Polychlorinated Biphenyls and Related Compound Interactions with Specific Binding Sites for Thyroxine in Rat Liver Nuclear Extracts

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Received April 25, 1986

Thyroid hormone analogues, polychlorinated biphenyls (PCBs), and their derivatives were shown to bind specifically to thyroxine-specific binding sites in rat liver nuclear extracts. The structure–binding relationship for thyroxine binding prealbumin was qualitatively similar to that for the nuclear receptor. In general for both binding proteins, increased binding affinity was seen for the more linear and in some cases rectangular shaped (as opposed to the angular shaped thyroid hormones) chlorinated aromatic hydrocarbons with chlorine concentrated in lateral positions (3,3′,5,5′-substitution on biphenyl nucleus). However two groups of compounds showed distinct quantitative differences. The relatively less polar and more lipophilic nonhydroxylated PCBs bound the nuclear receptor with signficantly lower affinities while two compounds that are structurally related by the potential for equilibrium interconversion to a rigid planar structure bound with significantly higher affinities. This latter class of compounds represents soluble dioxin (TCDD) approximate isosteres and has an extended (polarizable) π-system brought about by a planar structure (or conversion to the same) and lateral halogenation. These structure requirements are maximally expressed in 3,3′,5,5′-tetrachlorodiphenoquinone (TCDQ), which shows a remarkably high affinity ($K_a = 1.84 \times 10^{11}$ M^{-1}) for the nuclear receptor. Thus, the nuclear receptor shows the expected structural specificity and sensitivity for possible involvement in the high toxicity of these classes of compounds. The physiological significance of these binding results is supported by the dose-dependent regulation (increase) of the thyroxine nuclear receptor number by dioxin, although the mechanism responsible for this increase is not clear. The nuclear binding component was further analyzed by sucrose density gradient centrifugation and was found to have a sedimentation coefficient of 4.3 S under high salt conditions. A crude estimate of the molecular weight (45 200) was obtained from a linear plot of standard globular protein fraction number (sedimentation coefficient) vs. log molecular weight. Although direct evidence is not provided, the thyroxine nuclear receptor may cooperate with a second receptor in binding the TCDQ type ligand or exists as a multimeric species with binding properties of both prealbumin and the dioxin (or Ah) receptor.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (dioxin or TCDD) is the prototypical structure for a broad class of halogenated aromatic hydrocarbons with varying structure and toxic potency.[1] The biological activities of these classes of compounds vary remarkably among the various congeners, depending on a planar or coplanar structure in a shape approximating a rectangle and with both the number and position of chlorine substituents with lateral substitution being particularly important[2] (see Chart I). The toxicity and induction responses have both been proposed to involve specific, saturable, noncovalent binding of the soluble dioxin (Ah) receptor.[3] The receptor complex is accumulated in the cell nucleus, which is believed to be the primary site of action.[4-6] In the intact cell, the receptor may in fact have a nuclear origin.[7] Recently, several lines of evidence have indicated that thyroid hormones share many common molecular properties with TCDD and can modulate its toxicity[8-12] (see Chart I inset and ref 10,

Chart I

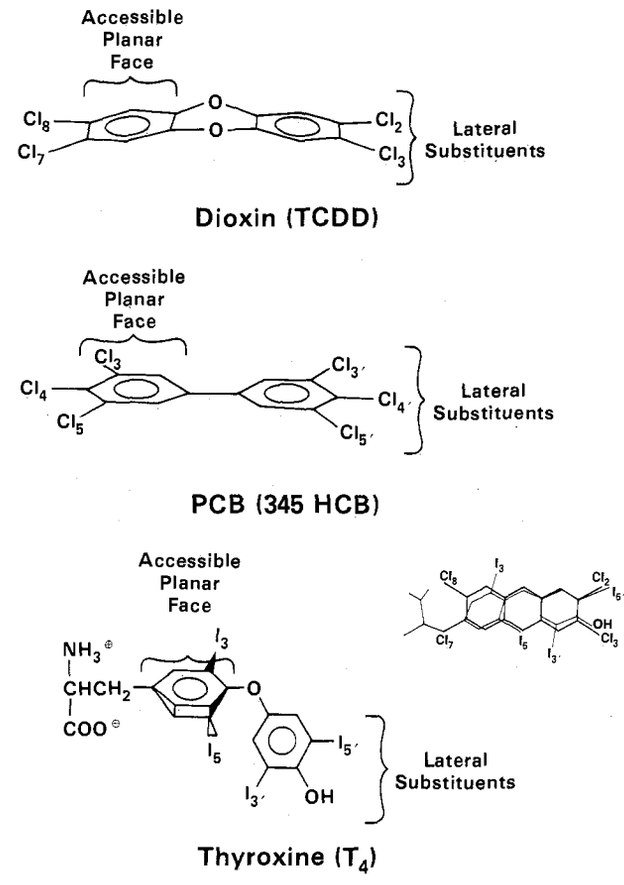


Figure 3, for a least-squares comparison of the TCDD and thyroxine (T_4) X-ray structures showing the close correspondence of the planar faces and lateral substituents). Toxicity may be (at least in part) the expression of potent

(1) *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Compounds*; Kimbrough, R. D., Ed.; Elsevier North Holland: New York, 1980; p 406 and reference therein.
(2) McKinney, J. D.; Chae, K.; McConnell, E. E.; Birnbaum, L. S. *EHP, Environ. Health Perspect.* **1985**, *60*, 57.
(3) Poland, A.; Knutson, J. C. *Ann. Rev. Pharmacol.* **1982**, *22*, 517.
(4) Greenlee, W. F.; Poland, A. *J. Biol. Chem.* **1979**, *254*, 9814.
(5) Okey, A. B.; Bondy, G. P.; Mason, M. E.; Kakl, G. F.; Eisen, H. J.; Guenthner, T. M.; Nebert, D. W. *J. Biol. Chem.* **1979**, *254*, 11636.
(6) Poellinger, L.; Kurl, R. N.; Lund, J.; Gillner, M.; Carlstedt-Duke, J.; Högberg, B.; Gustafsson, J.-A. *Biochim. Biophys. Acta* **1982**, *714*, 516.
(7) Whitlock, J. P., Jr.; Galeazzi, D. R. *J. Biol. Chem.* **1984**, *259*(2), 980.
(8) Rozman, K.; Rozman, T.; Greim, H. *Toxicol. Appl. Pharmacol.* **1984**, *72*, 372.
(9) McKinney, J. D.; Chae, K.; Oatley, S. J.; Blake, C. C. F. *J. Med. Chem.* **1985**, *28*, 375.
(10) McKinney, J. D.; Fawkes, J.; Jordan, S.; Chae, K.; Oatley, S.; Coleman, R. E.; Briner, W. *EHP, Environ. Health Perspect.* **1985**, *61*, 41.
(11) Bastomsky, C. H. *Endocrinology* **1977**, *101*, 292.
(12) Birnbaum, L. S.; Harris, M. W.; McKinney, J. D.; Lamb, J. *Pharmacologist* **1984**, *26*, 232.

and persistent thyroid hormone activity. A two-receptor mechanistic model for the toxic action has been proposed[10] in which the planar aromatic system controls the initial binding to the Ah cytosolic receptor, and halogen substituents control subsequent nuclear events involving the nuclear thyroxine receptors.

The two-receptor mechanism model for toxicity is supported by several lines of evidence. Comparison of the structure–toxicity and structure–binding relationships (involving the Ah receptor) for a wide range of both halogenated and nonhalogenated compounds shows that they are different and suggests that binding the Ah receptor may be a necessary but not sufficient condition for toxicity.[2] In addition, we have shown[13] that hexabromonaphthalenes bind the Ah receptor specifically and with high affinity but show little dependence on bromine substitution pattern and the toxic potency of the compound. This is further supported by the development of a theoretical model for these binding interactions that can account for the number and kinds of halogen substituents but not the preference for lateral substitution and rectangular shape required for high toxicity.[14] The model predicts that the sterically accessible tyrosyl ring (see Chart I) of the thyroid hormones can undergo stacking complexation and permit binding to the Ah receptor. The possibility exists that the binding proteins involved in mediating toxicity may be the same or similar to those involved in thyroid hormone action. It is interesting that recent independent studies[15,16] with the thyroid hormone nuclear receptor are suggesting that it may also be a multimeric species complexed with DNA. Such a comlex may represent a higher order of structural organization necessary for hormone action at the cellular level. We have recently demonstrated that myelotoxicity induced by thyroid hormones or TCDD can be mediated by similar molecular events[17] and the effects of both segregate with the Ah locus. A combination and specific ratio of triiodothyronine (T_3) and T_4 (4:1) is required to mimic the TCDD toxic result suggesting that separate and distinct binding proteins for T_3 and T_4 are cooperatively involved.

These results provide indirect evidence for the involvement of the Ah receptor in the mechanism of toxicity of both dioxin and thyroid hormones. However, it has not been possible to demonstrate specific binding of thyroid hormones to the Ah receptor using the established methods.[18] We believe that this is due to large differences in aqueous desolvation energies for thyroid hormones relative to dioxin which prevent the measurement of intrinsic binding activity for thyroid hormones by these methods.[19] A binding assay has been described[20] in which the radiolabeled dioxin is not free in solution but held in micelles of a nonionic detergent, which minimizes or eliminates desolvation energy differences. Under the conditions of this assay thyroid hormones have been shown[21] to effectively compete with labeled dioxin for specific binding sites in rat liver. In addition, we have also demonstrated[13] using the hydroxylapatite assay that diiodobenzenes, which serve as models for the accessible planar faces of the tyrosyl ring of thyroid hormones, bind specifically to these dioxin binding sites. These combined results are compatible with the proposal that binding of thyroid hormones to the Ah receptor can occur in vivo, but for technical reasons it is difficult to demonstrate it directly in vitro. The possibility exists that certain thyroid hormone binding proteins and the Ah receptor may be identical or bear a close structural relationship.

Because of the structural similarities between TCDD and T_4 and the consistent finding[10,11] of serum T_4 reduction in toxicity studies with dioxin and related compounds, we focused our attention on thyroxine-binding proteins as possible mediators and as sites for critical biochemical lesions in toxic responses. Initial studies[9,22] involved thyroxine binding prealbumin (TBPA), since it is a major transport protein for T_4 in blood, bears a close structural relationship to both thyroxine binding globulin[23] and thyroxine 5′-deiodinase,[24] and has been proposed[25] as a model for studying the interaction of thyroid hormones with the nuclear thyroxine receptor. Recent work[23] has established that human, rat, and rabbit prealbumin are more than 80% identical and all residues in the binding site are conserved. Competitive binding studies[9,22] using human prealbumin and polar (soluble) derivatives of dioxin and related polychlorinated biphenyl compounds have shown that simple halogenated hydrocarbons which contain only one aromatic ring or linear structures with multiple rings bind more strongly than the normal angular diphenyl–ether bridged system characteristic of thyroid hormone analogues. Furthermore, lateral chlorination was common to all compounds that showed high binding activity. The binding model also suggested a preference for a linear and symmetrical molecular shape. These structural properties are also characteristic of highly toxic structures of this type.[2]

We provide evidence in this work for specific binding sites for thyroxine in rat liver nuclear extracts that show the expected structural specificity and sensitivity for possible involvement in the toxicity of these compounds. However, as in the studies[9,22] with TBPA it was necessary to use polar (soluble) structural probes in order to obtain reproducible binding results. The results are briefly discussed in relation to the possible involvement of these specific binding sites in the mechanism of toxicity of these halogenated aromatic hydrocarbons and in the mechanism of thyroid hormone action.

Results

High salt solubilization of thyroid hormone receptors from rat liver tissue has been shown[26-28] to give rise to

(13) Cheung, E. N. Y.; McKinney, J. D., submitted for publication in *Mol. Pharmacol.*
(14) McKinney, J. D.; Darden, T.; Lyerly, M. A.; Pedersen, L. G. *Quant. Struct.-Act. Relat. Pharmacol. Chem. Biol.* **1985**, *4*, 166.
(15) Jump, D. B.; Seelig, S.; Schwartz, H. L.; Oppenheimer, J. H. *Biochemistry* **1981**, *20*, 6781.
(16) Perlman, A. J.; Stanley, F.; Samuels, H. H. *J. Biol. Chem.* **1982**, *257*(2), 930.
(17) Hong, L. H.; McKinney, J. D.; Luster, M. I. *Biochem. Pharmacol.*, in press.
(18) Poland, A.; Glover, E. *J. Biol. Chem.* **1976**, *251*(16), 4936 and unpublished observations, this laboratory.
(19) Long, G.; Pedersen, L. G.; McKinney, J. D., *Quant. Struct.-Act. Relat. Pharmacol. Chem. Biol.*, in press.
(20) Chae, K.; Albro, P. W.; Luster, M. I.; McKinney, J. D. *Int. J. Environ. Anal. Chem.* **1984**, *17*, 267.
(21) McKinney, J. D. *EHP, Environ. Health Perspect.* **1985**, *61*, 5.
(22) Rickenbacher, U.; McKinney, J. D.; Oatley, S. J.; Blake, C. C. F. *J. Med. Chem.* **1986**, *29*, 641.
(23) Sundelin, J.; Melhus, S.; Das, S.; Eriksson, U.; Lind, P.; Tragardh, L.; Peterson, P. A.; Rask, L. *J. Biol. Chem.* **1985**, *260*(10), 6481.
(24) Cody, V. *EHP, Environ. Health Perspect.* **1985**, *61*, 163.
(25) Blake, C. C. F.; Oatley, S. J. *Nature (London)* **1977**, *268*, 115.
(26) Latham, K. R.; Ring, J. C.; Baxter, J. D. *J. Biol. Chem.* **1976**, *251*(23), 7388.
(27) Eberhardt, N. L.; Ring, J. C.; Latham, K. R.; Baxter, J. D. *J. Biol. Chem.* **1979**, *254*(17), 8534.
(28) Eberhardt, N. L.; Ring, J. C., Johnson, L. K.; Latham, K. R.; Apriletti, J. W.; Kitsis, R. N.; Baxter, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5005.

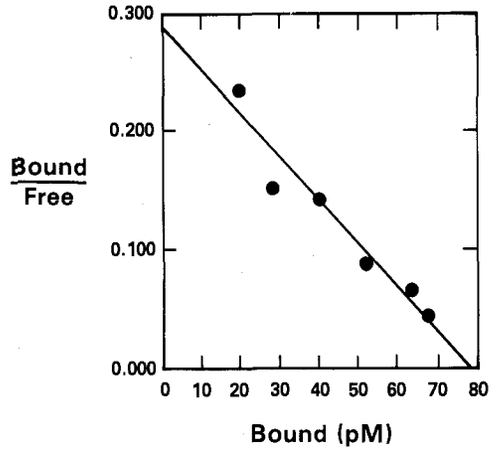


Figure 1. Scatchard analysis of [^{125}I]T_4 binding to rat liver nuclear extract. The preparation and in vitro labeling of nuclear extracts are described in the Experimental Section. The plot shown is a single analysis of nuclear extract from a pool of control rat liver and is representative of many experimental determinations.

multiple forms which differ in their affinity for T_3 and T_4. T_3 but not T_4 binding activity is markedly reduced on heating, dilution, or lowering the pH of the extract. Our method of solubilization as described in the Experimental Section is a modification of the procedure described by Heintz[29] and is similar to those used by others.[26,30] However, our binding assay is done under relatively more dilute conditions (10–100-fold) where T_4 binding specificity is seen. Scatchard analysis[31] of the [^{125}I]T_4 binding to nuclear extracts affords a linear plot (see Figure 1 for representative plot) indicating a class of independent sites of equal affinity. The equilibrium dissociation constant (negative reciprocal of the slope) for the thyroxine–receptor complex in a number of rat liver nuclear preparations varied between 0.1 and 0.9 nM. Such values are similar to those found with similar preparations from other sources.[26-28]

In this work, we compare the structure–binding relationships for human TBPA with the thyroxine nuclear receptor extracted from rat liver nuclei using selected structural probes for thyroxine hormone analogues and polychlorinated biphenyls (PCBs). Similar studies have been carried out with a limited number of dioxin and furan polar derivatives with similar results (data not shown) analogous to our previous work with TBPA.[9,22] A number of unsuccessful attempts were made to solubilize TCDD in the binding assays by use of detergents, other protein carriers, and further dilution. Because of the poor water solubility of TCDD and related (nonpolar) compounds and their high lipophilicity, the polar derivatives were technically necessary in order to minimize binding to high-capacity, low-affinity lipophilic sites and achieve sufficient solubility in the assay and access to the low-capacity, high-affinity sites. This solubility limitation in the binding assays is not likely to be a limitation in vivo since TCDD is soluble in blood up to a concentration of 0.3 mM.[32]

In our hands, perfused tissue gave improved (~10-fold) binding results (data not shown), but with the exception of the sucrose gradient experiments was not used routinely

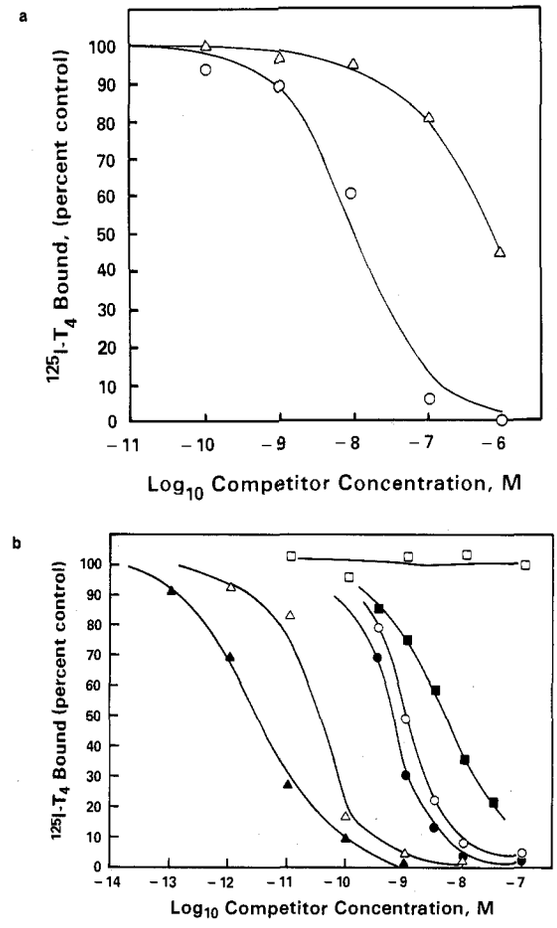


Figure 2. The competition bindng curves for [^{125}I]T_4 by unlabeled (a) T_4 (○) and T_3 (△) and (b) TCDQ (▲), TCDB-1 (△), TCDB-2 (●), 354-DHB (○), L-T_4 (■), and 352DHB (□) in rat liver nuclear extracts are shown. The preparation and in vitro labeling of nuclear extracts in the absence or presence of competitors are described in the Experimental Section. Results are the mean of duplicate assays with two or more independent experimental determinations.

in the competition experiments. These results suggest that these thyroxine binding proteins are characteristic tissue proteins and are not artifactually derived from tissue contamination by serum proteins. As in our previous work, gel filtration was used to separate protein-bound from free ligand in the competition binding experiments. Since TBPA and presumably the nuclear receptor protein completely "engulf" the radioligand, there is little opportunity for direct interaction of the bound radioligand with the gel, which could lead to erroneous results and complicate the interpretation of the binding data. In addition, nonspecific binding is expected to be small and relatively constant for these closely related competitiors (with the possible exception of the nonhydroxylated PCBs as discussed below). The binding constants obtained with thyroid hormones using the gel filtration procedure are also similar to those obtained by other[33] using equilibrium dialysis and fluorescence quenching methods.

The relative binding activities for each class of compounds for TBPA or the nuclear receptor are given in Table I. As illustrated in Figure 2a (top) this nuclear receptor preparation shows a clear preference for T_4 binding (over T_3) consistent with the results of others.[27,28] The relative binding activities of the compounds listed are expressed as the ratio of the apparent association constant

(29) Heintz, J. F.; Carew, L. B.; Murnane, A.; Heintz, R. I. *Eur. J. Biochem.* **1984**, *139*, 613.
(30) Surks, M. I.; Koerner, D. H.; Oppenheimer, J. H. *J. Clin. Invest.* **1975**, *55*, 50.
(31) Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660.
(32) Albro, P. W., unpublished observation using [^{14}C]TCDD of 150 mCi/mmol.
(33) Nilsson, S. F.; Peterson, P. A. *J. Biol. Chem.* **1971**, *246*, 6098.

Table I. Relative Binding Affinities

competitor (comp)		$K_{A(comp)}/K_{A(T_4)}$	
name	abbrev	prealbumin	nuclear receptor
L-thyroxine	L-T_4	1.00	1.00
L-3,3′,5-triiodothyronine	L-T_3	0.05	0.01
L-3,3′,5′-triiodothyronine	L-rT_3	0.65	0.60
DL-biphenylthyroxine	DL-BpT_4	1.12	0.74
2,4,6-triiodophenol	TIP	10.15	0.27
biphenyl	BP	*a*	*a*
2,2′,6,6′-tetrachlorobiphenyl	26TCB	*a*	*a*
3,5-dichloro-2-hydroxybiphenyl	352DHB	0.37	*a*
3,3′,4,4′,5,5′-hexachlorobiphenyl	345HCB	12.23	0.01
3,3′,5,5′-tetrachlorobiphenyl	35TCB	8.25	0.09
3,5-dichloro-4-hydroxybiphenyl	354DHB	15.17	3.42
2,3,5,6-tetrachloro-4,4′-dihydroxybiphenyl	TCDB-2	13.92	8.26
3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl	TCDB-1	13.27	183.23
3,3′,5,5′-tetrachlorodiphenoquinone	TCDQ	0.76	582.94

[a] No binding.

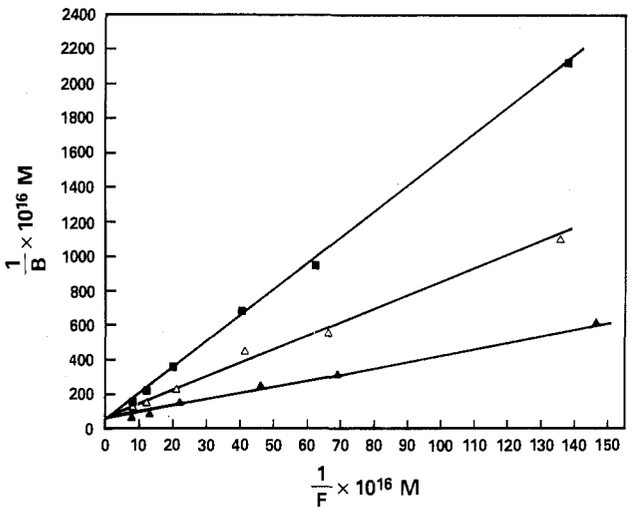
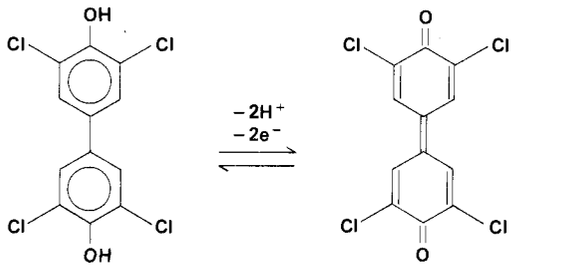


Figure 3. Lineweaver–Burk plot for 3,3′,5,5′-tetrachlorodiphenoquinone (TCDQ) interaction with nuclear extract: (▲) 0.0 pM, (△) 1 pM, (■) 2 pM. The preparation and in vitro labeling of nuclear extracts in the absence or presence of competitors are described in the Experimental Section. Results are the mean of duplicate assays with two or more independent experimental determinations.

of the competitor to that of L-T_4 as determined in the competitive binding experiments (see Figure 2b (bottom) for selected curves). The competitive behavior of these compounds was further substantiated by Lineweaver–Burk plot analyses[34] (see Figure 3 for representative plot). Nonspecific binding relative to unlabeled T_4 varied between 15 and 30% of the total [^{125}I]T_4 binding acitivity (data not shown). The binding data indicate that TBPA and the nuclear receptor bind thyroid hormone analogues and biphenyl systems with qualitatively similar binding specificities. The binding specificities substantiate the importance of lateral halogenation and some interesting stereochemical effects of ortho substitution in the case of the biphenyl compounds. Structures with no chlorine or only *o*-chlorine substitution on the biphenyl nucleus have relatively little or no binding activity to prealbumin or the nuclear T_4 receptor. In contrast *m*-chlorine substitution as in 3,3′,5,5′-tetrachlorobiphenyl leads to significant

Chart II



Tetrachlorodihydroxybiphenyl Tetrachlorodiphenoquinone

binding activity in both experimental systems. Two *m*-chlorines on the same ring appear to be sufficient for good binding since 3,5-dichloro-4-hydroxybiphenyl shows good binding behavior (with essentially identical results at pH 7.4 and 8.5) although hydrogen bonding can not be ruled out as a contributing factor especially in binding to prealbumin.[35] In contrast, the isomeric 3,5-dichloro-2-hydroxybiphenyl showed relatively little or no binding activity to prealbumin or the nuclear receptor. *o*-Chlorine substitution in the presence of *m*-chlorines does not preclude binding but can affect the strength of binding in the nuclear receptor preparation (compare isomers TCDB-1 and TCDB-2 in Table I).

However, some interesting quantitative differences are seen in comparing TBPA with the nuclear receptor which may reflect differences in nonspecific binding and solubility and/or differing requirements for molecular size and shape. Varying activities of 3,3′,4,4′-tetrachlorobiphenyl, 3,3′,4,4′,5,5′-hexachlorobiphenyl, and possibly 2,4,6-triiodophenol (brought about by shielding of the polar oxygen atom by the adjacent large iodine atoms) in the two assays are probably due to nonspecific binding differences. Because of the relatively more lipophilic character of these compounds, they may show extensive binding to low-affinity, high-capacity sites which would compete with equilibrium binding to the high-affinity sites. Such nonspecific binding to lipophilic sites would be greatest in the nuclear receptor preparation, which is consistent with the binding results for this group of compounds (see Table I). The strong binding activity of the tetrachlorodiphenoquinone (TCDQ) for the nuclear receptor suggests that an extended π-system brought about by a rigid planar structure may be a dominant factor in the binding of these types of compounds since TCDQ binding to prealbumin is similar to T_4 and the other nonplanar structures studied. The structurally related tetrachlorodihydroxybiphenyl ((TCDB-1) see Chart II) also consistently showed strong binding activity (but somewhat lower than TCDQ) for the nuclear receptor.

To further test that these classes of binding ligands are in fact binding to the same site as T_4, the [^{125}I]T_4-labeled nuclear extract was run on 10–40% (w/v) sucrose gradients, spun for 17 h at 250000*g*. In comparision with the competitive binding experiments, the protein concentration was increased 10-fold and the amount of [^{125}I]T_4 doubled in order to provide sufficient counts in the radioactive peak. Qualitatively similar competitive binding behavior was seen under these conditions. A single saturable radioactive peak was found when studied in the presence or absence of competitors including T_4, T_3, TCDQ, and 2,2′,6,6′-TCB (Figure 4a,b, (top and bottom, respectively)). It is clear that all the competitors (except

(34) Clark, J. H.; Peck, E. J. In *Receptors and Hormone Action I*; O'Malley, B. W., Birnbaumer, L., Eds.; Academic Press: New York, 1977; pp 383–410.

(35) Blaney, J. M.; Jorgensen, E. C.; Connolly, M. L.; Ferrin, T. E.; Langridge, R.; Oatley, S. J.; Burridge, J. M.; Blake, C. C. F. *J. Med. Chem.* **1982**, *25*, 785.

Table II. Number and Affinity of T_4 Receptors in Rat Liver Nuclear Extracts from TCDD-Treated Rats

TCDD treatment, μg/kg	no. of T_4 binding sites, nM/mg of protein[a]	K_d of $[^{125}I]T_4$,[d] nM	liver wt, g	body wt, g	serum T_4, μg/dL
control	3.24 ± 0.57	0.70 ± 0.18	15.1 ± 1.4	302 ± 19.9	7.6 ± 0.7
12	4.06 ± 0.14[a,c]	0.48 ± 0.21	18.9 ± 1.5[a,c]	293 ± 8.7	5.6 ± 0.4[a,b]
25	4.12 ± 0.41[a,c]	0.56 ± 0.19	18.3 ± 2.9[a]	273 ± 23.1[a]	4.4 ± 0.8[a,b]
37	8.71 ± 0.25[a,b]	0.69 ± 0.21	19.3 ± 2.0[a,c]	274 ± 15.0[a]	4.1 ± 0.6[a,b]
50	6.65 ± 0.03[a,b]	0.72 ± 0.01	19.9 ± 1.8[a,c]	271 ± 19.6[a]	4.2 ± 0.5[a,b]

[a] Significant dose-dependent effect by the William's (ref 55) dose test at $P < 0.01$. Rat livers from five rats were pooled for the analysis of each group. [b] Significantly different from control ($P < 0.01$). [c] Significantly different from control ($P < 0.05$). [d] Differences are not statistically significant and neither is the dose dependency.

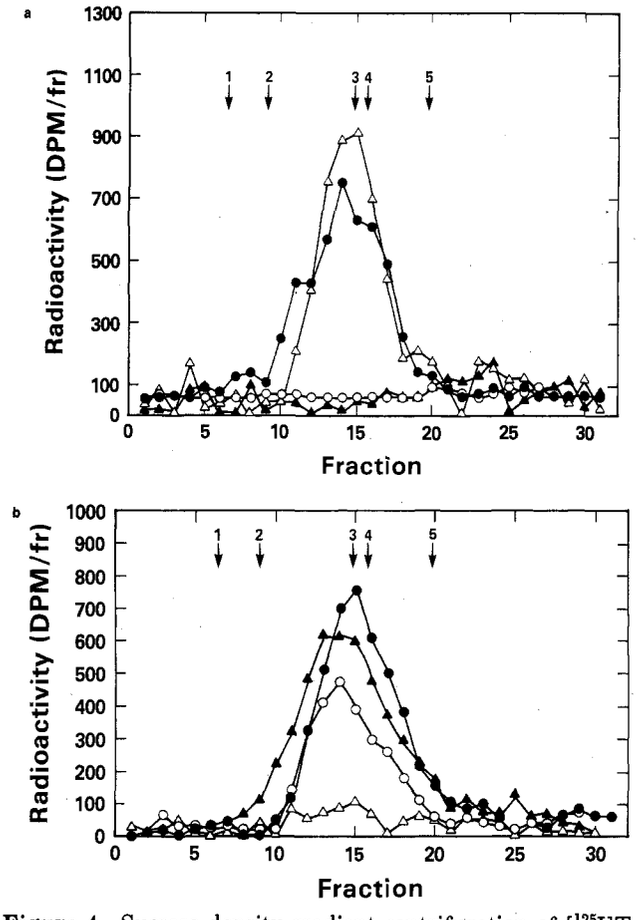


Figure 4. Sucrose density gradient centrifugation of $[^{125}I]T_4$ binding components in rat liver nuclear extracts in absence or presence of unlabeled competitors: (a) (●) control, (○) 1.0 μM T_4, (▲) 0.01 μM TCDQ, (△) 40 μM 26 TCB; (b) (●) control, (○), 0.3 μM T_3, (△) 0.01 μM T_4, (▲) 10^{-11} M TCDQ. Separate gradients were run with standard proteins: (1) catalase (11.15S), (2) aldolase (7.80S), (3) BSA (4.30S), (4) ovalbumin (3.55S), (5) ribonuclease A (1.82S). Methods for preparing labeled nuclear extract and analyses on sucrose gradient are given in the Experimental Section.

the noncompetitor 2,2′,6,6′-TCB) studied here competitively inhibit specific $[^{125}I]T_4$ binding, confirming involvement of the same site occupied by T_4. In addition, the relative inhibitory potencies under these conditions were in line with that found for the competitive binding experiments given in Table I. Of particular note is confirmation of the preference for T_4 (over T_3) and the strong binding activity of TCDQ.

Calibration of the gradient with standard proteins indicated that the saturable radioactive peak sedimented with an apparent sedimentation coefficient of 4.3 S (Figure 4).[36] It was possible to crudely estimate the molecular

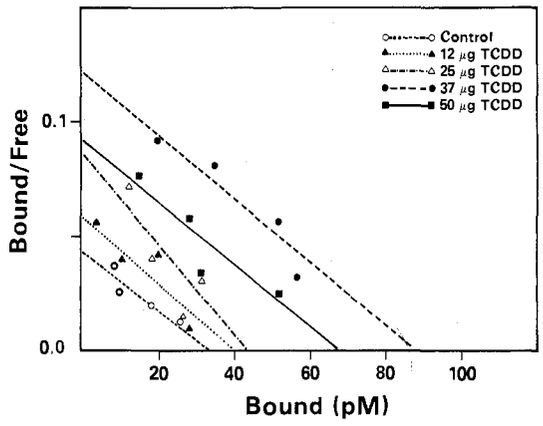


Figure 5. Scatchard analysis of $[^{125}I]T_4$ binding to rat liver nuclear extracts from control or TCDD-treated animals (see also Table II). Dosing of animals, preparation, and in vitro labeling of nuclear extracts are described in the Experimental Section. The plots shown are the mean of duplicate determinations. Similar results were obtained in independent experiments with rats dosed at 37 μg/kg and 25 and 50 μg/kg TCDD vs. controls.

weight of 45 200 directly from a plot of the log of the standard protein molecular weights vs. fraction number on sucrose gradient column ($R = -0.98$).[37] This crude estimate of molecular weight of the receptor protein is based on the assumption that the standard proteins are similar in shape to the receptor protein and that their reported molecular weights are reasonably accurate.

Since in vitro experiments could not be done directly with TCDD, Scatchard analysis was used to assess the possible in vivo effects of TCDD on the number and affinity of T_4 receptors in rat liver nuclear extracts from TCDD-treated rats (see Figure 5 and Table II). The animals treated with various doses of TCDD showed characteristic toxic responses.[11] These included decreases in body weight and total serum T_4 levels and an increase in liver weight. The results of Scatchard analysis show a significant dose-dependent effect on the number (intercept with abscissa) of T_4 binding sites in the rat liver nuclear extracts. Although the number of binding sites was seen to vary (3–8 nM/mg of protein) for nuclear extracts in independent experiments, an increase in receptor number was consistently seen when control and treated animals were analyzed under identical conditions as a part of the same experiment. Such values for T_4 binding capacity are also similar to those found with similar preparations by others.[26-28]

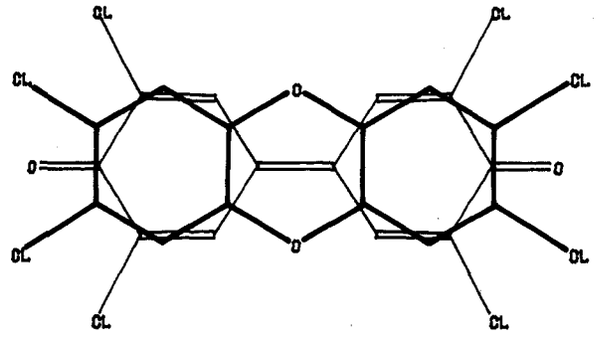
Discussion

In this work we have investigated the structure-binding relationship of the T_4 nuclear receptor in rat liver with two classes of halogenated aromatic hydrocarbon compounds and compared it with the results of similar studies using human prealbumin. The first class of iodinated com-

(36) Tanford, L. In *Physical Chemistry of Macromolecules*; Wiley: New York, 1961.

(37) Martin, R. G.; Ames, B. N. *J. Biol. Chem.* **1961**, *236*(8), 1372.

Chart III



pounds is thyroid hormone analogues or those compounds that bear a close structural relationship to all or part of the thyroid hormone structure. The second class of chlorinated compounds is PCBs or oxygenated derivatives of PCBs. Several of the PCB compounds studied are considered reasonably isosteric with the dioxin structure. Thus, the compounds studied in this work represent soluble structural probes for the binding sites of prealbumin and the T_4 receptor for general correlation with known structure–toxicity relationships for PCBs and related compounds.)[1,2] In general both prealbumin and the nuclear receptor site show increased affinity for the more linear and in some cases rectangular shaped (as opposed to the angular shaped thyroid hormone analogs) chlorinated aromatic hydrocarbons with chlorine concentrated in lateral positions (see Chart I). In contrast, chlorine replacement of iodine in the basically nonlinear thyroid hormone analogues normally leads to lowered binding activity.[38] In comparison with prealbumin, three compounds (TIP, 345 HCB, and 35 TCB) show significantly lower affinities for the nuclear receptor, while two compounds (TCDB-1 and TCDQ) show significantly higher affinities. The former group of compounds represents a relatively less polar and more lipophilic subclass that may undergo extensive binding to low-affinity lipophilic sites in the nuclear extract. The lipophilic properties of these types of compounds are well-documented.[1] The latter two compounds are structurally related by the equilibrium process shown in Chart II, which may operate under the influence of oxidizing–reducing equivalents in the nuclear extract.[39] The high binding activity of TCDB-1 in the nuclear extract may be due to partial conversion to TCDQ during the incubation process. However, when the binding of the dihydroxybiphenyl is studied at pH 8 (conditions which should promote diphenoquinone formation), the binding result was essentially identical to that observed at pH 7.4. In contrast, the isomeric dihydroxybiphenyl TCDB-2, which because of ortho substitution cannot form the diphenoquinone system or otherwise achieve a coplanar state, shows a binding activity with the nuclear receptor similar to that found with TBPA where the coplanar state is less important.

The strong binding activity of preformed (see Experimental Section) TCDQ for the nuclear receptor suggests that an extended π-system brough about by a rigid planar structure may be a dominant factor in the nuclear receptor binding of these types of compounds since TCDQ binding to prealbumin is similar to T_4 and other nonplanar structures studied. TCDQ can be viewed as an experimental binding model that is reasonably isosteric (also has identical elemental composition) with dioxin but has greater aqueous solubility (see Chart III superposition). This is consistent with the fact that TCDQ has a total surface area similar to TCDD (231 Å^2 vs. 271 Å^2 for TCDD) but has significantly more polar surface area (46.7 Å^2 vs. 10.9 Å^2 for TCDD). The molecule contains the somewhat more favorable lateral chlorine positions of the toxic 3,3′,4,4′,5,5′-HCB, but since it is also a conformationally restricted isostere of the HCB, it now contains in its rigid coplanar structure an approximately rectangular placement of these four lateral chlorines as in TCDD. In addition, it is significantly more polarizable than TCDD ($\bar{\alpha}$ = 40.81 vs. $\bar{\alpha}$ = 33.73 for TCDD). Polarizability could be important in the expression of a coplanar (or planar) requirement by facilitating dispersive interactions with protein constituents.[40] The complete lack of binding for 3,5-dichloro-2-hydroxybiphenyl to the nuclear receptor can also be explained in part on the basis of the inaccessibility of the coplanar state.[41] Thus, a consistent picture emerges supporting the requirement for an extended (polarizable) π-system in addition to lateral halogenation for high binding activity.

This property of the thyroxine-specific binding sites in the nucleus was not recognized by the previous workers who studied the binding of the basically noncoplanar thyroid hormone analogues.[42] However, this preliminary work showed that the nuclear binding site is similar to prealbumin but is more rigid. It was suggested that the nuclear T_4 receptor may be composed of prealbumin-like subunits. Our results (Table I) with thyroid hormone analogues are in good agreement with their preliminary findings and interpretation. We have not been able to find further published reports from these workers to substantiate their early results.

The thyroxine nuclear receptor shows the expected structural specificity and sensitivity for possible involvement in the toxicity of these types of compounds. Since the receptor has a nuclear origin, it may function as a gene regulatory protein. Scatchard analysis of rat liver nuclear extracts in TCDD-treated animals shows a significant dose-related increase in the T_4 receptor number. The apparent drop in receptor number in the high-dose (50 μg/kg body weight) group may reflect overriding toxic effects since this dose is close to the lethal dose in this animal species.[1] This effect may be due a thyroid-hormone-like-induced augumentation of receptor synthesis,[43] or it may be the result of a more general increase in protein synthesis possibly brought about by other nuclear events which produce hyperplasia and hypertrophy of parenchymal cells, especially a proliferation of the smooth endoplasmic reticulum.[1,44] The resulting increase in liver weight appears to be a sensitive and useful indicator of toxicity for dioxin and related compounds.

Although we do not provide direct evidence, the results are consistent with the involvement of multiple receptors or a multicomponent receptor with which TCDD or thyroid hormones may interact to produce thyroid hormone activity. Previous workers[45] have provided experimental evidence for a prealbumin-like receptor which performs a gene-activating function in cell cytosol sensitive to thy-

(38) Jorgensen, E. C. In *Thyroid Hormones*, (Li, C. H., Ed.); Academic Press: New York, 1978; pp 107–204.
(39) Carlson, B. W.; Miller, L. L. *J. Am. Chem. Soc.* **1985**, *107*, 479.
(40) McKinney, J. D.; Gottschalk, K. E.; Pedersen, L. *J. Mol. Struct.* **1983**, *105*, 427.
(41) McKinney, J. D.; Gottschalk, K. E.; Pedersen, L. *J. Mol. Struct.* **1983**, *104*, 445.
(42) Blake, C. C. F. *Proc. R. Soc. London B* **1981**, B*211*, 413.
(43) von Overbeck, K.; Lemarchand-Beraud, Th. *Mol. Cell. Endocrinol.* **1983**, *33*, 281.
(44) Dickins, M.; Seefeld, M. D.; Peterson, R. E. *Toxicol. Appl. Pharmacol.* **1981**, *58*, 389.
(45) Abdukarimov, A. *Int. Rev. Cytol. Suppl.* **1983**, *15*, 17.

roid hormones. In the case of TCDD, potent and persistent agonist activity could result in toxicity. This hypothesis advances a two-receptor or multimeric receptor model in which the planar aromatic face (see Chart I) controls the initial binding to the dioxin or Ah cytosolic receptor, and halogen substituents control subsequent nuclear events involving the nuclear thyroxine receptors (see ref 10, Figure 4, for a schematic representation of this model). We have reported a theoretical model for the Ah receptor which supports the availability of an accessible and polarizable planar face for good binding activity.[14]

The nuclear thyroxine receptor may be a multimeric species with properties of both prealbumin and the Ah receptor. This possibility is consistent with previous independent studies[15,16] suggesting that the thyroid hormone nuclear receptor may exist as an oligomer in chromatin or in association with other discrete proteins complexed with DNA. The smallest form of the receptor has properties[16] that are remarkably similar to those of prealbumin (relatively globular structure, 3.8 S form, M_r 54000). An abundant 6.5 S form, M_r 149000, which contains the 3.8 S form, appears to have the expected properties of a prealbumin(like)–Ah receptor complex. The 3.8 S receptor form studied by others[14,15] and the thyroxine binding protein studied in this work appear to have similar properties to the two major 45- and 56-kDa thyroxine binding receptor proteins recently identified[46] in rat liver nuclear extracts using photoaffinity labeling techniques. These T_4-specific binding proteins may constitute a family of structurally similar proteins which are structurally distinct from T_3-specific binding proteins. Previous experimental findings[20,47,48] have already suggested striking similarities in the Ah and T_3 receptors. In addition, in experimental studies just completed[49] we find that thyroid hormones undergo stacking complexation involving their tyrosyl rings and donor aromatic molecules with binding energies consistent with their known relative binding affinities for the T_3 nuclear receptor. In contrast to the apparent importance of the phenolic ring and lateral halogenation of thyroid hormones in binding to T_4-specific binding proteins, the sterically accessible and polarizable planar face of the tyrosyl ring may be important in binding to T_3-specific binding proteins. Thus, a mechanism for hormone action which involves cooperative protein binding of the hormone involving both the phenolic and tyrosyl rings would build in considerable structural specificity for the control process. Further studies are needed to clarify the role of both T_3 and T_4 receptors in the regulation of thyroid hormone activity and their relationship to prealbumin and the Ah receptor and possible involvement in the mechanism of toxicity of TCDD and related compounds.

Experimental Section

Materials. L-T_4, T_3, and rT_3 free acids were purchased from Sigma Chemical Co. (St. Louis, MO), and 2,4,6-triiodophenol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Biphenyl T_4 (T_4 (DL) without the ether oxygen) was kindly provided by Dr. M. Bolger, School of Pharmacy, University of Southern California, Los Angeles, CA. [^{125}I]T_4 (L) with a specific activity of 1250 μCi/μg and a radiochemical purity of >99% was purchased from New England Nuclear Corporation. Hydroxylated PCBs were obtained from Ultra Scientific (Hope, RI), with the exception of TCDB-2 which was synthesized in our laboratory by adaptation of methods in the PCB synthetic literature.[50] TCDQ was synthesized from TCDB-1 by oxidation with concentrated HNO_3 in ether at 0 °C. The product precipitates as an orange-red solid, which can be washed free of starting reactants with additional ether. Synthetic compounds were characterized by ^{1}H NMR and MS analysis. TCDQ (8.29 ppm in dioxane-d_8) could be converted back to TCDB-1 (7.61 ppm) by simple protonation (HCl) in the NMR tube. The TCDQ product gave the expected four-chlorine isotope pattern at m/e 320 and a characteristic 2Cl + CO loss at m/e 222 on mass spectral analysis with evidence of thermal reduction to TCDB-1. The remaining PCBs used were available from our previous work.[22]

Human prealbumin (80% pure) was obtained from Calbiochem Behring Corp. (La Jolla, CA) and further purified by preparative gel electrophoresis to homogeneity. Sephadex G-25, particle size 50–150 μm, was purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex was deaerated by boiling for at least 1 h in buffer. For gel filtration, minicolumns (Pasteur pipet, 2-mL bed volume) were used. The buffer used in gel filtration was 0.1 M imidazole adjusted to pH 7.4 with glacial acetic acid. Buffer A was 0.25 M sucrose, 20 mM Tris·HCl, 2 mM $MgCl_2$, pH 7.6. Buffer B was 20 mM Tris·HCl, 0.4 M KCl, 5% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 2 mM $MgCl_2$, pH 7.6.

Male Sprague–Dawley rats (200–250 g, 6 weeks old) were used in all experiments requiring liver tissue. Animals were sacrificed by carbon dioxide treatment.

Preparation and in Vitro Labeling of Nuclear Extracts. Approximately 4 g of liver was homogenized with 35 mL of buffer A. The homogenate was filtered through four layers of cheesecloth and centrifuged again at 1000g for 15 min. The pellet was resuspended in 10 mL of 2.1 M sucrose with 1 mM $MgCl_2$ and layered onto a solution of 25 mL of 2.1 M sucrose with 1 mM $MgCl_2$. The mixture was centrifuged at 24000g for 90 min, repelleted, and centrifuged at 3000g for 10 min. The supernatant solution was discarded, and the pellet was stored at −70 °C. The nuclear protein was solubilized by use of a modification of a procedure described by Heintz et al.[29] The nuclear pellet was thawed by addition of 20 mL of buffer B. The suspension was subjected to vigorous vortex mixing for 1 min followed by a 5-min period in an ice bath. The vortexing was repeated nine times for 15 s each time with 5 min between each mixing. The suspension was centrifuged at 20000g for 20 min, and the supernatant solution was used for the incubation. All operations were carried out at 4 °C. Protein concentrations were determined by Lowry et al.[51]

The binding assay using gel filtration procedures was described previously.[9,22] The standard binding mixture contains 0.3 nM [^{125}I]T_4, various concentrations (10^{-12}–10^{-6} M) of unlabeled competitors (added in 10 μL of dimethyl sulfoxide), and 10 μg of protein in a final volume of 0.5 mL of buffer B. After 2 h of incubation at 25 °C (conditions are based on time and temperature course studies, data not shown), the reactions were assayed in the following way. The mixture was then cooled to 0 °C, and protein-bound [^{125}I]T_4 was isolated by gel filtration on Sephadex G-25 columns (bed volume of 2.0 mL) equilibrated with buffer B at 4 °C. A 0.4-mL aliquot of the incubation mixture was applied to the column. The protein fraction was eluted with an additional 1.4 mL of buffer. The following 2.6-mL fraction contained free, radioactive iodide. Free hormone binds tightly to the gel matrix and does not elute in the volumes used. The remaining gel in the column was poured into a tube, and the amount of ^{125}I was determined with a Packard Prias auto γ counter (60% counting efficiency). Specific binding was calculated by subtracting nonspecific binding (incubation containing excess T_4) from total binding (incubation without competitor). Free T_4 concentration was calculated by subtracting the total binding from the total hormone concentration in the incubation. Results are the mean of duplicate assays with two or more experimental determinations.

A Lineweaver–Burk plot for TCDQ interaction with the nuclear extract was obtained in the following way. Ten micrograms of rat liver nuclear extracts was incubated with [^{125}I]T_4 (L) (0.1–1.5

(46) Dozin, B.; Cahnmann, H. J.; Nikodem, V. M. *Biochemistry* **1985**, *24*, 5197.
(47) Seelig, S.; Oppenheimer, J. H. *J. Biol. Chem.* **1982**, *257*(3), 1378.
(48) Poellinger, L.; Guelberg, D. *Mol. Pharmacol.* **1985**, *27*, 271.
(49) Chae, K.; McKinney, J. D., manuscript in preparation.
(50) Kato, S.; McKinney, J. D.; Matthews, H. B. *Toxicol. Appl. Pharmacol.* **1980**, *53*, 389.
(51) Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

nM) alone or in the presence of 1 or 2 pM unlabeled TCDQ. Incubations were allowed to achieve equilibrium at 25 °C for 2 h, and bound and free hormone were separated by gel filtration as described above. Results are the mean value of two experimental determinations. Linear regression analysis gave correlation coefficients of >0.9 for all lines.

In sucrose gradient experiments, 100 μg of perfused rat liver nuclear extract protein, various concentrations of competitors in 10 μL of Me_2SO, and 0.6 nM [^{125}I]L-T_4, all in a final volume of 0.5 mL of buffer B, were incubated for 2 h at 25 °C. [^{125}I]L-T_4 bound to protein was separated from free ligand by gel filtration on Sephadex G-25 columns (bed volume of 2.0 mL, equilibrated in buffer B at 4 °C) as follows: a 0.4-mL aliquot of the incubation mixture cooled to 4 °C was applied to the column. Elution with 1.4 mL of buffer B gave the bound fraction (termed "labeled nuclear extract"). Free iodide was eluted with an additional 2.6 mL of buffer. Free hormone remains tightly bound to the gel and does not elute with volumes used. One milliliter of the labeled nuclear extract was layered onto a linear 10–40% (w/v) sucrose gradient made by allowing 0.6 mL each of 40, 30, 20, and 10% sucrose to diffuse at room temperature for 2 h. The gradients were spun for 17 h at 250000g in a SW 60$_{Ti}$ rotor. Fractions (3 drops ~ 110 μL) were collected by puncturing the bottom of the tubes.

For Scatchard analysis, groups of five rats were administered TCDD at doses of 0, 12, 25, 37, or 50 μg/kg body weight. Each animal was given TCDD in corn oil (0.1 mL/kg body wt) by gavage and housed and maintained according to standard procedures. Control animals were dosed similarly with corn oil alone. The rats were sacrificed after 9 days, and the livers were frozen in liquid nitrogen and stored at −70 °C until use. The number of T_4 binding sites was estimated by Scatchard analysis. Rat livers were pooled for these analysis. Nuclear extracts (10 μg of nuclear protein) were incubated for 2 h at 25 °C with [^{125}I]T_4 (0.1–2.4 nM), and specific binding was determined as described above. The lines were determined by linear regression analysis and gave correlation coefficients >0.9. The intercept with the abscissa indicates the number of binding sites (nM/mg of protein), and the negative reciprocal of the slope provides an estimate of the equilibrium dissociation constant for the interaction of [^{125}I]T_4 with the binding sites. Values shown represent the mean ± SE of duplicate determinations. Similar results were obtained in independent experiments with rats dosed at 37 μg/kg and 25 and 50 μg/kg TCDD vs. controls. The Scatchard plot presented in Figure 1 is from control rats that were not treated with corn oil.

Calculations. A single binding site model was used to determine the $K_{A(T_4)}$ and $K_{A(PCB)}$ by nonlinear least squares for prealbumin binding.[52] For this purpose we used the SAS program DUD (*D*oesn't *U*se *D*erivatives) adapted for the VAX-11/780. A similar binding model was used to determine the $K_{A(PCB)}$ for nuclear receptor binding. In this case, since the total protein concentration is not known, it was necessary to fix the $K_{A(T_4)}$ at 0.142×10^{10} M^{-1} as determined independently (see Table II) by Scatchard analysis. The following equation for the fraction of the receptor bound (without and with PCB competitor, f_1 and f_2, respectively) was derived:[53]

$$\frac{f_2}{f_1} = \frac{[T_4]_2}{[T_4]_1} \frac{1 + [T_4]_1 K_{A(T_4)}}{1 + [T_4]_2 K_{A(T_4)} + [PCB] K_{A(PCB)}}$$

$K_{A(PCB)}$ was similarly calculated by nonlinear least-squares analysis.

The molecular modeling program MODEL 1.3 kindly provided to UNC—CH by W. Clark Still (Columbia University) was used to compute molecular surface areas and for molecular comparisons of the TCDD and TCDQ structures (Chart III). This program was modified in our laboratory to utilize a Tektronix 4107 color display terminal with graphics tablet input and the MM2p force field. In our hands,[54] MM2p has given energy-minimized structures for π-systems in excellent agreement with X-ray. MM2p was used to derive the starting geometry for TCDQ using standard parameters,[14] and the X-ray structure of TCDD was used.[9] The TCDD structure shown in Chart I (top) is from MM2p optimization and shows some deviation ($\phi_{min} \cong 142°$) from planarity. The method used to calculate their molecular polarizabilities has been previously reported.[40]

Acknowledgment. We thank Dr. P. Albro for helpful comments and suggestions and Dr. T. Darden for assistance with the molecular modeling work.

Registry No. L-T_4, 51-48-9; L-T_3, 6893-02-3; L-rT_3, 5817-39-0; DL-BpT_4, 100837-36-3; TIP, 609-23-4; BP, 92-52-4; 26TCB, 15968-05-5; 352DHB, 5335-24-0; 345HCB, 32774-16-6; 35TCB, 33284-52-5; 354DHB, 1137-59-3; TCDB-2, 100702-98-5; TCDB-1, 13049-13-3; TCDQ, 27728-29-6; TCDD, 1746-01-6.

(52) Pedersen, L. G.; Darden, T.; Oatley, S. J.; McKinney, J. D. *J. Med. Chem.* **1986**, *29*, 2451.
(53) Levitzki, A. *Receptor. A Quantitative Approach*; Benjamin-Cummings: Menlo Park, CA, 1984; p 24.
(54) Darden, T.; McKinney, J. D.; Gottschalk, K.; Maynard, A. T.; Pedersen, L. G. *J. Am. Chem. Soc.* **1986**, *108*, 207.
(55) Williams, D. A. *Biometrics* **1971**, *27*, 103.

5-Fluoro- and 8-Fluorotrimetoquinol: Selective β_2-Adrenoceptor Agonists

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The 5-fluoro and 8-fluoro analogues of trimetoquinol, TMQ, have been synthesized and evaluated for β_2- and β_1-adrenoceptor activity in guinea pig trachea and atria, respectively. The fluoro analogues of TMQ maintained potent β_2-adrenoceptor agonist activity but had reduced β_1-adrenoceptor agonist activity. The changes in β_1-activity of these compounds were correlated to differences in phenolic pK_a's. The β_1- and β_2-adrenoceptor actions of **2** and **3** were blocked in a competitive manner by propranolol. The enhanced β_2/β_1 selectivity for the analogues was found to be 8-fluoro analogue **3** > 5-fluoro analogue **2** > trimetoquinol (**1**).

Kirk et al.[1] have reported that fluorine substitution at the 2-position of norepinephrine produces a selective β-adrenergic agonist, while the 6-fluoro analogue of norepinephrine provides a selective α-adrenergic agonist. Several explanations have been presented for the selectivity conferred by the fluorine substitution on norepinephrine. One explanation suggests[2,3] that hydrogen-bond formation between the benzylic hydroxy group and the aromatic fluorine atom at the 2- or 6-position provides

(1) Kirk, K. L.; Cantacuzene, D.; Nimitkitpaisan, Y.; McCulloh, D.; Padgett, W. L.; Daly, J. W.; Creveling, C. R. *J. Med. Chem.* **1979**, *22*, 1493.
(2) Goldberg, L. I.; Kohli, J. D.; Cantacuzene, D.; Kirk, K. L.; Creveling, C. R. *J. Pharmacol. Exp. Ther.* **1980**, *213*, 509.
(3) Cantacuzene, D.; Kirk, K. L.; McCulloh, D. H.; Creveling, C. R. *Science (Washington, D.C.)* **1979**, *204*, 1217.

0022-2623/87/1830-0086$01.50/0