Ivanovas strain were kept in single cages with 14 h of light, 23 °C, and water and food ad libitum. On the third day postpartum, the number of pups was standardized to eight per female. The litter of each female was weighed daily at 10 a.m. to the nearest gram. Each pup was checked for the presence of milk in its stomach which is apparent as a "milk spot" through the translucent body wall. From day 5 to 8 inclusive the mothers were treated orally with test compound or vehicle (0.5 mL/100 g of body weight). The weight gains of the litters and the presence of milk spots were checked until the 12th day. Suckling mother animals were checked daily for signs of abnormal nursing behavior. Experimental animals which lost more than two pups during this time were excluded from subsequent evaluation. Differences in growth rate between the pups of treated and untreated females were evaluated by means of probit analysis. The ID_{50} is the dose of test compound which given once daily from day 5 to 8 produces a 50% inhibition.

Binding Studies. Displacement studies were carried out on various brain homogenates as previously described. Typically four to six different concentrations of test compound were incubated in triplicate, and the IC_{50} value, expressed in nM, was determined by appropriately weighted regression analysis. The ligands were as follows: for dopamine receptors, [^{3}H]spiperone[35] and [^{3}H]dopamine,[36] both in calf caudate; for serotonin receptors, [^{3}H]spiperone in rat frontal cortex[37] and [^{3}H]serotonin in whole rat brain;[38] and for α-adrenoceptors,[39] [^{3}H]clonidine in rat brain minus cerebellum and [^{3}H]WB4101 in whole rat brain.

Acknowledgment. We thank P. Graff, Ch. Jaeggi, and M. Kaiser for their capable technical assistance in the synthetic work. Thanks are also due to Dr. H. Braunschweiger and F. Seemann for the supply of ample quantities of intermediates. We appreciate the valuable help of H. R. Loosli, M. Ponelle, and T. Zardin (NMR spectra), W. Pfirter (analysis), C. Quiqerez (MS), and L. Thielemann (IR). We are further grateful to our colleagues U. Briner and H. R. Wagner for pharmacological testing and to Drs. W. Frick and A. Closse for performing the binding studies.

Registry No. **5**, 1212-08-4; **6**, 32940-15-1; **7b**, 87056-67-5; **8**, 53913-96-5; (±)-**9**, 94324-15-9; (±)-**10**, 94324-16-0; (±)-**11**, 94324-17-1; (±)-*cis*-**12a**, 87056-71-1; (±)-*trans*-**12b**, 87057-13-4; (±)-**13a**, 87056-72-2; (±)-**13b**, 87098-97-3; (±)-**13c**, 87098-98-4; (±)-**13d**, 87098-99-5; (±)-**14b**, 87056-66-4; (±)-**14c**, 87479-83-2; (±)-**15**, 87056-65-3; (±)-**15**·HCl, 94424-47-2; (±)-**16**, 87056-74-4; (±)-**16**·HO_2CCO_2H, 94424-48-3; (±)-**17**, 87056-77-7; (±)-**18**·2HCl, 94424-49-4; (±)-**19**, 87056-75-5; (±)-**20**, 87056-78-8; (±)-**20**·HCl, 94424-50-7; (±)-**21**, 87056-81-3; (±)-**22**, 87056-82-4; (±)-**23**, 87056-80-2; (±)-**24**, 87056-79-9; (±)-**25**, 87056-83-5; (±)-**26**, 87056-84-6; PhSSPh, 882-33-7; CH_3CH_2CHO, 123-38-6; Et_2NSO_2Cl, 20588-68-5; MeSH, 74-93-1.

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Molecular Interactions of Toxic Chlorinated Dibenzo-*p*-dioxins and Dibenzofurans with Thyroxine Binding Prealbumin

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The interactions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds with prealbumin, a model for the nuclear thyroid hormone receptor, have been studied with use of computer graphics and predictions made regarding relative binding affinities for such structures. These modeling predictions were tested by experimentally measuring the binding affinities of dioxin and furan analogues. The results were in general agreement with the modeling predictions and demonstrated that such compounds could be effective competitive binding ligands for thyroxine-specific binding sites in prealbumin. The computer modeling work also demonstrates the importance of lateral chlorine substitution in the binding of these toxic compounds. The prealbumin interaction model should be of use in investigating the structure–toxicity relationships of these classes of toxic compounds. Thus, if prealbumin is a model for the nuclear thyroid hormone receptor, this work would also have major implications bearing on the mechanism of dioxin toxicity and the potential of these compounds to function as potent and persistent thyroxine agonists. A new cooperative receptor mechanism for dioxin toxic action is proposed.

A number of compounds contained in the broad class of halogenated aromatic hydrocarbons including the polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs) produce a characteristic toxic syndrome.[1] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) are two of the most toxic compounds of this type (Chart I, drawn and numbered to correspond with figures and tables). These highly toxic compounds are also inducers of cytochrome P-448 mediated mixed function oxidase enzyme systems. The toxicity and induction response are both thought to involve initial binding of the hydrocarbons to the same cytosolic receptor (Ah receptor), but the subsequent events are not understood.[2]

In previous work[3] from this laboratory, the structure–induction relationship was found to be different from the structure–toxicity relationship. However, both depended,

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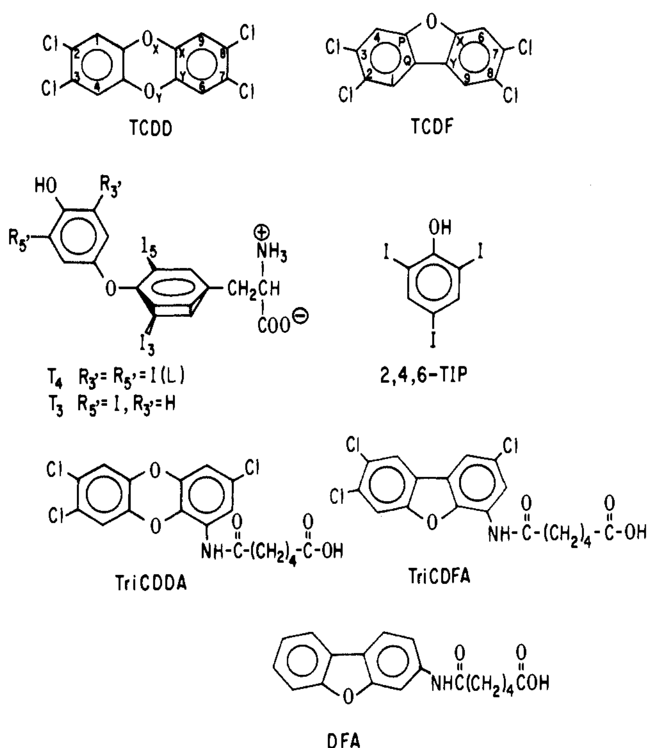
Table I. Interatomic Contacts Less than 4 Å for the TCDD–Prealbumin Model (See Figure 2a)

TCDD	amino acid	contact, Å	TCDD	amino acid	contact, Å
Cl-2[a] (≡ Cl-3)	CD,[b] Lys-15	3.92	Cl-1[c] (≡ Cl-4)	CA, Lys-15	3.84
	CE, Lys-15	3.77		CB, Lys-15	2.51
	NZ, Lys-15	3.72		CG, Lys-15	1.62
	OE2, Glu-54	3.49		CD, Lys-15	2.33
C-1 (≡ C-4)	CG, Lys-15	3.35		CE, Lys-15	2.58
	CD, Lys-15	3.85		NZ, Lys-15	3.72
	CE, Lys-15	3.56	Cl-9[c] (≡ Cl-6)	CA, Ala-108	3.85
Ox (≡ Oy)	CG, Lys-15	3.96		CB, Ala-108	3.23
	CD1, Leu-17	3.94		C, Ala-108	3.67
	CB, Ala-108	3.74		N, Ala-109	3.31
Cx (≡ Cy)	CB, Ala-108	3.91		CA, Ala-109	3.95
C-9 (≡ C-6)	CB, Ala-108	3.68		C, Ala-109	3.45
Cl-8 (≡ Cl-7)	N, Leu-110	3.86		O, Ala-109	3.24
	CA, Leu-110	3.97		N, Leu-110	3.90
	CB, Leu-110	2.94			
	CG, Leu-110	3.96			
	CD, Leu-110	3.90			
	OG, Ser-117	3.50			

[a] See numbering system in Chart I; equivalent atoms in parentheses make identical contacts with other side of binding site (residue numbers > 500). [b] Atom contacted in given amino acid residue; A, B, G, D, E, and Z denote α, β, γ, δ, ϵ, and ζ position of atom in side chain. [c] Contacts if peri positions (1, 4, 6, 9) were substituted with chlorine (no other changes made in atomic positions).

Chart I



in part, upon polarizability properties of the ligands. Structural considerations and experimental models for receptor interactions further suggested that binding to the Ah receptor involves a stacking interaction while binding to the hypothetical "toxic" receptor may involve dispersive interactions with halogen substituents. The existence of this second receptor for toxicity was postulated to account for the differences in the structure–activity relationships. Although halogenation is not necessarily required for binding to the Ah receptor, its presence can enhance the stacking interaction through increased molecular polarizability.[3] This work also suggested that a more toxicologically relevant binding protein should be sought because of the requirements of planarity and lateral halogenation for toxicity, as opposed to induction.

We have been seeking protein binding models consistent with the known structural requirements for toxicity and molecular interactions involved in the receptor-mediated binding events. The thyroid hormone binding proteins are likely candidates as receptors for these halogenated hydrocarbons because of their specificities for binding the biologically relevant halogenated aromatic hydrocarbon hormones (Chart I), thyroxine (T_4) and triiodothyronine (T_3). Since thyroxine depletion[4] in plasma is associated with the toxic effects of TCDD, it was reasonable to focus our attention on proteins which bind T_4 with greater affinity than T_3. Thyroxine binding prealbumin is a major carrier protein for T_4 in blood.[5] Interactions with thyroxine binding prealbumin (PA) have also been studied[6] as a model for the interaction of thyroid hormones with the nuclear thyroid hormone receptor and as a well-defined and relatively simple model for a drug–receptor interaction.

In this work, we first describe the potential interactions of PA with TCDD and TCDF using computer-assisted molecular graphics based on X-ray crystallographic measurements and make predictions about the relative binding affinities of these compounds to PA. Next we test our predictions experimentally using in vitro competitive T_4–PA binding assays with the dioxin and furan molecule rendered water soluble through addition of an adipamide linkage (TriCDDA, TriCDFA, and DFA in Chart I).

Results

The interatomic contacts less than 4 Å for the TCDD–prealbumin fit are given in Table I. Also included are the contacts for chlorine substitution in peri positions (1, 4, 6, 9). These more highly substituted dioxins are also of toxicological interest, and the 1-chloro substituent contacts provide an estimate of the relative effects on binding of the 1-adipamide linkage. Cl-1 (or Cl-4) substitition does lead to some close contacts with the Lys-15 residue, but as mentioned earlier, this flexible side chain assumes a different conformation on hormone binding and a similar behavior would be expected in this case in order to relieve these short contacts. A slightly short contact also occurs between the Cl-8 and CB of Leu-110, but again the local peptide chain is seen to move in hormone complexes to relieve the short contact. In general terms, hormone

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Table II. Interatomic Contacts Less than 4 Å for the TCDF-Prealbumin Model (See Figure 2b)[a]

TCDF	amino acid	contact, Å	TCDF	amino acid	contact, Å
Cl-7[a]	N,[b] Leu-110	3.91	Cl-2	CG, Lys-515	3.82
	CB, Leu-110	2.98		CD, Lys-515	3.71
	CG, Leu-110	3.98		CE, Lys-515	3.50
	CD1, Leu-110	3.90		NZ, Lys-515	3.75
	OG, Ser-117	3.52	Cl-4[c]	CA, Lys-15	3.36
Cl-8	CB, Leu-610	3.36		CB, Lys-15	2.06
C-6	CB, Ala-108	3.41		CG, Lys-15	1.20
Cx	CB, Ala-108	3.42		CD, Lys-15	1.74
	CD1, Leu-17	3.79		CE, Lys-15	2.33
C-9	CB, Ala-608	3.92		NZ, Lys-15	3.41
O	CB, Lys-15	3.94		C, Lys-15	3.94
	CG, Lys-15	3.18	Cl-1[c]	CB, Lys-515	3.33
	CG, Leu-17	3.88		CG, Lys-515	2.49
	CD1, Leu-17	3.88		CD, Lys-515	3.43
	CB, Ala-108	3.40		CE, Lys-515	3.36
Cp	CG, Lys-15	3.32	Cl-9[c]	CB, Ala-608	3.15
	CE, Lys-15	3.92		CG, Leu-517	3.87
C-4	CB, Lys-15	3.49		CD1, Leu-517	3.76
	CG, Lys-15	2.71	Cl-6[c]	CA, Ala-108	3.80
	CD, Lys-15	3.25		CB, Ala-108	3.44
	CE, Lys-15	3.10		C, Ala-108	3.37
C-3	CG, Lys-15	3.87		N, Ala-109	2.81
	CE, Lys-15	3.71		CA, Ala-109	3.29
Cl-3	CD, Lys-15	3.96		C, Ala-109	2.72
	CE, Lys-15	3.80		O, Ala-109	2.59
	NZ, Lys-15	3.75		N, Leu-110	3.22
	OE2, Glu-54	3.53		CA, Leu-110	3.75
				CB, Leu-110	3.89

[a] See numbering system in Chart I. [b] Atom contacted in given amino acid residue; A, B, G, D, E, and Z denote α, β, γ, δ, ϵ, and ζ position of atom in side chain. [c] Contacts if peri positions (1, 4, 6, 9) were substituted with chlorine (no other changes made in atomic positions).

binding induces a number of slight shifts in side chain and main-chain groups within the binding site. However, apart from the discrete change in the positions of Lys-15 and Lys-515, we have not attempted to include these in model building work. There is twofold symmetry in the ligand binding site; in the case of TCDD this corresponds to a rotation of 180° about its own twofold symmetry axis and results in exact superposition of the two bound states. The contacts for the TCDF–prealbumin fit are given in Table II and similarly reflect some short contacts. Contacts for the symmetry-related mode of binding of TCDF correspond to interchange of residue numbers to the symmetry-related subunit (e.g., Leu-110 becomes Leu-610). This interaction is otherwise equivalent to that for TCDD, both indicating good contacts with the protein (i.e., contacts closer than van der Waals). If one simply compares the number of contacts for TCDD and TCDF from Table I and II, the TCDD fit may be slightly better than the TCDF fit, but the short contacts particularly at C-4 are shorter in TCDF (compared to C-1 in TCDD) and may lead to some repulsion.

In comparison with the normal hormone binding contacts, two of the lateral chlorines in TCDD are occupying at least two of the three highly polar and polarizable phenolic ring binding pockets showing a number of favorable contacts, and the other two chlorines are showing favorable contacts with Lys-15 and Glu-54 which are normally involved in polar interactions with the hormone side-chain carboxyl and amino groups.

The relative binding affinities for the dioxin and furan analogues tested in this study are listed in Table III. The experimentally determined binding affinities for TriCDDA, TriCDFA, and DFA are in qualitative agreement with the predictions based on molecular modeling in that significant binding can be demonstrated for the chlorinated dioxin and, to a lesser extent, the structurally related chlorinated furan. However, in the absence of chlorination, it was not possible to demonstrate binding. The relatively high binding affinity of the triiodophenol compound demonstrates the importance of the phenolic ring binding pockets in binding and when coupled with the DFA binding result supports the argument that the polar carboxylic acid side chains in these dioxins and furan analogues do not contribute in a major way to their binding. This result is also consistent with the very high binding affinity of single-ring compounds previously reported.[7]

Table III. Binding Affinities of Test Compounds to Prealbumin

compd[a]	binding affinities[b]	compd[a]	binding affinities[b]
T_4	100	DFA	ND[c]
TriCDDA	15.0	2,4,6-TIP	384.0
TriCDFA	0.32		

[a] TriCDDA ≡ trichlorodibenzodioxin adipamide; TriCDFA ≡ trichlorodibenzofuran adipamide; DFA ≡ dibenzofuran adipamide; 2,4,6-TIP ≡ 2,4,6-triiodophenol (see Chart I). [b] The relative binding affinities of the compounds were determined from the competition binding assays and are expressed relative to L-T_4 (assigned a value of 100). [c] ND = not detectable; at the highest concentration tested (3.8×10^{-5} M), there was no measurable binding.

These control experiments (with 2,4,6-TIP and DFA) also provide validity to our simplified modeling experiments which position the dioxin and furan structures in the plane of the phenolic ring of T_4 in the binding site. While it is possible that these analogues may also bind at other sites on the protein, there is clear competition for the binding site normally occupied by T_4 as demonstrated by reproducible displacement of the labeled T_4 in the competition experiments. The T_4 binding site in PA is unique in that it matches the structure and the chemistry of the hormone with great precision and PA almost completely "engulfs" the hormone. For these reasons, we feel that these analogues must also bind the site in a way that closely matches the structure and chemistry of the hormone in order to effectively compete for the site. Thus, there were no compelling reasons to suggest significant

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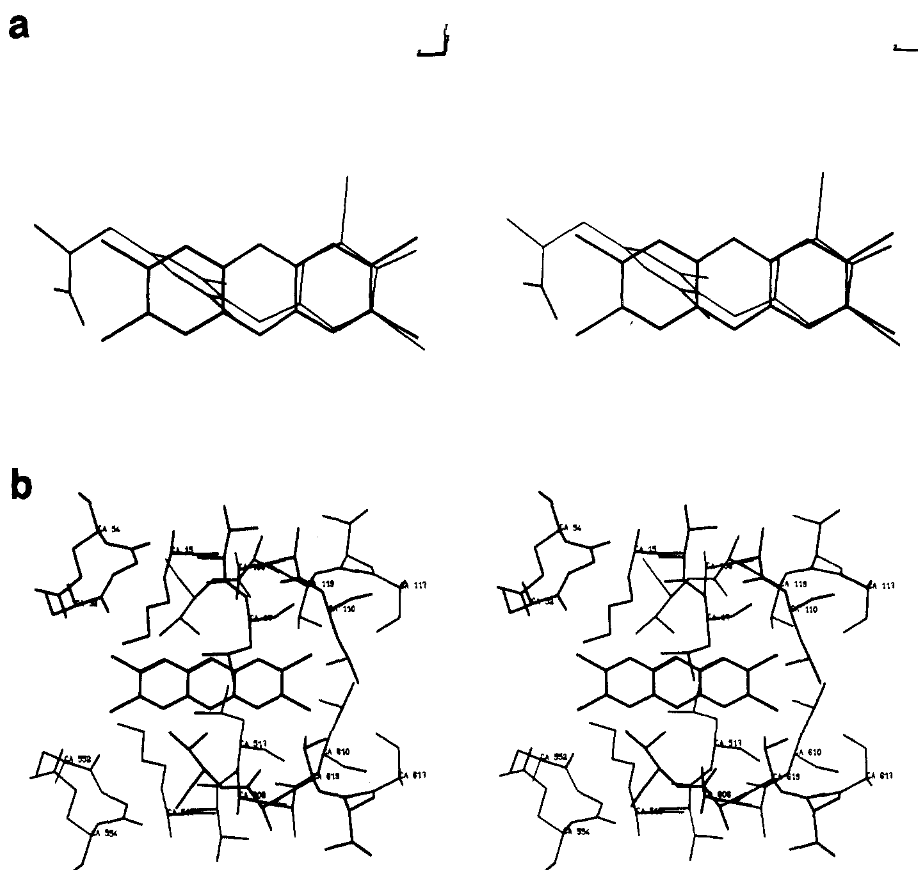

Figure 1. Stereopair superposition of (a) "bound" TCDD (heavy line) and T_4 (light line) and (b) model-built structure of TCDD in the hormone binding channel of prealbumin.

differences in the positioning of TCDD or TriCDDA in the binding channel of PA since the position is largely determined by the inner ring which can be the same in both.

However, because of the magnitude of the binding constant of this protein with T_4 (10^7–10^8 M^{-1}) and the poor water solubility of these compounds (nanomolar), the polar adipamide groups were technically necessary in order to achieve sufficient solubility in the binding assay medium. We made several attempts to increase the solubility of the hydrocarbons in the assay medium, but the extreme insolubility of these compounds made this an intractable problem with this system. Similar attempts to incorporate the hydrocarbons into PA for X-ray crystallographic studies have so far also been unsuccessful. This solubility limitation in the binding assay is not likely to be a limitation in vivo, since TCDD is soluble in blood up to a concentration of 0.3 mM.[8] We assume that the polar adipamide linkage extends toward the mouth of the protein binding channel as has been demonstrated for the hormone side chain.[9] The presence of the large adipamide group at C-1 and the absence of a lateral chlorine would be expected to lower somewhat the binding affinity of TriCDDA relative to TCDD. However, in spite of these differences TriCDDA binds with significantly greater affinity than L-T_3.

The approximately 50-fold lower binding affinity of TriCDFA (relative to TriCDDA) was a somewhat unexpected result. However, inspection of the data for interatomic contacts in Tables I and II involving contacts for substituents in the C-4 peri position may offer an explanation. The data suggests that the Lys-15 contacts with chlorine at C-4 (Cl-4) would be shorter in TriCDFA than in TriCDDA (compare Cl-1) and could not easily be relieved by conformational changes in the protein. Because of synthetic difficulties other derivatives were not available to further test this argument experimentally.

Discussion

As indicated earlier,[3] we seek protein binding models consistent with the known structural requirements for high toxicity in this series of compounds. In this work, we have modeled the interaction of TCDD and TCDF, prototypical halogenated aromatic hydrocarbons, and shown experimentally through competitive binding assay studies that effective interaction with PA is possible. The work further demonstrates a requirement for halogenation in binding to PA and the modeling emphasizes the importance of lateral halogen substitution in filling the binding pockets normally occupied by the phenolic ring of the thyroxine molecule. The structural complementarity of TCDD (and TCDF) and T_4 in this portion of the molecule can also be seen in their superposition shown in Figure 1a (and Figure 2a). There is no specific requirement for numbers of halogen atoms provided there is a sufficient number to fill or partially fill the thyroxine phenolic ring binding pockets.

Inspection of the modeling data for TCDD (in Table I) reveals that some substitution of chlorine in peri positions can be tolerated and would not be expected to greatly reduce binding. Consistent with this argument and the possible toxicological relevance of this model is the essentially equipotent toxicity of 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin and 20–30-fold lowering of toxicity for hexa isomers where at least two of the peri positions are occupied.[10] A similar pattern is also found for the toxicity of

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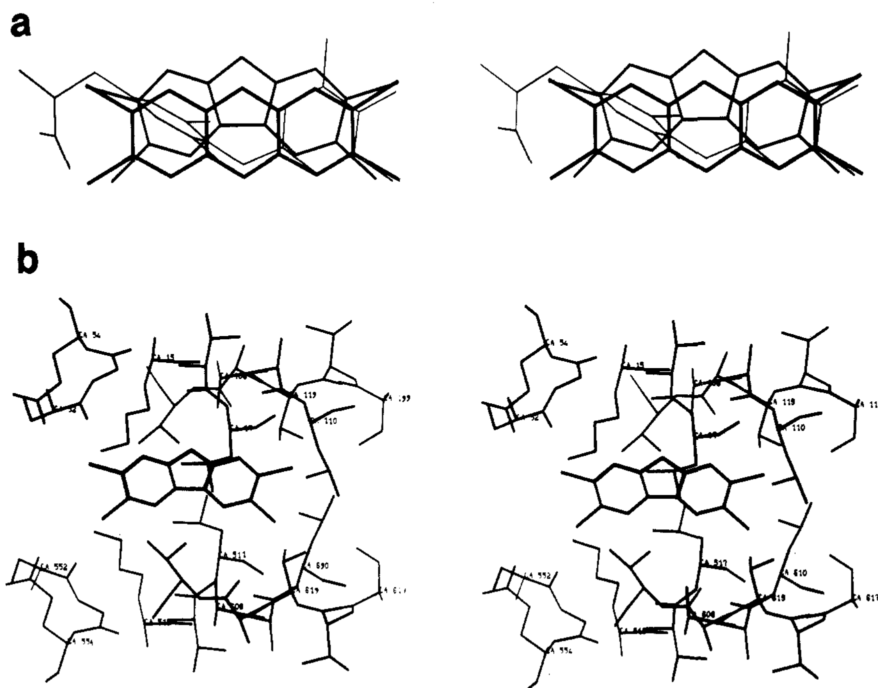

Figure 2. Stereopair superposition of (a) "bound" TCDD (heavy line), TCDF (medium line), and T_4 (light line) and (b) model-built structure of TCDF in the hormone binding channel of prealbumin.

chlorinated dibenzofurans.[11] This model for TCDD binding to PA is in contrast to our previous model[12,13] for the Ah receptor where the numbers of halogens is important in effecting a dispersion–stacking type interaction which depends upon sufficient molecular polarizability and planarity of structure. Chlorine substitution in peri positions results in lower binding affinity to the Ah receptor,[2] possibly due to loss of stabilizing hydrogen bond formation with the receptor. In the absence of X-ray data to fix the positions, we feel this simple model is adequate for making qualitative predictions about relative binding affinities of these toxic compounds to prealbumin.

In addition to the potential of the PA interaction model for investigating structure–toxicity correlations with both halogenated hydrocarbon hormones and related xenobiotics, our earlier findings[14] of PA depletion in rat sera of TCDD treated animals coupled with our preliminary findings[15] of a parallel increase in retinol binding protein and vitamin A suggest that the serum thyroid hormone binding proteins may be directly involved in the mechanism of toxicity of these compounds. The structural resemblance of certain chlorinated aromatic hydrocarbons in the DDT series to the thyroid hormones and the potential of these compounds to interact with thyroxine binding sites and produce aberrant thyroid activity was first recognized over 15 years ago.[16] Future work will investigate the relative binding affinities of the toxic and relatively nontoxic molecular conformations of selected polychlorinated biphenyls.

We do not claim that this work proves that TCDD and related compounds bind to PA and related proteins in vivo. However, we do feel that the work directly and logically links the structure of these toxic halogenated hydrocarbons to the structure of thyroxine. These studies also show that TCDD and related compounds can be competitive binding ligands for T_4 specific binding proteins like PA exhibiting similar structure–binding relationships for thyroid hormone analogues. If PA is indeed a model for certain binding components of the nuclear thyroid hormone receptor, this work could have major implications bearing on the mechanism of TCDD toxicity and the potential of this class of compounds to function as potent and persistent T_4 agonists.

Conclusion

In our earlier work[3] we proposed the involvement of multiple receptors with structurally distinct binding properties in the mechanism of toxicity of TCDD and related compounds. The molecular modeling work for PA interactions with TCDD and TCDF described here fulfills the requirements for one of the receptors which can account for the need for halogenation in lateral positions. Our previous theoretical model[12,13] for Ah receptor interactions can account for the need for sufficient numbers of halogens and planarity or coplanarity of structure. Thus the structural requirements for binding the Ah receptor, combined with the structural requirements for binding PA, a model for a nuclear protein receptor, correspond with those required to produce toxicity for this class of compounds.[3] It has also been demonstrated that thyroid hormones can interact with the Ah receptor.[17] Furthermore, recent workers[18] studying the effects of thyroidectomy and thyroxine on TCDD induced toxicity have sug-

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gested that thyroid hormones are involved in toxicity. Finally, we have completed experimental studies[19] which support the T_4 agonist activity of TCDD in vivo and suggest that the high toxicity of this environmentally important compound is the expression of potent and persistent thyroid hormone activity.

This leads us to propose a new cooperative receptor mechanism for TCDD action, possibly involving the same receptor proteins responsible for thyroid hormone action. In this hypothesis the Ah receptor can be viewed as a storage and translocating protein which is in equilibrium with the nuclear binding events. The equilibrium movement of these proteins in and out of the nucleus can be controlled by a concentration gradient determined by available binding ligands and capacity. This initial binding event may be charge transfer in nature.[3,13] Once in the nucleus a two-protein activated complex could be generated by association of the Ah receptor protein–TCDD complex with a second chromatin-bound protein receptor. This second protein binding event would be assisted by the lateral halogen interactions similar to those found in PA binding and perhaps by protein–protein interactions. This binding event is stablized by favorable halogen to protein contacts and two adjacent lateral halogens appear to be sufficient for high affinity.

Thus, an activated complex resulting from the cooperative binding of two proteins to TCDD and related structures could control the subsequent nuclear events leading to a biological response. The cooperative binding event introduces considerable structural specificity to the control process. This would have its analogy[20] in some steroid hormone action where certain structural features of the steroid molecule control initial receptor binding and others the subsequent nuclear events. In this mechanism, binding the Ah receptor is a necessary but not sufficient condition for toxicity. Therefore, it would not be surprising to find that all toxic structures can bind both receptors, but not all structures that can bind one or the other receptor alone need be toxic. This is being examined as a working hypothesis for further investigation of the molecular mechanism of TCDD toxicity as well as insight into the mechanism of thyroid hormone action.

Experimental Section

Molecular Modeling. All the model building was carried out on the Evans and Sutherland Picture System 2 at the Laboratory of Molecular Biophysics, University of Oxford. The prealbumin coordinates were those obtained from the X-ray crystallographic refinement at 1.5-Å resolution,[21] improved by further refinement at 1.8 Å, leading to a standard crystallographic R factor of 0.18.[22] The basis of the modeling was the experimentally determined interaction of the thyroid hormones (T_4 and T_3) and hormone analogues with prealbumin.[9] The only change made in the refined native protein coordinates was adjustment of the torsional angles of the Lys-15 and Lys-515 side chains so that they corresponded to the typical values observed when its range of ligands is bound. The coordinates of TCDD[23] and TCDF[24] were those previously published. Standard geometries were used to examine the effects of substituents in the peri positions (1, 4, 6, 9) of the dioxin molecule. The interaction of TCDD was modeled so that its symmetry matched that of the prealbumin binding site: the twofold axis along its lengths is coincident with that of the binding site, and its plane almost coplanar with that of the phenolic ring of T_4, so that the Cl-2 and Cl-3 of TCDD are positioned close in space to the 5′-I and 4′-OH of the hormones.

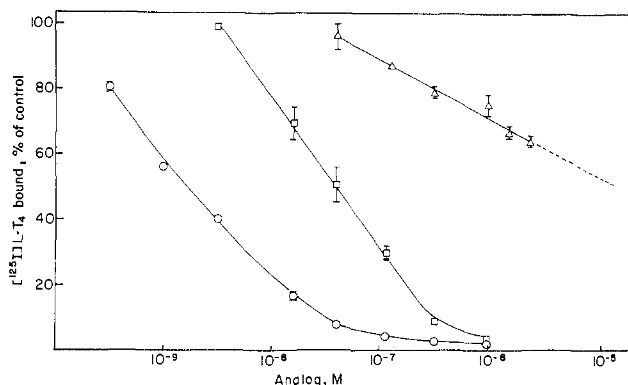

Figure 3. Competitive binding curves for 2,4,6-TIP, TriCDDA, and TriCDFA against L-[^{125}I]thyroxine. 100% = cpm with no unlabeled analogue added. Each point represents the mean of two determinations: (○) 2,4,6-TIP, (□) TriCDDA, (△) TriCDFA.

Figure 1a shows the superposition of "bound" TCDD (heavy line) and T_4 (light line) and Figure 2a the superposition of the "bound" TCDD (heavy line), TCDF (medium line), and T_4 (light line). Figure 1b shows the model-built TCDD in the hormone binding channel of prealbumin. The entrance to the channel is on the left; atoms in one subunit are labeled 1–127 and in the other symmetry-related subunit 501–627. The list of contacts are given in Table I. The assumption was made for TCDF that the chlorine atoms would again be the major sites of interaction, and hence it was reasonable to model its interaction by analogy with TCDD. It was therefore oriented on the basis of a least-squares fit between the Cl-2, Cl-3, Cl-7, and Cl-8 atoms of the two molecules. The distance between the Cl-2 and Cl-8 and Cl-3 and Cl-7 pairs of atoms was 0.05 Å and between the Cl-3 and Cl-7 and Cl-2 and Cl-8 pairs was 0.77 Å (Figure 2b). Table II gives the list of contacts for this fit.

Biochemical Methods. Materials. L-T_4 and T_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). [^{125}I]T_4 (L) with specific activity of 800–1200 μCi/μg was purchased from New England Nuclear and contained less than 1% unbound iodide. 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, bead size 50–150 μm, was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). The adipamide free acid derivatives (see Chart I) of 1-amino-3,7,8-trichlorodibenzo-*p*-dioxin,[25] 4-amino-2,7,8-trichlorodibenzofuran,[26] and 3-aminodibenzofuran[27] were prepared according to the previously reported procedures.[28,29] The absence of detectable impurities in the adipamide preparations was confirmed by gas chromatography–mass spectrometry of their methyl esters.

Competition Binding Assays. The binding assay using the gel filtration procedure described by Somack et al.[30] was used to

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measure the ability of various halogenated hydrocarbons to displace [^{125}I]T_4 from the high-affinity prealbumin binding site. The assay mixture contained 10 nM prealbumin, 0.4 nM [^{125}I]T_4 containing approximately 0.15 μCi, and varying concentrations of unlabeled compounds (10^{-5}–10^{-9} M) in 0.1 M imidazole acetate buffer containing 1 mM EDTA (pH 7.4) in a final volume of 0.5 mL. After incubation for 1 h at 25 °C, 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 imidazole acetate buffer 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 1.2 mL of buffer. The following 1.6-mL fraction contained free, radioactive iodide. Free hormone binds tightly to the gel matrix[30] 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 Gamma Counter (60% counting efficiency).

The relative binding affinities of the various halogenated hydrocarbons for the prealbumin were obtained from the competitive binding assays shown in Figure 3.

The binding potency of each compound relative to that of T_4 (assigned a value of 100) was calculated as follows:

$$\text{binding potency} = \frac{\text{concn of } T_4 \text{ at } \tfrac{1}{2} \text{ maximal of control}}{\text{concn of compd at } \tfrac{1}{2} \text{ maximal of control}} \times 100$$

The control value was obtained from incubation with [^{125}I]T_4 alone. The concentration of T_4 at half-maximal of control was 9.6×10^{-9} M at L-T_4 concentrations between 1.2×10^{-9} and 4.8×10^{-6} M. We determined the K_a of L-T_4 to be 5.8×10^8 M^{-1}, which is in agreement with the generally accepted range 10^7–10^8 M^{-1}. In this work, L-T_3 was found to have 5.3% the affinity of L-T_4, which compares favorably with the 9% affinity reported by others.[31]

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Registry No. TCDD, 1746-01-6; TCDF, 51207-31-9; T_4, 51-48-9; 2,4,6-TIP, 609-23-4; TriCDDA, 93943-01-2; TriCDFA, 93943-02-3; DFA, 93943-03-4.

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[^{3}H]Batrachotoxinin A 20α-Benzoate Binding to Voltage-Sensitive Sodium Channels: A Rapid and Quantitative Assay for Local Anesthetic Activity in a Variety of Drugs

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[^{3}H]Batrachotoxinin A benzoate ([^{3}H]BTX-B) binds with high affinity to sites on voltage-dependent sodium channels in a vesicular preparation from guinea pig cerebral cortex. In this preparation, local anesthetics competitively antagonize the binding of [^{3}H]BTX-B. The potencies of some 40 classical local anesthetics and a variety of catecholamine, histamine, serotonin, adenosine, GABA, glycine, acetylcholine, and calcium antagonists, tranquilizers, antidepressants, barbiturates, anticonvulsants, steroids, vasodilators, antiinflammatories, anticoagulants, analgesics, and other agents have been determined. An excellent correlation with the known local anesthetic activity of many of these agents indicate that antagonism of binding of [^{3}H]BTX-B binding provides a rapid, quantitative, and facile method for the screening and investigation of local anesthetic activity.

The sodium channel of the plasma membrane of nerve or muscle cells functions as a voltage-sensitive gate for sodium ions.[1] Transitions between various resting, open, and inactivated states function to permit a specific increase in the permeability of the plasma membrane allowing influx of sodium ions followed by a return to a state which is relatively impermeable to sodium ions.[1] Local anesthetics modify the properties of the sodium channel resulting in a decrease in the flux of sodium ions. It has been proposed that local anesthetics bind to a site in the sodium channel complex, thereby promoting inactivation of the channel.[2] Local anesthetics appear to interact more rapidly with open conducting forms of the channel and to bind with higher affinity to inactivated forms of the channel.

Investigation of the nature of local anesthetic action has been aided by the discovery of neurotoxins which exhibit both specificity and high affinity for binding sites directly associated with the sodium channel. Radiolabeled derivatives of such toxins have provided molecular probes for at least three separate binding sites associated with the sodium channel. Two of these sites, the tetrodotoxin site and the scorpion toxin site, do not appear to be influenced by local anesthetics.[3,4] A third site, the batrachotoxin (BTX) binding site, is associated with the gating mechanism of the channel. The binding of BTX to the sodium channel prevents inactivation of the channel resulting in a massive influx of sodium ions and a persistent membrane depolarization.[5] Binding of a [^{3}H]BTX analogue is inhibited by local anesthetics in a competitive or mutually exclusive manner.[6] Recent studies suggest that the binding of local anesthetics promotes an allosteric inhibition of binding of the [^{3}H]BTX analogue, thus increasing the "off-rate" of BTX and shifting the sodium channel to an inactive form.[7] In the present report, [^{3}H]batrachotoxinin A 20α-benzoate ([^{3}H]BTX-B) has been utilized to

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