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Estrogen Receptor-Binding Activity of Polychlorinated Hydroxybiphenyls: Conformationally Restricted Structural Probes

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SUMMARY

A series of polychlorinated hydroxybiphenyls (PCBs) has been tested for their binding activity to soluble uterine estrogen receptor protein. Competitive binding analysis was performed on 0–40% ammonium sulfate-enriched uterine cytosol receptor preparations which improved the binding activity for the PCB compounds by a factor of 10–40, by decreasing the nonspecific binding. The binding activities have been correlated to molecular properties supported by molecular modeling studies which emphasize the importance of conformational restriction. The estrogen receptor bound 4-hydroxy-2′,4′,6′-trichlorobiphenyl (4H2′,4′,6′TCB) with the greatest affinity, with the concentration of unlabeled inhibitor yielding half-maximal specific binding relative to estradiol (C_{50}) being ~42 compared to estradiol, C_{50} ~1.0. PCB compounds that demonstrated appreciable receptor-binding activity were also active *in vivo* in stimulating uterine weight increases, whereas weak binders were inactive. The 4H2′,4′,6′TCB compound represents a high degree of conformational restriction around the interring bond due to the presence of two *ortho*-chlorine atoms. The other PCBs in this series, which show lower receptor-binding activity, vary in position of chlorine substituents and can assume multiple low energy conformations as a result of less hindrance to rotation around the interring bond.

Certain halogenated aromatic hydrocarbons have been shown to exhibit estrogenic hormonal activity (1). One of the most widely recognized compounds of this type is *o,p′*-DDT (2), which binds to the ER protein. Studies have indicated a similar intracellular mechanism of action for *o,p′*-DDT and the endogenous hormone, estradiol (3). There are, however, significant differences in the biological activity of the various hydrocarbons (4). For instance, the estrogenic activity of *o,p′*-DDT compared with the inactive *p,p′*-isomer is due to differences in receptor-binding activity and may have its origin in the unique conformational properties and reactivity associated with the *o*-chlorophenyl group. Furthermore, it has recently been demonstrated (5) that the interaction of *o,p′*-DDT with the estrogen receptor is stereospecific, and the binding activity resides with the levo enantiomer. In studies of the molecular properties of PCBs (6), *o*-chlorobiphenyl structures show similar conformational properties. Interestingly, a PCB mixture (Aroclor 1221) rich in *o*-chlorobiphenyl has been shown to have estrogenic activity in female rats (7). Mixtures of this type (with low chlorine content) have been proposed as alternatives to the more highly chlorinated PCB mixtures where environmental persistence and bioaccumulation become serious problems. In early studies (8) of the uterotropic activity of commercial mixtures and pure individual chlorobiphenyls, only those mixtures or compounds with a significant (one or more) degree of *ortho*-substitution were active. However, these compounds may be rendered active as estrogens through metabolic hydroxylation at vacant *para*-positions. Although it is unlikely that these compounds will persist and bioaccumulate in mammalian tissues, steady state concentrations may exist as result of continuous exposure and maintenance of body burdens of PCBs.

The steroidal estrogens are relatively rigid, polycyclic molecules with pronounced asymmetry, and their binding to the estrogen receptor generally reflects a high degree of stereospecificity. In contrast, the nonsteroidal synthetic estrogens usually have elements of symmetry and conformational flexibility which make it difficult to determine the exact nature of the binding interaction with the receptor and structure of the active conformer (9). Because of the obvious relationship to the phenolic A ring in estradiol, several classes of synthetic estrogens have been based on phenyl-substituted hydrocarbons. These include the biphenyl methanes (e.g., DDT derivatives) (1), the biphenyl ethanes and ethylenes (e.g., DES) (10), and

ABBREVIATIONS: *o,p*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane; ER, estrogen receptor; PCB, polychlorinated biphenyl; DDT, dichlorodiphenyltrichloroethane; DES, diethylstilbestrol; TEG buffer, 10 mM Tris, 1.5 mM EDTA, 10% glycerol, pH 7.6; EDTA, ethylenediaminetetraacetic acid; 4H2′,4′,6′TCB, 4-hydroxy-2′,4′,6′-tetrachlorobiphenyl (RPM-19); 4,4′DH3,5,3′,5′TCB, 4,4′-dihydroxy-3,5,3′,5′-tetrachlorobiphenyl (RPM-24); 4,4′DH2′,3′,5′,6′TCB, 4,4′-dihydroxy-2′,3′,5′,6′-tetrachlorobiphenyl; TCHB, 2,3,4,5-tetrachloro-4′-hydroxyphenyl; 4,4′DH2′CB, 4,4′-dihydroxy-2′-chlorobiphenyl.

the triphenylethylenes (e.g., tamoxifen and nafoxidine) (11). Interestingly, the biphenyls themselves have not received a great deal of attention, presumably because early derivatives were devoid of any estrogenic activity (12).

A close examination of the structure-activity relationships which may contribute to the estrogenic activity of this compound class suggested that an important factor for estrogen receptor-binding activity and hence estrogenic potency might be the degree of conformational restriction inherent in any given structure. Conformational restriction (13) has been used effectively in drug design to increase potency and selectivity, provided the conformationally restricted analog bears a close structural resemblance to the original molecule and has similar pKa and partitioning properties. Conformational restriction is often brought about by introducing additional ring structure, the introduction of double bonds or steric bulk in key positions of the molecule. For example, in *o,p'*-DDT versus *p,p'*-DDT, the *ortho*-chlorophenyl group brings about conformational restriction through the steric effects of *o*-chlorine substitution on the phenyl ring rotations (6).

A series of PCBs was selected for initial study because a number of congeners, available from previous synthetic work, have basic structures that have been well defined from both experimental (primarily X-ray crystallographic) and theoretical studies (14). These compounds were used to examine the potential of conformationally restricted PCBs to bind the estrogen receptor and for studying the topography of the receptor-binding site and the associated molecular mechanism of action. Therefore, this work and approach may assist in delineating the unique molecular determinants of estrogenic activity of a compound and in providing a molecular basis for understanding and predicting the observed hormonal activity of xenobiotics.

Experimental Procedures

Materials. Hydroxylated PCBs were obtained from Ultra Scientific (Hope, RI) with the exception of 2,6-dichloro-4'-hydroxybiphenyl, 2-chloro-4,4'-dihydroxybiphenyl, and 2,3,5,6-tetrachloro-4,4'-dihydroxybiphenyl, which were synthesized in our laboratory by adaptation of methods in the literature (15). Structures and purities (>98%) of synthetic compounds were established by spectroscopic and gas chromatographic analyses, respectively. All other reagents were obtained from commercial sources. Commercial PCBs were also determined to be >98% pure by gas chromatographic analysis. [^{3}H]-2,4,6,7-Estradiol was obtained from New England Nuclear (Boston, MA) and purified (>98% radiochemical) as previously described (16).

Receptor-binding activity. Competitive binding analyses of ER binding of PCB compounds were performed as previously described (17) for studies involving stilbestrol compounds, with the exception that the analyses were performed with receptor from unfractionated cytosol as well as ammonium sulfate-enriched receptor preparations. Cytosol was prepared from uteri of ovariectomized mice as follows. Uterine tissue was homogenized with a Polytron homogenizer for 10 sec at a ratio of 75 mg wet weight/ml of TEG buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, pH 7.6). The homogenate was filtered through 125-μm Nitex and the filtrate was centrifuged at 1,000 × *g* for 10 min to separate nuclei. The supernatant was recentrifuged at 105,000 × *g* for 45 min at 4° to form the cytosol fraction. Cytosolic estrogen receptor was used either directly or enriched by a 0–40% ammonium sulfate fractionation. Ammonium sulfate fractionation was achieved by stirring the uterine cytosol at 4° and slowly adding ultrapure crystalline ammonium sulfate (Schwartz-Mann) to a final concentration of 40%. The solution was allowed to stir for an additional 60 min at 4° and the resulting solution was centrifuged at 20,000 × *g* for 30 min at 4°. The supernatant was decanted and the precipitate resuspended in TEG buffer, and aliquots were used directly for the binding assay. In the latter case, the precipitate containing ER was resuspended in TEG buffer. A 100-μl aliquot of ER preparation was incubated for 18 hr at 4° with 5 nM [^{3}H]estradiol and 2.5–25,000 nM concentrations of the unlabeled competitors. Receptor-bound fractions were assayed using the hydroxylapatite absorption procedure (18). Competitive binding data were analyzed and the C_{50} values (the concentration of unlabeled inhibitor yielding half-maximal specific binding relative to estradiol) were determined by the method of Korenman (19) using a commercial CDATA computer program (EMF Software, Knoxville, TN).

Uterotropic assay. Adult ovariectomized (5-day) CD-1 mice were injected for 14 days subcutaneously with 50 μl of unlabeled 4H2',4',6'TCB (RPM-19) and 4,4'DH3,5,3',5'TCB (RPM-24) at concentrations listed in the figure legends. Uteri were removed, trimmed of fat and adhering connective tissue, and weighed. Results are expressed as mean ± standard deviation of the uterine weight to body weight ratio from two experiments ($N = 8$). Statistical results were from analysis of variance, and *p* values are given in the figure legends.

Molecular modeling. A modified version of the program MODEL 1.3, kindly provided to the University of North Carolina at Chapel Hill by W. Clark Still (Columbia University), was used to perform approximate force field calculations and make least squares comparison of structures. The program was modified in our laboratory to include the MM2p molecular mechanics force field (20) and to utilize a Tektronix 4107 color display terminal with graphics tablet input. The MM2p program gave an interring torsional angle of 56.5° for TCHB. This structure was used in comparisons with estradiol and DES by matching the 6-carbon phenolic aromatic rings and remaining hydrophobic bulk. Stereopair superposition structures are shown with the phenolic ring in the plane of the paper or rotated by 90°. The X-ray crystallographic structures of estradiol (21) and DES (22) were used in the comparisons. Surface areas of the molecules were also computed using the MODEL 1.3 program. The estimated surface areas are a combination of the polar and non-polar areas without the inclusion of hydrogen atoms.

Results

A group of PCB compounds was investigated for their ability to bind to the uterine ER protein. The analyses were initially performed using a competitive equilibrium binding assay with cytosol receptor preparations (10), and a representative curve is shown in Fig. 1. The *numbers* on the curve correspond to the structures for some of the compounds listed in Table 1. The results from these assays indicated that the cytosol ER had poor binding affinity for these compounds. In earlier studies investigating the activity of certain dichlorodiphenylether and DDT compounds, it was shown that binding activity was increased by using an enriched cytosol receptor preparation formed by 0–40% ammonium sulfate precipitation.[1]

The compounds were reanalyzed using the enriched preparation, and the competitive binding curves are shown in Fig. 2. Binding activity of the PCBs is increased by a factor of 10–40 times in this preparation; however, the affinity for DES or estradiol is not altered. There is a range of binding affinities illustrated in Fig. 2 for the different PCBs. The curves all show a shape similar to that of estradiol and consistent with that of a competitive inhibitor. A competitive inhibitor interaction was confirmed by Lineweaver-Burke analysis of the unlabeled compounds and is illustrated for 4H2',4',6'TCB (RPM-19) in Fig. 3. The C_{50} binding value was determined from the competition curves in Fig. 2 and reported in Table 1. The lower the value, the stronger the binding interaction. As reported in the past,

[1] P. Sarver and K. S. Korach, unpublished observations.

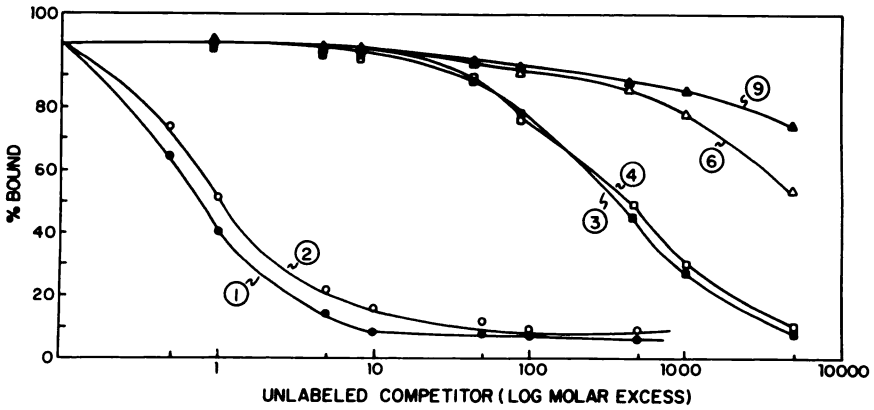
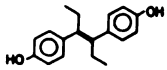
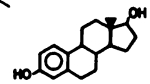
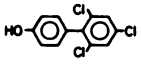
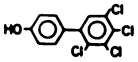
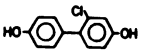
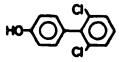
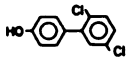
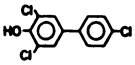
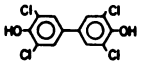
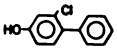
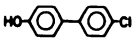
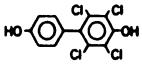
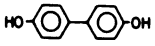
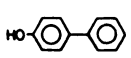


Fig. 1. A-100-μl aliquot of mouse uterine cytosol was incubated with 5 nM [^{3}H]estradiol and a 2.5–2,500 nM concentration of unlabeled DES (**1**) or estradiol (**2**). Unlabeled compounds 4H2′,4′,6′TCB (**3**), 4H2′,3′,4′,5′TCB (**4**), 4H2′,6′DCB (**6**), or 4,4′DH3,5,3′,5′TCB (**9**) were incubated at concentrations of 5–25,000 nM. Bound fractions were assayed and analyzed as described under Experimental Procedures. Results are the mean of duplicate samples and are representative of four independent experiments.

TABLE 1

Receptor binding activity of DES, estradiol, and PCBs

RECEPTOR BINDING ACTIVITY OF DES, ESTRADIOL AND POLYCHLORINATED BIPHENYL COMPOUNDS

	STRUCTURE	NAME	C_{50}
1.		DES	0.4
2.		ESTRADIOL	1.0
3.		4-hydroxy, 2′,4′,6′-trichloro biphenyl (4H2′,4′,6′TCB)	42
4.		4-hydroxy, 2′,3′,4′,5′-tetrachloro biphenyl (4H2′,3′,4′,5′TCB)	95
5.		4,4′ dihydroxy 2′-chloro biphenyl (4,4′DH 2′CB)	90
6.		4-hydroxyl 2′,6′-dichloro biphenyl (4H2′,6′DCB)	388
7.		4-hydroxy 2′,5′ dichloro biphenyl (4H2′,5′DCB)	506
8.		4-hydroxy 3,5,4′-trichloro biphenyl (4H3,5,4′TCB)	1000
9.		4,4′-dihydroxy 3,5,3′,5′-tetrachloro biphenyl (4,4′DH3,5,3′,5′TCB)	1354
10.		4-hydroxy 2-chloro biphenyl (4H2CB)	2500
11.		4-hydroxy 4′-chloro biphenyl (4H4′CB)	3900
12.		4,4′-dihydroxy 2′,3′,5′,6′-tetrachloro biphenyl (4,4′DH2′,3′,5′,6′TCB)	5000
13.		4,4′-dihydroxy biphenyl (4,4′DHB)	>5000
14.		4-hydroxy biphenyl (4HB)	>5000

DES completes 2–3 times better than the endogenous ligand, estradiol, for the estrogen receptor.

There is a progressive decrease in binding affinity for the related PCB structures listed in Table 1. Compounds with the stronger affinities also possess either single or multiple *ortho*-chlorine substitutions. The PCB with the strongest binding affinity, compound **3**, had two *ortho*-chlorines and a *para*-substituent as well. When *ortho*-chlorine substitution was absent (compounds **8**, **9–11**, **13**, and **14**), binding activity decreased significantly (~10–100 fold). *ortho*-Chlorine substitution on the phenolic ring may be less effective than on the nonphenolic ring (compare compounds **5** and **10**). As a test for this, an additional compound, 2,2′,5′-trichloro-4-hydroxybiphenyl, was studied. This compound differs structurally from compound **7** by only the 2-chloro group on the phenolic ring. The binding activity (C_{50} = 654) was similar to that of compound **7**, suggesting that the difference between compounds **5** and **10** may be in large part associated with the absence of a *para*-substituent in compound **10**. The importance of a *para*-substituent in enhancing binding activity is further supported by comparing the results of compounds **3** and **6**. Compound **6** was 9 times weaker than compound **3** and differed structurally by only the 4′-chloro group. In the absence of *ortho*-substitution, the effect of the number of substituents on binding depended on the position as well as the nature of the substituent (compare compounds **8** and **9** and compounds **11** and **13**). *meta,para*-Substitutions alone were apparently less effective than *ortho* or *ortho,para*-substitutions but are apparently more effective than *para*-substitutions alone. Study of additional compounds would be necessary to establish the importance of these more subtle structural differences. Our result with 4,4′DH2′,3′,5′,6′TCB (compound **12**) would appear to be inconsistent with the structure-binding relationship just described. However, this is the only *ortho*-substituted compound studied which should be expected (23) to have a significantly different pK_a (6.5–7.0) from that of the others (pK_a values of 8–10). Thus, this compound will undoubtedly be highly ionized under the binding assay conditions (pH 7.6) and cannot readily be compared with the other compounds studied. Other workers (13) have noted similar problems.

The *in vivo* biological activity of two of the PCB compounds was determined to evaluate whether the receptor-binding activity correlated. As shown in Fig. 4, treatment of mice for 2 weeks with the active binder (compound **3**) produced a dose response which doubled uterine weight at 5 mg/kg. The weak binder (compound **9**) showed no significant increase above control. In other uterotropic assays (data not shown), a shorter injection regimen with compound **3** for 5 days also produced significant responses. These results using two different PCB compounds suggest a correlation between *in vivo* uterotropic activity and receptor-binding affinity.

The conformation of TCHB, determined using molecular

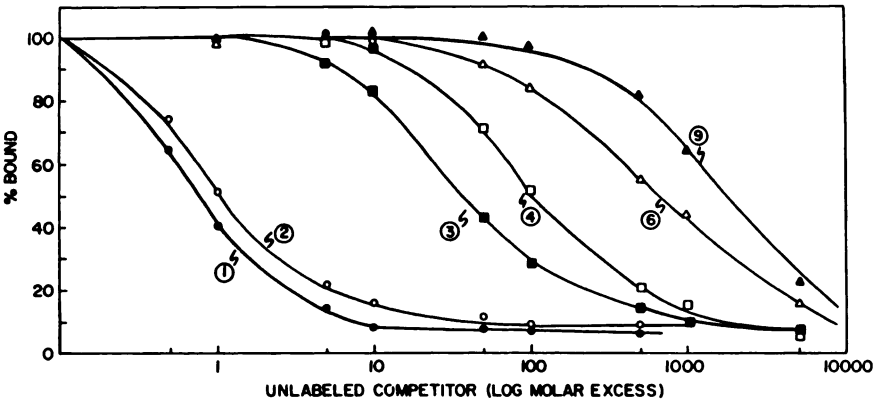


Fig. 2. Uterine cytosol was fractionated by 40% ammonium sulfate precipitation at 4° for 60 min. The precipitate was centrifuged at 20,000 × *g* for 60 min at 4° and resuspended in TEG buffer. A 100-μl aliquot of the cytosol was incubated identically to those assays described in Fig. 1. Results are the mean of duplicate samples and are representative of three independent experiments.

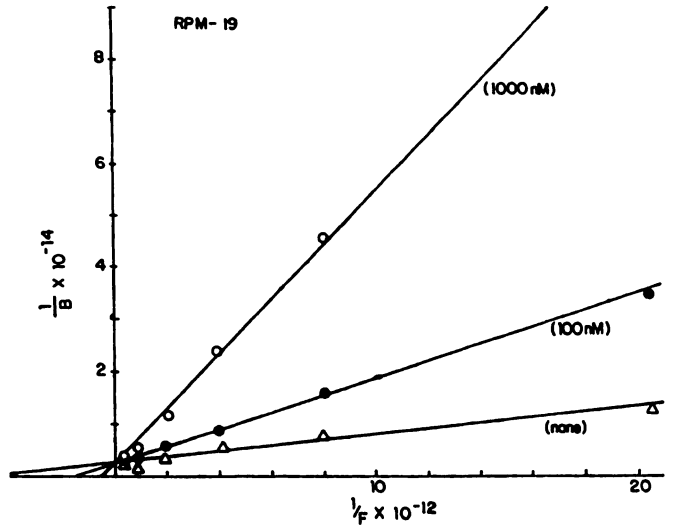


Fig. 3. Ammonium sulfate uterine cytosol fraction prepared as described in Fig. 2 legend was used in these experiments. A 100-μl aliquot was incubated with labeled estradiol (0.1–2.0 nM) alone (△) or in the presence of 100 nM (●) or 1000 nM (○) unlabeled 4H2′,4′,6′TCB (RPM-19). Incubations were performed as described under Experimental Procedures. Results are the mean value of triplicate determinations and represent two independent observations. Unweighted linear regression analysis of the lines gave correlation coefficients >0.9.

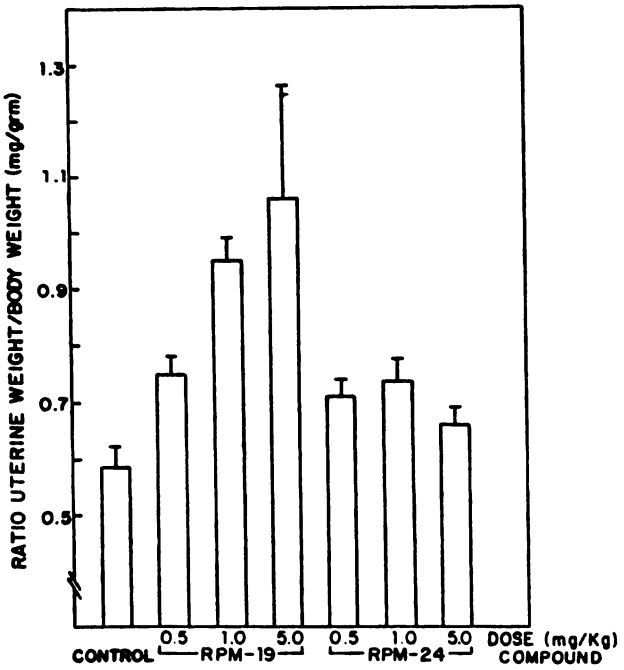


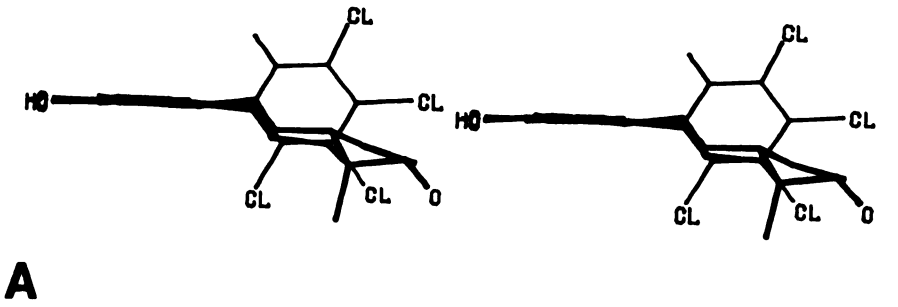
Fig. 4. Adult ovariectomized mice were injected daily for 14 days with vehicle (corn oil) as control or 0.5–5 mg/kg of either 4H2′,4′,6′TCB (RPM-19) or 4,4′DH3,5,3′,5′TCB (RPM-24). Uterine tissue was removed and weighed as described under Experimental Procedures. Analysis of variance gave $p < 0.001$ for the RPM-19 group and $p < 0.35$ for the RPM-24 group.

mechanics calculations, was compared to the previously determined X-ray structures of estradiol and DES. The overall structural match between TCHB and estradiol is shown in Figs. 5A and 6A. For simplicity, the A-ring in estradiol, an important factor in binding to the receptor (24), was matched with the corresponding ring in the other structures (although other comparisons are possible). The TCHB and estradiol structures are seen to be similar in overall molecular size and shape and hydrophobic bulk. For example, the estimated surface area of estradiol was 205 Å² compared to 221 Å² for TCHB. The *ortho*-chlorine appeared essential in determining receptor-binding activity, probably because of decreased conformational flexibility brought about by restricted rotation about the interring bond. In addition, the *ortho*-chlorine provides additional hydrophobic bulk in the vicinity of the B-ring of estradiol, both factors probably contributing favorably to the free energy of binding and thus increased binding affinity. The adjacent *meta*-chlorine also appears to provide hydrophobic bulk in the vicinity of the D-ring of estradiol; however, the other *meta*-chlorine does not appear to be important and may even hinder binding.

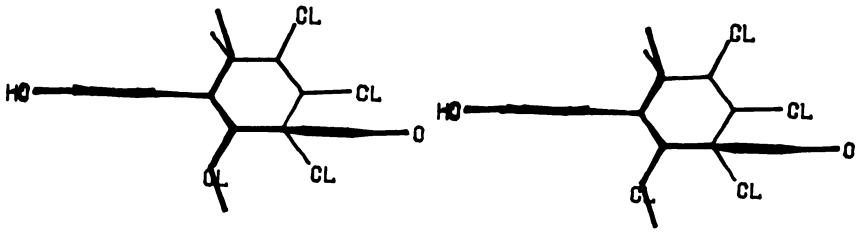
Similar results are obtained in comparing the overall conformational match between TCHB and DES (Figs. 5B and 6B). In this case, there is a poorer match because the DES molecule is larger (surface area 250 Å²). However, similar arguments apply in terms of overall similarity in molecular size and shape and placement of hydrophobic bulk. Thus, the experimental binding results are supported by molecular modeling studies which indicate that PCBs which are conformationally restricted and contain a phenolic ring system show the greatest similarity to estradiol in overall structural properties.

Discussion

X-ray crystallography and computational chemistry (energy calculations) have been shown to be complementary techniques for investigating the various possible conformations (and their relative energies) of a given PCB molecule, which may be

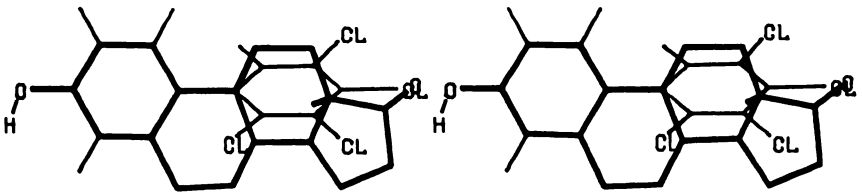


A

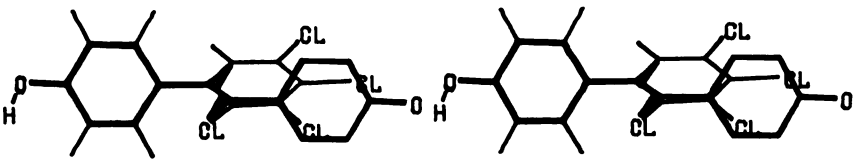


B

Fig. 5. Stereopair superposition structures of estradiol (A) or DES (B) (from X-ray) and 2′,3′,4′,5′-tetrachloro-4-hydroxybiphenyl (from energy minimization).



A



B

Fig. 6. Stereopair superposition structures of estradiol (A) or DES (B) (from X-ray) and 2′,3′,4′,5′-tetrachloro-4-hyroxybiphenyl (from energy minimization). This is the same presentation as in Fig. 5 except that the structure has been rotated 90°.

important in protein interactions in biological systems. *Ab initio* quantum mechanics, molecular mechanics, and X-ray crystallography can give similar results for the interring dihedral angle in PCB structures (25).

It has been demonstrated through X-ray crystallographic and theoretical studies that *ortho*-chlorine substitution severely limits the accessible dihedral angle range in the biphenyl molecule. This range for a *diortho*-substituted biphenyl is ~55 to ~100°. With mono-*ortho* substitution the range widens, but only in the non-*ortho*-substituted case is a fully conformationally flexible molecule achieved, i.e., relatively free rotation about the interring bond. Thus, the *ortho*-substituted derivatives have a considerable degree of conformational restriction associated with their structures. It appears that conformational restriction improves the estrogen receptor-binding activity of the biphenyl molecule. The degree of enhancement appears to be near maximum with one *ortho*-chlorine and with slight improvement with two *ortho*-chlorines.

Our study illustrates that certain biphenyl compounds can interact effectively with the estrogen receptor. Early work with hydroxylated biphenyls indicated that these compounds had estrogenic activity with regard to uterotropic stimulation (26). Conformationally restricted hydroxy PCBs are shown to be particularly effective binding ligands for the ER. This binding affinity is based on assays in which the receptor protein has been enriched due to ammonium sulfate fractionation of the cytosol, while lower binding activities are observed in unfractionated cytosol. The differences are probably due to the presence of metabolic enzymes or components in the unfractionated preparations which have appreciable nonspecific binding for

these biphenyls. The effect of the enrichment is not directly on the receptor protein because the binding affinity for DES or estradiol was unchanged in the two preparations. The enhanced activity of the *ortho*-substituted biphenyls is believed to be associated not only with the close match of the phenolic ring with the A-ring in estradiol, but also with the reduced conformational flexibility and possibly increased hydrophobic bulk of the molecule brought about by the presence of the *ortho*-chlorine and other substituents. These compounds may be useful for probing the topography of the receptor site since many synthetic variations are possible, including the kinds, numbers, and positions of the phenyl substituents. The work also suggests the potential for an affinity label, possibly involving an appropriately substituted aminobiphenyl system.

In addition, structures of this type derived from metabolism of PCBs may reasonably account for the estrogenicity of certain PCB mixtures in laboratory animals and possibly unexplained episodes of estrogenic activity in human populations exposed to complex mixtures of polyhalogenated aromatic hydrocarbon residues in the environment (7, 27). Results of our studies clearly show a relationship for the PCB compounds between receptor binding affinity and biological activity. This is similar to earlier reports with steroidal compounds showing a relationship between receptor affinity and uterotropic activity (19). A major difference is that an extended treatment period was required to acquire a response, which may be explained by the rapid clearance and metabolism of these compounds from a single daily injection. This is similar to studies with Kepone, another environmental estrogenic chemical, which showed uterotropic stimulation at doses of 15–60 mg/kg (28). The relationship of PCB structure and hormonal activity has been shown (29) with 2-chlorobiphenyl, which is the major component (~32 mol %) of the estrogenic PCB mixture Aroclor 1221 in rats (7). Furthermore, 4,4′-dihydroxy-2′-chlorobiphenyl (4,4′DH2′CB), compound **5** in this study, has been identified (30) as a major urinary metabolite of 2-chlorobiphenyl.

Conformational restriction in phenyl-substituted compounds is not only demonstrable in the hydroxy PCB compounds described in this study but is evidenced from studies investigating the antiestrogenic effects of ring-substituted alkylated hydroxyphenyl ethanes (31). These compounds showed a higher estrogen receptor-binding activity when the phenyl substituents were in *ortho*-positions. However, these compounds are already thought to be conformationally restricted to some degree because of the crowded tetramethyl-substitution near the center of their molecular structure. Thus, *ortho*-substitution may not bring about large enhancements, as seen in this work.

Thus, in effect, this work is compatible with the concept that the nonsteroidal synthetic estrogens which are most effective in binding the ER are those which combine an overall structural resemblance to estradiol with some degree of conformational restriction. Conformational restriction of course appears to be an intrinsic property of steroidal estrogens.

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