°) that alter overall molecular shape and size.

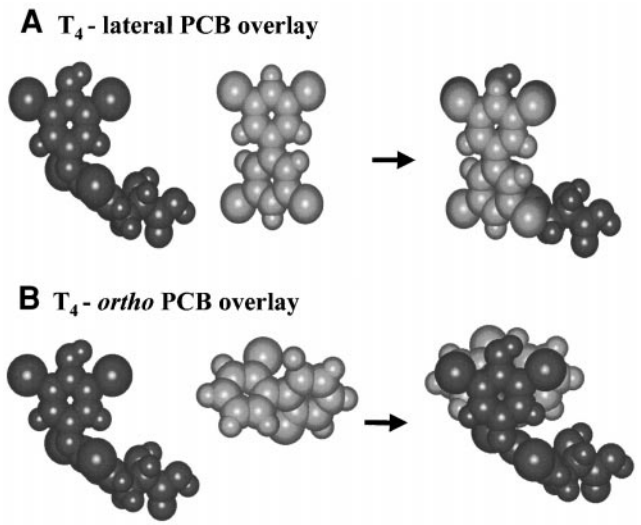


**FIG. 7.** Proposed mode of binding for lateral-substituted (primary, A) and *ortho*-substituted (secondary, B) PCBs. The primary binding mode is shown by superpositioning lateral PCB on $T_4$ phenolic ring system, whereas the secondary binding mode is shown by superpositioning $T_4$ phenolic ring system on *ortho*-PCB.

action of concentration and the tested PCBs. When the 3,5 (*meta*-substituted PCB) congener was tested for the effect of *ortho*-substitution (Fig. 9A), decreased binding activity was observed (3,5 > 2,2′,3,5 > 2,2′,3,5,6′), thus showing the effect of *ortho*-substitution adjacent to *meta*-chlorines. However, increased binding activity could be observed when *ortho*-substitution was introduced in positions not adjacent to *meta*-chlorines, such as seen (4,4′ < 2,4,4′ ≪ 2,2′,4,4′ ≪ 2,2′,4,4′,6) when the 4,4′ congener is substituted in *ortho* positions (Fig. 9B). In this case, the binding potency of the fully *ortho*-substituted compound 2,2′,4,4′,6,6′ decreased compared to 2,2′,4,4′,6 and 2,2′,4,4′. This trend toward reversal of binding properties depending on adjacency or nonadjacency of chlorine was confirmed by studying the effects of *ortho*-substitution on the 3,3′,4,4′ pattern, i.e., 2,2′,3,3′,4,4′ was less active, while 2,2′,4,4′,5,5′ was more active (Fig. 9C).

### *Relative Potency of Tested PCBs Compared to* L-$T_4$ *on* [$^{125}$I]$T_4$ *Binding to TTR*

The relative potency of PCBs on [$^{125}$I]$T_4$ binding to TTR was calculated by dividing the IC50 of L-$T_4$ by the IC50 of the tested PCB, which exhibited parallel behavior (Table 1). PCBs which did not produce 50% inhibition, even at 1000 nM, were not directly compared with PCBs which produced more than 50% inhibition. Also, the activity of each congener at 1000 nM expressed as a percentage of control was presented in Table 1. Significant binding activity (>0.04 relative potency) was found among the congeners tested in all three binding classes,

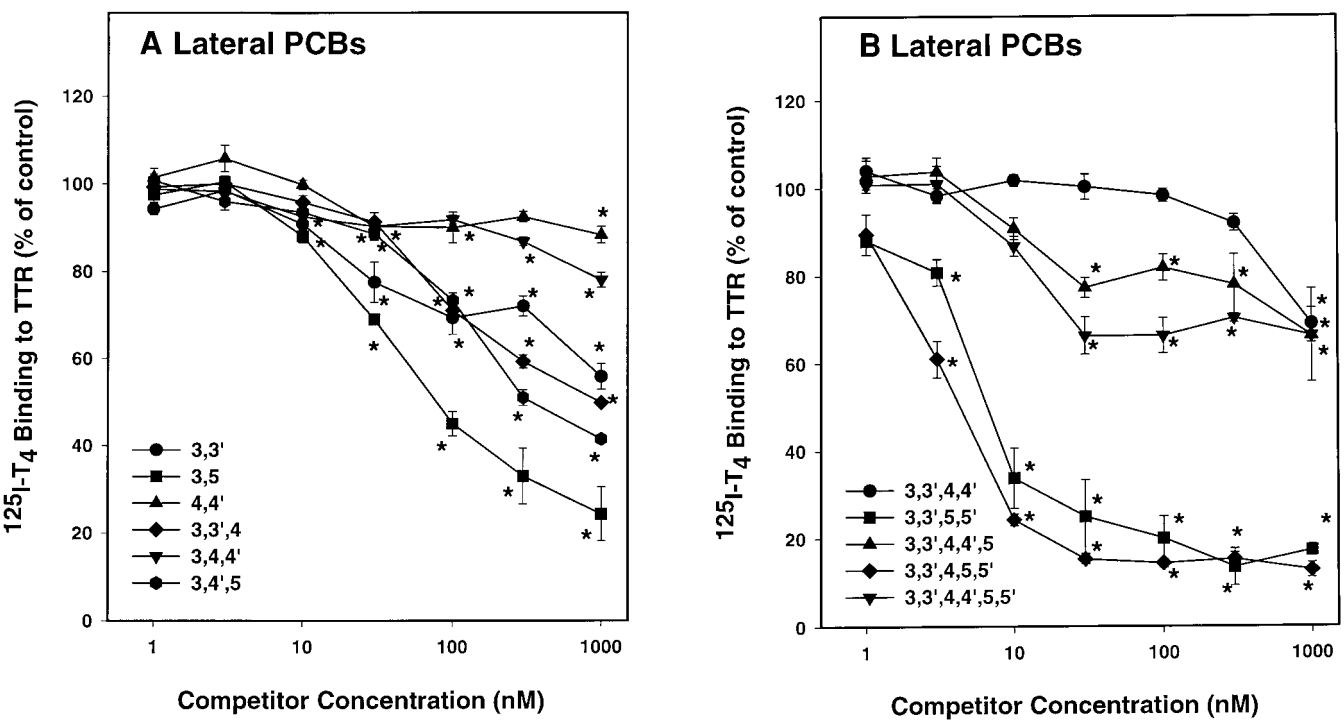


**FIG. 8.** Competitive binding of lateral-only PCBs with low (A) and high (B) chlorination, as evaluated by TTR-$T_4$ competitive binding assay. Data points are mean values of three experiments done in duplicate. All other details are as mentioned in the legend to Fig. 2. Asterisks indicate that these values are significantly different from the respective controls at $p < 0.05$.

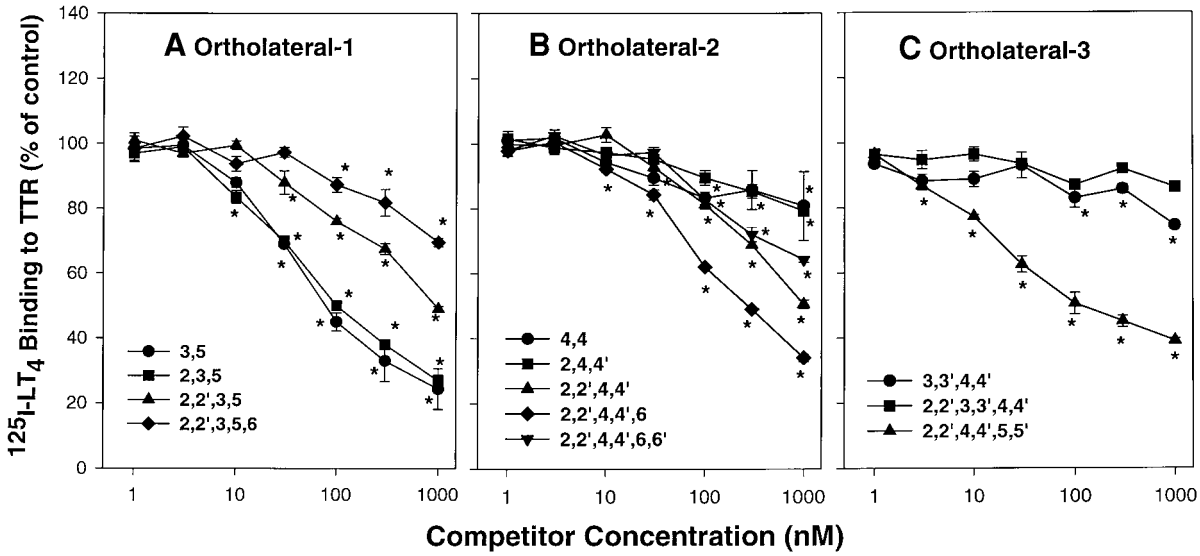


**FIG. 9.** Competitive binding of *ortho*-lateral PCBs, as evaluated by TTR-$T_4$ competitive binding assay. (A) Substitutions at *ortho* and *meta*. (B) Substitutions at *ortho* and *para*. (C) Substitutions at *ortho*, *meta*, and *para*. Data points are mean values of three experiments done in duplicate. All other details are as mentioned in the legend to Fig. 2. Asterisks indicate that these values are significantly different from the respective controls at $p < 0.05$.

the *ortho* and lateral-only and the mixed *ortho*-laterals. Table 2 supports the view that good binders tend to show structural resemblance to $T_4$ in terms of the substitution pattern of the phenolic ring.

## DISCUSSION

Initial studies aimed at optimizing the conditions for the competitive binding assay indicated that Tris buffer and incubation temperature at RT (25°C) for 1 h are optimal for the competititive binding of PCBs to TTR. Reproducible competitive binding activity was observed for all the three basic PCB structural classes tested (i.e., *ortho*, *ortho*-lateral, and lateral) as demonstrated by equilibrium binding assay analysis (Fig. 4). Current results demonstrate that parent PCBs can also bind to TTR, but generally have less affinity compared to the hydroxy-PCBs, reported previously (Rickenbacher *et al.*, 1986). However, some PCBs such as 3,3′,5,5′-TeCB (IC50 = 7 nM) and 3,3′,4,5,5′-PeCB (IC50 = 6 nM ) are several times more potent in binding to TTR when compared to the natural ligand, L-$T_4$ (IC50 = 49 nM). Although present data support the interaction of all three classes of PCBs with the same high-affinity binding site in TTR, our results also suggest that the *ortho*-only class may be adopting a different binding mode (see secondary binding mode, Fig. 7B) within that site, since all the other congeners tested contain some degree of *meta*, *para*-substitution analogous to the di-*meta*-iodine, *para*-hydroxy substitution pattern of $T_4$ (see primary binding mode, Fig. 7A). It is clear that binding can occur in the absence of hydroxy substitution on the biphenyl ring system. Hydroxybiphenyls in the absence of chlorine substitution also failed to show any significant binding activity (data not shown).

**TABLE 2**
**Classification of PCBs Resembling $T_4$- and $T_3$-like Patterns Based on Their Structure**

| IUPAC No. | PCB | $IC_{50}$ (nM) | Relative potency[a] | % Control at 1000 nM |
|---|---|---|---|---|
| | | $T_4$-like | | |
| 14 | 3,5-DCB | 84 | 0.59 | 22 |
| 34 | 2′,3,5-TCB | 199 | 0.25 | 25 |
| 38 | 3,4,5-TCB | 26 | 1.9 | 15 |
| 39 | 3,4′,5-TCB | 289 | 0.17 | 41 |
| 43 | 2,2′,3,5-TeCB | 884 | 0.06 | 50 |
| 80 | 3,3′,5,5′-TeCB | 7 | 7.05 | 16 |
| 94 | 2,2′,3,5,6′-PeCB | >1000 | <0.04 | 68 |
| 111 | 2,3,3′,5,5′-PeCB | 18 | 2.74 | 15 |
| 126 | 3,3′,4,4′,5-PeCB | >1000 | <0.04 | 92 |
| 127 | 3,3′,4,5,5′-PeCB | 6 | 8.23 | 14 |
| 133 | 2,2′,3,3′,5,5′-HCB | >1000 | <0.04 | 62 |
| 156 | 2,3,3′,4,4′,5-HCB | >1000 | <0.04 | 69 |
| 162 | 2,3,3′,4′,5,5′-HCB | 21 | 2.35 | 35 |
| 169 | 3,3′,4,4′,5,5′-HCB | 43 | 1.15 | 73 |
| | | $T_3$-like | | |
| 12 | 3,4-DCB | >1000 | <0.04 | 56 |
| 33 | 2′,3,4-TCB | 796 | 0.06 | 50 |
| 35 | 3,3′,4-TCB | 854 | 0.06 | 50 |
| 37 | 3,4,4′-TCB | NA | <0.01 | 74 |
| 77 | 3,3′,4,4′-TeCB | NA | <0.01 | 60 |
| 105 | 2,3,3′,4,4′-PeCB | >1000 | <0.04 | 92 |
| 118 | 2,3′,4,4′,5-PeCB | >1000 | <0.04 | 70 |
| 128 | 2,2′,3,3′,4,4′-HCB | NA | <0.01 | 90 |

[a] Relative potencies are compared to that of $T_4$ at 1.00.

The ostensibly unique binding properties of the *ortho*-only PCBs are not easy to explain in the context of its resemblance to the phenolic ring in the usual way (see Fig. 7A) as indicated earlier. However, rotating the ring 90 degrees to place the phenyl rings in an overlay proximity to the iodines in $T_4$ provides another interpretation of these data (Fig. 7B). This is further supported by the lack of binding activity of the fully *ortho*-only-substituted congener, which maximizes the steric constraints which might limit access to the site, fit in the site, or both. The increase in binding with fewer *ortho*-bromines is consistent with the larger size of bromine and more rapid widening of torsional angles. Previous work has suggested that the phenolic-ring iodine binding pockets in TTR can be occupied by other organic groups in certain classes of nonhalogenated aromatic compounds (Cody, 1978), as suggested in this work for the phenyl rings of certain *ortho*-only PCBs. The inactivity of the mono- and fully *ortho*-substituted congener, while the di- and tri-*ortho* congeners are active binders of TTR, may represent a signature SAR that predicts specific biological/toxic effects.

Evaluation of the lateral-only series revealed that having both *para* positions (4,4′) filled, in general, contributes to decreased competitive binding, while having all *meta* positions (3,3′,5,5′) filled with halogens contributes to increased activity. Although occupied *para* positions are not essential for binding, they do not prevent binding, and in some cases can increase binding (compare 3,4,5-TCB with 3,5-DCB). Two *para*-chlorines appear to decrease aqueous solubility of congeners, which in turn may affect availability to the binding site under assay conditions. Most of the PCBs studied can be classified in this way as $T_4$-like (chlorine substitutions at 3 and 5 positions on one or both phenyl rings) or $T_3$-like (chlorine substitutions at 3 or 5 positions only on one or both rings) in their behavior. In this context, *para*-chlorine-substitution is the steric equivalent of *para*-hydroxylation.

When *ortho*-substitution was introduced to the lateral-only PCBs, decreased competitive binding was observed for most of the compounds tested. The variability in the magnitude of decreased activity was dependent on the substitution pattern. However, in some cases significant binding activity could be restored by *ortho*-substitution, especially in situations where it is introduced in nonadjacent positions (as in introducing *ortho*-substitution on the 3,3′,4,4′ structure to generate the 2,2′,4,4′,5,5′ congener or on the 3,3′ structure to generate the 2,2′,5,5′ congener). This difference, depending on adjacency or nonadjacency of added *ortho*-chlorines, is undoubtedly the result of introducing additional steric constraints on binding, which in some cases favors and in others discourages it. The importance of this observation lies in the fact that patterns such as the 2,5 and 2,4,5 are favored in commercial PCB mixture preparations (Frame *et al.*, 1996; Kodavanti *et al.*, 1998) as well as preferred for accumulation in several target organs (Nims *et al.*, 1994; Johansen *et al.*, 1996; Kodavanti *et al.*, 1998b). It is also interesting to note that the 2,2′,4,4′,5,5′-hexachlorobiphenyl congener has been shown to account for as much as 22% of the total PCB residue in human tissue (Jensen and Sundstrom, 1974). The 2,2′,4,4′,5,5′-hexabromobiphenyl can account for as much as 55% of commercial polybrominated (PBB) mixtures, which may be of interest in view of the apparent increased activity of the bromine-substituted congeners. These types of lateral-substituted congeners as a group are significantly better binders than the *ortho*-only series.

Experimental evidence (Brouwer *et al.*, 1990; Darnerud *et al.*, 1996) and molecular modeling (Rickenbacher *et al.*, 1986) studies provide additional support for the direct binding of PCBs to TTR, as suggested by the displacement studies in this work. We believe the results of the present study provide definitive evidence that nonhydroxylated PCBs can effectively bind to TTR at physiological concentrations. Total PCB levels in maternal and fetal cord blood were reported to be 2.4 to 3.5 ppm (equivalent to 7 to 12 $\mu$M) in mothers from Upstate New York (Bush *et al.*, 1984, 1985). Similar blood concentrations were also reported in animals (offsprings and dams) exposed to PCBs (Takagi *et al.*, 1986; Nims *et al.*, 1994). Specific PCB congeners such as PCBs 138 and 153, which bind to TTR effectively (IC50 = 28–90 nM), were detected as high as 1–2 ppm (equivalent to 3000–6000 nM) in the plasma of mothers from industrialized areas in the Netherlands (Koopman-Esseboom *et al.*, 1994a) and in the Aland/Turku Archipelago (Hagmar *et al.*, 1998). Current results strongly support the hypothesis that binding of PCBs to TTR is a major contributing factor for the reduction in circulating thyroid hormones and possibly for certain biological effects related to PCB exposure.

A predictive model based on TTR binding activity might be useful in screening other classes of compounds for their potential to interfere with TH transport and availability. Such a model might also be useful in a broader sense for screening compounds with potential for binding the larger family of $T_4$-specific binding proteins with TTR-like properties. Such studies could, in turn, help link TH antagonism at the level of specific binding proteins with certain biologic/toxic effects. In this regard, it is interesting to note that certain classes of $T_4$ antagonists (e.g., certain flavones and flavonols) have also been shown to be dioxin-receptor antagonists (Auf'mkolk *et al.*, 1982; Gasiewicz *et al.*, 1996). The significance of this connection remains to be shown.

The combined, unique, and subtle binding properties (a possible signature pattern) with the *ortho*-only and *ortho*-lateral PCB series parallel the proposed neuroactive properties of these PCBs as determined under *in vitro* conditions(Shain *et al.*, 1991; Kodavanti *et al.*, 1995, 1996; Fischer *et al.*, 1998). Parallel features include (1) the size and/or shape limited binding properties/activities of the *ortho*-only PCBs; (2) the strong binding properties/activities of the di-*meta*-only (in same ring) PCBs; (3) the different effects of adding *ortho*-chlorines to poor and strong binding/activity patterns; and (4) the consistent finding of decreased binding/activity for any patterns, which also contained two *para* substituents. These

parallel features suggest involvement of an intracellular site(s) for neuroactivity that may have similar properties to the TTR binding domain. These results further support the hypothesis that certain neurological effects of PCBs may be the result of altered thyroid hormone availability to critical tissues and cellular systems. The overall findings have implications for predicting new classes of chemicals with potential for neurotoxicity as well as for providing new insights into mechanisms of action for these classes of chemicals.

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