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Multifunctional Receptor Model for Dioxin and Related Compound Toxic Action: Possible Thyroid Hormone-Responsive Effector-Linked Site

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Molecular/theoretical modeling studies have revealed that thyroid hormones and toxic chlorinated aromatic hydrocarbons of environmental significance (for which dioxin or TCDD is the prototype) have similar structural properties that could be important in molecular recognition in biochemical systems. These molecular properties include a somewhat rigid, sterically accessible and polarizable aromatic ring and size-limited, hydrophobic lateral substituents, usually contained in opposite adjoining rings of a diphenyl compound. These molecular properties define the primary binding groups thought to be important in molecular recognition of both types of structures in biochemical systems. Similar molecular reactivities are supported by the demonstration of effective specific binding of thyroid hormones and chlorinated aromatic hydrocarbons with four different proteins, enzymes, or receptor preparations that are known or suspected to be involved in the expression of thyroid hormone activity. These binding interactions represent both aromatic-aromatic (stacking) and molecular cleft-type recognition processes. A multiple protein or multifunctional receptor-ligand binding mechanism model is proposed as a way of visualizing the details and possible role of both the stacking and cleft type molecular recognition factors in the expression of biological activity. The model suggests a means by which hormone-responsive effector-linked sites (possible protein-protein-DNA complexes) can maintain highly structurally specific control of hormone action. Finally, the model also provides a theoretical basis for the design and conduct of further biological experimentation on the molecular mechanism(s) of action of toxic chlorinated aromatic hydrocarbons and thyroid hormones.

Introduction

Halogenated aromatic hydrocarbons have added much to our modern living and have become an important part of everyday life. Because of their production on a large scale and widespread usage for the last 30 years or more, many of these persistent chemicals such as the DDT family of insecticides, polychlorinated biphenyl (PCB) dielectric fluids, etc., are commonly present in the environment and its biota, including humans (*1*). Some of these compound classes (e.g., dioxins and furans) occur as unwanted contaminants of the useful chemicals. These classes of compounds are usually found as complex mixtures of various congeners and isomers, each with its own independent toxic potential. The concern about the health and ecological effects (*2*) of these persistent lipophilic chemicals is due to their acute toxicity, e.g., the chlorinated dibenzodioxins and dibenzofurans that occur as contaminants of chlorinated phenol preparations, or their chronic toxicity resulting from bioconcentration over time in body tissues, e.g., PCBs, chlorinated benzenes, and naphthalenes, as well as the dioxins and furans.

The toxicological and ecological effects of these chemicals have been studied extensively in many laboratories (*1-3*). Signs and symptoms include chloracne, weight loss, general malaise, nausea, loss of appetite, impairment of liver function, hepatic porphyria, and sensory neuropathy. The entire group of chlorinated aromatic hydrocarbons (CAHs) show many similarities in toxicity, with considerable variation in dose-response. The contamination with these chemicals is very extensive, and much of the toxicological work has been rather empirical and descriptive in nature. However, knowledge about their effects on laboratory animals is advancing rapidly.

Treatment of animals with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the prototype for certain halogenated aromatic hydrocarbons that effect a similar pattern of toxicity, produces divergent effects on circulating levels of L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3) concentrations (*4-7*). These results, along with the lack of in-

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formation on thyroid hormone-modulated responses in these animals, have caused uncertainty regarding their functional and biochemical thyroid status. For example, the presence of goiter (*4*) and depressed circulating levels of total and free T_4 (*4–7*) in TCDD-treated rats led Pazdernik and Rozman (*8*) to propose that the animals are hypothyroid. On the other hand, administration of TCDD to rats increased (*9*) the thyroid hormone-responsive hepatic enzyme activity of malic enzyme, an effect seen in hyperthyroidism. The hormone response in TCDD-treated animals may reflect an adaptation to a toxic biochemical lesion that occurs very early after TCDD exposure (*10*). This in turn may reflect a direct effect of TCDD on thyroid hormones themselves, e.g., in functioning as agonists and antagonists with respect to their specific binding interactions in biochemical systems.

Because of their relative low toxicity, the PCBs have been a useful model class for studying the toxicological effects (*3*) of these chemicals in the laboratory. The action of foreign compounds such as PCBs in biological systems can be produced by a variety of specific molecular interactions and combinations of nonspecific processes. All of these actions reflect a close relationship between physiochemical properties encoded in the molecular structure of the compounds and the responses they evoke in biological systems (*11*). Using primarily PCBs as well-characterized structural models, our approach was simply to determine the important structural features and the unique stereoelectronic conditions in these molecules that determined toxicity. This in turn should provide insight into the nature of their binding sites and associated possible endogenous ligands in toxicologically relevant biological systems.

Because of the exquisite toxicity of the parent compounds, it was reasonable to anticipate that much of their toxic properties may be the result of over- (or under-) expression of normal life-controlling biological activity. In this article we review our findings and some supporting information that we believe indicate an important role for thyroid hormone binding sites in the expression of toxicity of CAHs. We first examine the structural relationship of toxic CAHs and the thyroid hormones (THs). Model systems are described that explore the molecular recognition processes of both types of structures in biochemical systems. Molecular determinants of binding interactions with proteins and model systems and the expression of biological activity are shown to be similar, if not identical, for both the toxic compounds and thyroid hormones. Finally, we propose a multifunctional receptor model for CAH toxic action consistent with its normal function as a critical thyroid hormone-responsive effector-linked site.

General Structural Relationship

The toxic CAHs and THs can be more broadly classified as halogenated aromatic hydrocarbons. Thyroid hormones have the additional feature of being amino acid analogs. The possible importance of this difference will be discussed later. The structural resemblance of certain CAHs to THs, e.g., the DDT family of insecticides, was recognized many years ago (*12*), but the importance and similarity of specific molecular features for activity were not clear. The toxic potency of the CAHs varies considerably and is remarkably dependent on the number and position of halogen atoms in their molecular structure. Qualitative structure requirements for high toxicity include planarity or coplanarity of structure in a shape approximating a rectangle and a sufficient degree of halogenation concentrated about lateral positions (e.g, 2,3,7,8-chlorines in TCDD) of the molecule. Halogenation, for example, in nonlateral positions (e.g., *ortho*-substitutions in PCBs) in variably decreases the biological and toxic potency. This has led to the 3×10 Å rectangular box generalization for the structure-activity relationship (*2,13–15*). On the basis of this and other observations, we advanced the following working hypotheses: a) There is a common structural basis for the toxicity of many different halogenated aromatic hydrocarbon classes; b) a polarizable aromatic ring and lateral halogen substituents are the primary binding groups whose topographical arrangement is fundamental to the toxicity of these compound classes; c) stereoelectronic factors determine the extent of their relevant binding interactions which in turn determine toxic potency; d) these same structural features are represented by the polarizable tyrosyl ring and the diiodophenolic moiety of the thyroid hormones.

Clearly, if these hypotheses (Fig. 1) could be verified, then they would provide the basis for an extremely useful common toxicophore for use in mechanism-based toxicity prediction and assessment. We provide evidence in the form of molecular and theoretical modeling studies that common structural features between THs and CAHs do exist and are represented in their binding interactions with proteins and related model systems. Such interactions between CAHs and thyroid hormone binding proteins in biological systems may mediate the expression of potent and persistent thyroid hormone agonist and antagonist activity that could underlie toxicity.

It was recognized some time ago (*16*) that specific structural and stereochemical features of the THs were responsible for their hormone action. Thyromimetic activity is directly related to the ability of the sterically large 3,5 substituents to constrain the diphenyl ether thyronine nucleus to the two approximately energetically equal, readily interconvertible, proximate, and distal conformers (two rings are disposed nearly perpendicular and bisecting, and the 3′ and 5′ positions become nonequivalent with respect to the alanine-bearing ring). This gives rise to the concept of a preferred, though not rigid, orientation of the molecule, described as a skewed conformation (*17*). Early work (*18*) has shown that aromatic character for the alanine-bearing (tyrosyl) ring may be important for thyromimetic activity, and it was suggested (*16*) that the bulky 3,5 substituents, in addition to positioning the outer (phenolic) ring, together with the methylene group of the alanine side chain provide enhancement of the binding characteristics of the tyrosyl

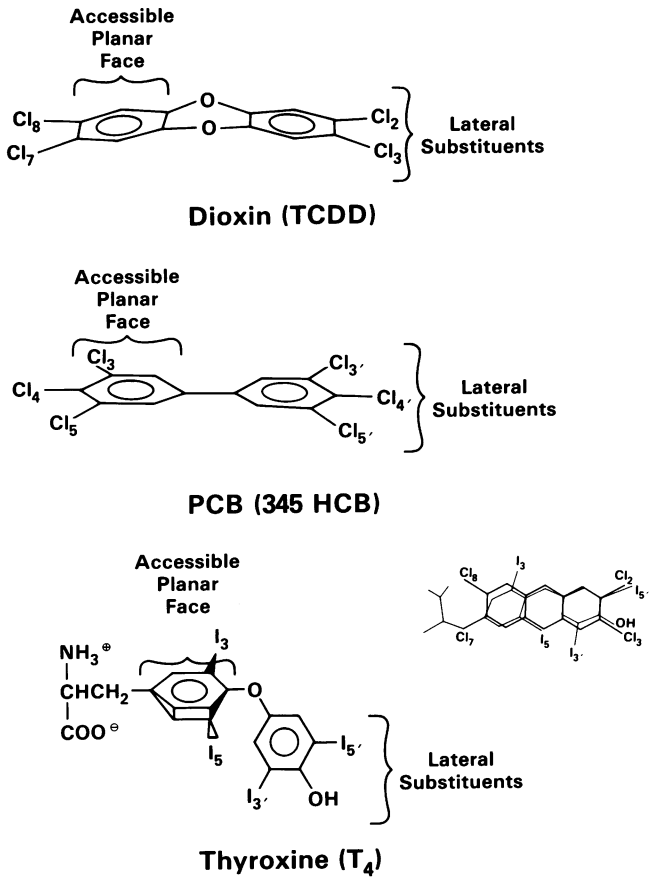

FIGURE 1. Common structural properties for thyroxine and toxic chlorinated aromatic hydrocarbons. Reprinted with permission (*69*).

ring to some biologic receptor through their electronic contributions. We have recently suggested that the polarizability of the 3,5 substituents may contribute to the overall hydrophobic property of the tyrosyl ring (*19*). The 3,5-iodines may serve both to restrict the overall conformation and increase molecular polarizability of the tyrosyl ring, specifically facilitating hydrophobic (perhaps stacking in nature) interactions. Thus, although in the overall structure the tyrosyl and phenolic rings are not coplanar, the tyrosyl ring does present a sterically and energetically accessible planar face for possible stacking interactions with other aromatic moieties. In addition, it is clear that size-limited hydrophobic associations involving phenolic ring substituents are also important in the expression of hormonal activity (*16*). There are also definite structure-activity relationships associated with variations in the amino acid side chain, but it is not clear to what extent these are direct effects on binding interactions versus indirect effects, e.g., on the hydrophobic properties of the tyrosyl ring (*20*).

For highly active (or toxic) structures in both the CAH and TH series, there is an apparent need for a sterically and energetically accessible, polarizable aromatic ring coupled with hydrophobic substituents in the lateral positions of the adjoining rings. If these features of the molecules are aligned (Fig. 1), there is an overall similarity in geometrical size and shape of the molecules and close correspondence of the planar faces and lateral substituents. On the basis of these preliminary molecular comparison studies, it was possible to postulate two major types of molecular recognition processes in biochemical systems that might mediate their biological activities. The first would involve dispersion-stacking type interactions between polarizable planar faces of aromatic rings. Likely binding sites in proteins for stacking interactions are the rigid side chains of aromatic amino acids (*21*), as well as the heme prosthetic group in hemoproteins. Recent crystallographic studies (*22–23*) have shown that stacking interactions between aromatic amino acids and DNA bases are important recognition factors in the interactions of proteins and nucleic acids. The second type of interaction would involve molecular cleft-type interactions (*24*) between highly polarizable lateral halogen atoms and the hydrophobic interior of the cleft provided by amino acid side chains that converge on the halogen substituents. The importance of C-shaped molecular cavities or clefts with viselike properties in molecular recognition has been recognized (*25*).

The question arises as to whether both of these structurally distinct recognition sites are components of a single, multifunctional binding protein or whether they are found in different proteins, perhaps involving the lipid phase that cooperates in the binding process and ultimate expression of biological activity. For example, it has recently been suggested (*26*) that the direct ligand receptor model might be replaced in some cases by a multiple sequential step model in which the binding energy from membrane interaction is used to overcome the entropy requirement for bringing the ligand-receptor together. Regardless of which of these possibilities is correct, there are likely to be molecules that are efficiently recognized (perhaps cooperatively) by both sites, while others are efficiently recognized by only one of the sites. Such site selection differences (*27*) might provide a plausible basis for understanding partial agonist and antagonist properties of some compounds. We now examine some molecular models that have been developed to support the importance of these molecular recognition processes in the expression of biological activity by CAHs and THs.

Stacking Models

The toxic and induction responses of CAHs at the cellular level have been proposed to involve specific, saturable, noncovalent binding of the soluble *Ah* (or dioxin) receptor. The role of the *Ah* receptor protein in the mechanism of action of toxic CAHs has been throughly investigated (*13–15,28*) and satisfies most of the specific criteria that support a receptor-mediated cellular process. A planar (or energetically favorable coplanar) aromatic system is the primary structural feature that is found in all known high-affinity ligands for this receptor, which include both halogenated and nonhalogenated aromatic compounds. Theoretical modeling (*29–32*) of CAH-*Ah* receptor interactions based on experimental competitive binding studies support a stacking interaction model in

which the CAH molecular polarizability and CAH to receptor equilibrium separation distance are the important molecular parameters. For two structurally related CAHs (one being a reference compound, CAH_1), the ratio of equilibrium binding constants for receptor binding simplifies to give

$$K_i/K_1 \cong \exp\,[3\alpha_R\,\bar{E}/(4kT)(-\alpha_i/r_{e,i}^6 - \alpha_1/r_{e,1}^6)] \quad (1)$$

Here k is Boltzmann's constant, T is absolute temperature, $\bar{E}$, and average excitation energy, is given by

$$\bar{E} = E_i E_R/(E_i + E_R) \quad (2)$$

where E_i and E_R are excitation energies for the CAH class and receptor, respectively, and α_R, α_1 and α_i are the polarizabilities of the receptor, a reference CAH and CAH_i, respectively; r_e is the equilibrium receptor to CAH separation distance. Modeling results have been interpreted in terms of linear free-energy relationships. Since polarizability and distance parameters are exponential functions in the equilibrium equation, small differences can have large effects on binding. The polarizability component in the lateral direction of these basically rectanglar-shaped molecules is usually dominant and can be enhanced significantly by the addition of lateral halogens. Therefore, it is understandable in this model how lateral halogenation can lead to increased binding activity. The advantage of this model is that it incorporates the stereoelectronic effects of chlorine substitution on binding free energy (Fig. 2).

Although the molecular structure of the *Ah* receptor binding site is not known, the modeling results support the importance of a stacking mechanism in the molecular recognition process. The PCB *ortho* effect is readily apparent in such a model because it can have a significant effect on the PCB-to-receptor separation distance that affects binding activity. We have used porphine as a model for the receptor binding domain, although an alternative hydrophobic region on a protein may be the rigid indole side chain nucleus of the tryptophan residue. Such a stacking model is reminiscent of those drawn for charge-transfer complexes, and polarization of the PCB molecule by the electronic environment of the binding site would be relevant. Evidence that these halogenated aromatic hydrocarbons may act as electron-acceptors in charge transfer complex with the *Ah* receptor had been provided (*14,33*).

What can be predicted from such a model in terms of the types of structures that should show good binding activity? A somewhat rigid and accessible planar face characteristic of a phenyl ring is of obvious importance in effecting close contact with a similar face in the receptor binding site. The importance of aromatic-aromatic interactions in protein stability and function has been realized (*13*). Both parallel stacking and perpendicular stacking arrangements are energetically favorable and

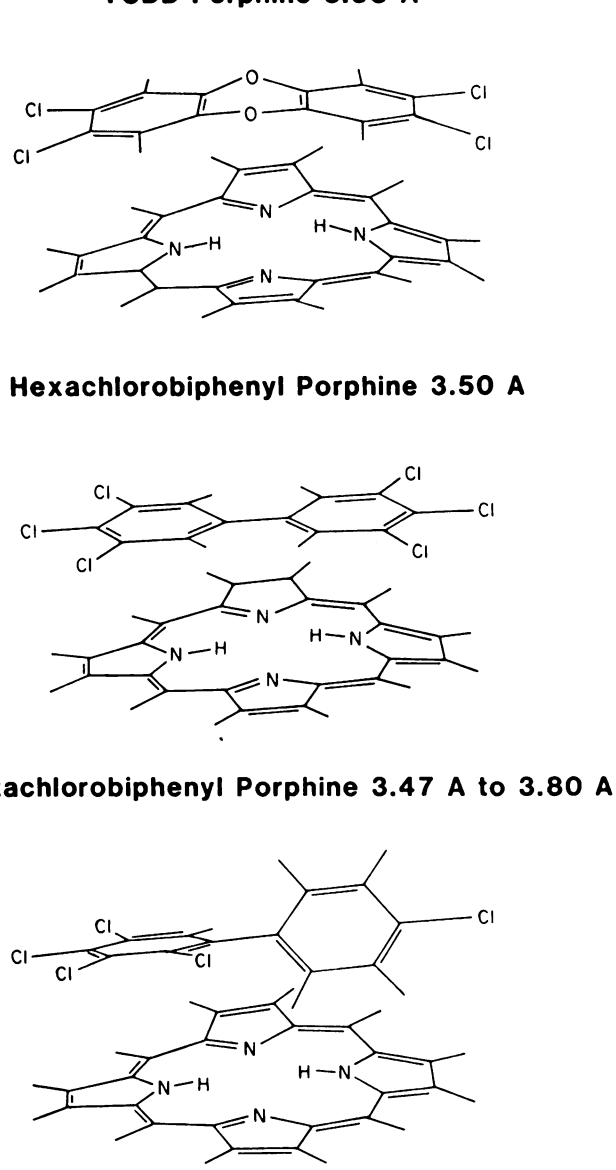

FIGURE 2. Computer graphics model from molecular mechanics minimization of porphine interactions with various chlorinated aromatic hydrocarbons with equilibrium separation distances in angstroms. Reprinted with permission (*31*).

observable (*34,35*). Of equal importance is the polarization of this face in a direction that will bring about a good donor-acceptor relationship as in a charge-transfer complex. The inductive and resonant effects of halogen substitution can effectively polarize the aromatic system. CAHs could be viewed overall as having electron-poor faces with areas of significant hole density along the edges, particularly in hydrogen substituted positions; conversely, possible donor aromatic compounds could be viewed as having electron-rich faces with areas of significant electron density along the edges, particularly in association with nitrogen and oxygen atoms. For example, a more favorable donor-acceptor interaction may account

in large part for the relatively much lower toxicity of the chlorinated naphthalenes as compared with the significantly more polarizable brominated naphthalenes (*14*). Directional fusing of phenyl rings as in polynuclear aromatic hydrocarbons can also effect similar polarization of the molecule (*36*).

The fundamental requirement for good binding activity is a conformationally restricted (somewhat rigid), sterically accessible and highly polarizable planar (aromatic) ring. The critical factors are apparently the direction and degree of polarizability necessary to facilitate efficient electron acceptance by the ring system (or electron acceptance by ring edges). This can be accomplished in smaller ring systems with the larger and more polarizable halogen atoms, i.e., bromine and iodine. These predictions are consistent with a range of experimental competitive binding studies involving various *Ah* receptor preparations and a variety of both nonhalogenated and halogenated aromatic hydrocarbons, some significantly smaller than PCBs (*13,37,38*). Other workers (*39*) have also stressed the importance of aromatic-aromatic interactions in the binding sites of cytochromes P-448 (to which the *Ah* receptor is related). Although experimentally, hydroxy-CAHs have relatively much lower binding affinities than their halogen substituted counterparts, the model would predict that this is due to large differences in aqueous desolvation energy (because of the potential for hydrogen bonding) rather than to differences in intrinsic binding properties (*29*). This technical problem would limit the range of ligands that could be studied in *in vitro* in experiments.

We have also examined the possibility that THs might also be able to bind by a stacking complexation mechanism (*19*). The potential of thyroid hormone analogs to form charge-transfer complexes involving the substituted phenolate ion and selected acceptors was recognized some time ago (*40*), but the physiological importance of such complexes remains unclear. In contrast, the potential of THs to have large electron affinities was also recognized early (*41*). Using methods based on nuclear magnetic resonance (NMR) spectroscopy, we showed that selected, structually distinct thyroid hormone analogs with widely different hormonal activities functioned as electron acceptors in molecular complexes with aromatic donors involving the nonphenolic or tyrosyl ring (*19*) of THs. Interestingly, relative binding free energies for these complexes correlated well with those previously reported (*42*) for the triiodothyronine (L-T_3) nuclear receptor binding interaction with the same compounds. As predicted by the *Ah* receptor modeling work, smaller ring systems that have a highly polarizable planar face can effectively participate in equilibrium stacking interactions.

Of particular interest to structure-activity similarities between CAHs and THs was the recognition that although iodine is not directly involved, it can facilitate binding interactions of a charge-transfer type in the tyrosyl rings of thyroid hormone analogs (*43*). The 3,5-iodines serve not only to sterically constrain (making it more rigid) the diphenyl ether thyroxine nucleus to facilitate a stacking interaction with the tyrosyl ring but also to increase the electron-acceptor strength of the ring through their polarization properties. Electron-accepting ability may also be further enhanced by the hydroquinone-type property of the adjoining ether-oxygen-phenolic ring system (*16*). Therefore, in diphenylether systems (such as the chlorinated diphenylethers) related to THs, it is reasonable to expect that the more biologically active compounds will also contain substitutions (in positions equivalent to the 3,5 positions in THs) that restrict conformational space through hindering rotation about the ether linkage. The toxic CAHs generally are more rigid structures that already have an entropy advantage in stacking-type binding interactions. In fact, an accessible and highly polarizable aromatic ring system characteristic of toxic CAHs is also characteristic of the tyrosyl ring of THs and represents the fundamental molecular element of importance in our NMR binding model (*19*) as well. Binding ligands for the T_3 nuclear receptor and the *Ah* receptor may share common molecular parameters in the expression of their binding activities. The tyrosyl ring could be envisioned as presenting a polarized surface to the receptor, which then adheres to the receptor somewhat like Velcro, analogous to the CAH interaction shown in Figure. 2.

Cleft Models

As polarizability is roughly proportional to the number of electrons, the stacking model can reasonably account for the number and kinds of halogen substituents in highly toxic structures. It cannot account for the preference for lateral substitution and rectangular shape. It was recognized (*20*) some time ago that the *o*-diiodophenolic structure of THs is very important in their binding interactions with serum transport proteins. Considerable knowledge has been gained of the high-resolution X-ray crystallographic structure of one of the transport proteins, thyroxine binding prealbumin (TBPA) or transthyretin. A well-developed picture at the molecular level of the binding interactions between hormone and TBPA (*44*) has also provided a model against which all other binding associations of the hormones and their analogs will be compared and contrasted. Studies of TBPA and the thyroid hormone nuclear receptor suggested the possibility of a closer relationship between the two proteins than would have been anticipated, which suggests that the studies on TBPA may have a direct bearing on the properties of the nuclear receptor (*45,46*).

Prealbumin from human plasma is a tetramer of identical subunits of 127 amino acid residues of known sequence (*47*) giving an M_r of the tetramer as 54 980. Aromatic pair amino acid interactions in TBPA that appear to play an important role in protein structure stabilization (*21*) and DNA interactions (*22,23*) may also facilitate the ability of these types of proteins to sequester CAHs. Recent work (*48*) has established that human, rat, and rabbit TBPA are more than 80% identical and all residues

in the binding site are conserved. The four identical subunits are oriented to form a central channel containing two identical T_4 binding sites. The molecule has 2-fold symmetry, and the binding site is lined primarily with hydrophobic amino acid side chains that form polarizable pockets for halogen interactions. The binding site matches the structure and chemistry of the hormone with great precision, which together with the fact that TBPA almost completely engulfs the hormone, probably accounts for the high association constant. Thus the hormore binding site in TBPA represents an almost idealized case of a molecular cleft with complementarity in size, shape, and functional groups for efficient molecular recognition (*24,25,49*). Competitive binding studies (*50,51*) using human TBPA and polar (soluble) derivatives of dioxin and related polychlorinated biphenyl compounds have shown that simple halogenated hydrocarbons that contain only one aromatic ring or linear structures with multiple rings bind more strongly than the normal angular diphenylether bridged system characteristic of thyroid hormone analogs. Furthermore, lateral chlorination was common to all compounds that showed high binding activity. The binding model in fact suggested a preference for a linear and symmetrical molecular shape like TCDD (Fig. 3*A*). These structural properties are also characteristics of highly toxic CAHs (*13-15*).

Computer graphics modeling of the ligand-TBPA complexes has been particularly useful in visualizing the importance of lateral substitution and a linear and symmetrical molecular shape. In view of the highly hydrophobic/polarizable nature of the binding site, it was anticipated that the van der Waals/hydrophobic interactions would be dominant in controlling the binding strength of these compounds. In this simple modeling (docking) exercise, a correspondence between the binding efficacy of each compound and the total number of contacts less than 4 Å between the compound and protein molecular surface was assumed. Figure 3*A* shows a close correspondence of the 2,3-lateral chlorine substituents in TCDD with the diiodophenolic moiety of thyroxine in the binding site. A similar fit is found for the 2,3-lateral chlorine substituents in TCDF or 3,5-lateral (*meta*) chlorine substituents in 3,3′,4,4′,5,5′-hexachlorobiphenyl (Fig. 3*B*), whereas the 2,2′,4,4′,6,6′-hexachlorobiphenyl lacking *meta*-substituents (Fig. 3*C*) shows a relatively poor fit. Outer ring (nearest to entrance of binding channel) lateral substitution is not critical for PCB binding to TBPA and may even lower binding affinity (*50*). Deviation from a linear shape as in the 1,4,6,9-substituted dioxin case (Fig. 3*D*) could lead to bad (repulsive) contacts due to the colliding or short contacts between molecular surfaces. A theoretical model based on molecular mechanics energy minimization procedures (*52*) was developed (*53*) for this binding interaction with TBPA which permits one to estimate the differential free energies of complex formation for compounds of this type for which it may be difficult or impossible to obtain experimental binding data. The theory correctly separates strong, intermediate, and nonbinders in cases where experimental values were known. In addition, the theory predicts that TCDD and TCDF would be strong binders and that octachlorodibenzodioxin, a 1,4,6,9-substituted dioxin, would not bind at all. These results also suggest that the assumptions made in our earlier molecular modeling exercises are reasonable and support the use of such models for preliminary screening purposes.

Model Predictions and Tests

No endogenous physiologic ligand for the *Ah* receptor has yet been clearly identified. The *Ah* receptor system enhances enzyme activities (*37*) that often are far more important in toxification than in detoxification, and thereby contributes to a process ostensibly contrary, rather than advangtageous, to life. The *Ah* receptor seems likely to have beneficial functions that outweigh its disadvantageous effects. The stacking models described above for CAHs suggests that the nonphenolic (tyrosyl) ring of THs appears to be suitable (somewhat rigid, sterically accessible aromatic ring that is polarizable) for undergoing a stacking interaction of the type described for the *Ah* receptor interaction (*19*). For technical reasons (*29*), it has not been possible to demonstrate directly that THs will compete with ^{3}H-TCDD using conventional *Ah* receptor binding assays. Under more physically favorable conditions based on the use of nonionic detergent micelles (*54*), the THs can compete with dioxin-type ligands specifically bound to sites in rat liver cytosol preparations. We have recently demonstrated (*38*) moderate specific binding affinity of diiodobenzenes (as models for THs) with *Ah* receptor preparations. In addition, both the *Ah* and T_3 receptors show a strong dependence of binding activity on hydrophobic properties of the binding ligands (*55,56*) and on dilution, temperature, and pH properties of the assay itself (*57,58*). Tissue distribution of the two receptors in the rat is also similar (*59,60*), and the relative levels of receptors in the various tissues are consistent with the known thyroid hormone responsiveness of these tissues (*59*). Finally, certain toxic effects of both THs and CAHs segregate with the *Ah* gene locus (for which the *Ah* receptor is the gene product) in cell culture systems (*61*). Thus, several lines of evidence are suggesting that the *Ah* receptor has properties in common with the nuclear triiodothyronine (T_3) receptor.

Because of the structural similarities between certain CAHs and THs and the consistent finding of serum T_4 reduction in toxicity studies (*4-7,62*) with dioxin and related compounds, our results with the serum binding protein TBPA are not surprising. In addition to the modeling aspects of the TBPA interactions presented above, it is likely that interactions of CAHs with thyroid hormone serum-binding proteins play a direct role in mediating certain toxic effects of dioxin and related compounds, e.g., in mediating vitamin A depletion in tissues (*63,64*). Certain CAHs may interfere with a major function of TBPA in facilitating uniform distribution of T_4 among all cells within each tissue (*65*). Previous workers (*66*) have also provided evidence for a structural similar-

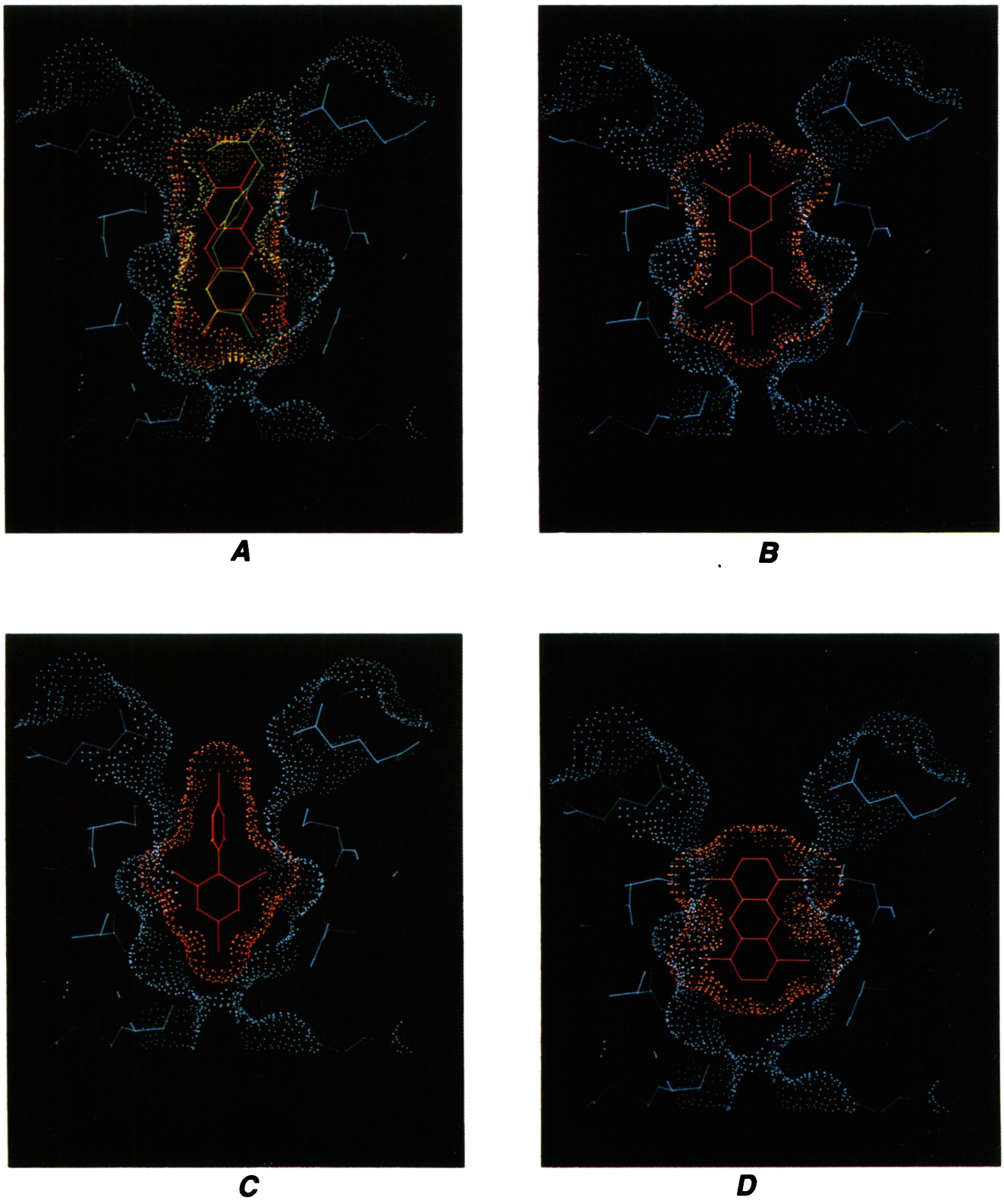

FIGURE 3. Computer graphic models of ligand-TBPA binding pocket complexes. Pictures show cross-section of solvent-accessible molecular surfaces of the model-built binding site (view is perpendicular to plane of inner ring). The TBPA surface is blue. (*A*) Superposition structure of T_4 (green) from X-ray and TCDD or dioxin (red) from docking; (*B*) docked 3,3,',4,4', 5,5'-hexachlorobiphenyl; (*C*) docked 2,2',4,4',6,6'-hexachlorobiphenyl; (*D*) docked 1,4,6,9-tetrachlorodibenzo-*p*-dioxin.

ity between the binding sites of prealbumin and thyroxine-5′-deiodinase found in tissues. The structure-activity relationship for 11 iodothyronine analogues acting as inhibitors for rat liver deiodinase activity was statistically correlated with their binding affinities to human TBPA. Similarly, recent preliminary work (*67*) in our laboratory has demonstrated potent inhibition of deiodinase activity in rat liver microsomal fractions by certain CAHs in the dioxin and PCB series. This inhibition depended on the presence of lateral chlorination consistent with our competitive binding studies using TBPA. Interestingly, flavone natural products derived from plant pigments are also inhibitors of deiodinase activity (*11*), as well as binding ligands for the *Ah* receptor (*37*).

As pointed out earlier, TBPA and at least one thyroid hormone nuclear receptor have a considerable number of their properties in common or have aspects of similarity (*44,45*). These similarities include similar molecular masses, similar overall chemistry of the thyroid hormone binding sites, similar binding affinities for the hormones and analogs, and both may be DNA binding proteins. Families of proteins do exist in which, within a single broad function, individual members are subtly or grossly modified to fulfill specific functions, and the identification and analysis of such families is of much importance in understanding biological processes. In this regard, previous workers (*68*) have provided experimental evidence for a prealbuminlike receptor that performs a gene-activating function in cell cytosol sensitive to thyroid hormones. These workers found that the specific cytosol protein acquired the necessary properties for interacting with certain chromatin DNA sites after binding thyroid hormone. Binding of the hormone-receptor complex with the DNA sites resulted in the initiation of gene transcription.

In our laboratory (*69*), we have demonstrated that prealbuminlike thyroxine-specific binding sites in rat liver nuclear extracts have the expected structural specificity and sensitivity for possible involvement in the high toxicity of CAHs. In addition to the requirement for lateral chlorination for high binding activity, the nuclear receptor showed a remarkably enhanced affinity for laterally substituted compounds that were also planar (or had energetically favorable coplanar conformations) and highly polarizable. For example, 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl showed a remarkably high affinity ($K_a = 5.86 \times 10^{10} M^{-1}$) while the isomeric, but *diortho*-substituted compound, 2,3,5,6-tetrachloro-4,4′-dihydroxybiphenyl, that cannot achieve coplanarity, showed an affinity about 22 times weaker. The rigid coplanar structure 3,3′,5,5′-tetrachlorodiphenoquinone (reasonably isosteric with TCDD) showed an affinity about 3.2 times stronger. As in our previous work with TBPA (*50,51*) the use of polar derivatives was technically necessary in order to achieve sufficient solubility in the assay medium and reduce nonspecific binding interactions probably involving lipid-rich sites. Therefore, a nuclear thyroxine receptor appears to have properties in common with both TBPA and the *Ah* receptor, which is also compatible with its possible role in mediating some of the toxic effects of these compounds.

Possible Biological Significance—Multifunctional Ligand-Receptor Complex

Molecular modeling studies have revealed that CAHs and THs have similar structural properties that could be important for molecular recognition in biochemical systems. These molecular properties include a somewhat rigid, sterically accessible aromatic ring that is polarizable and size-limited, hydrophobic lateral substituents, usually contained in opposite adjoining rings of a diphenyl compound (but may be found in both rings). These properties are most often highly expressed (in the case of CAHs) in fairly rigid, rectangular-shaped molecules like TCDD. Molecular size can vary as long as there is sufficient expression of these fundamental molecular properties to effect significant binding interactions. Similar molecular reactivities are supported by the demonstration of effective specific binding of both types of structures (CAHs and THs) with four different proteins, enzymes, or receptor preparations). Three of these systems are known thyroid hormone-binding proteins. This observation, along with other evidence presented, raises the possibility that the *Ah* receptor may also be a thyroid hormone binding protein. The structural relatedness of TCDD to THs has recently been suggested (*70*) in support of the likelihood that the *Ah* receptor will ultimately be part of the hormone-responsive transcription factor super-family.

The overall structural requirements revealed in these modeling and binding studies are compatible with the known structure-toxicity relationships for the CAHs. The question remains as to whether these distinct molecular recognition properties for activity are associated with multiple binding sites in different acceptor proteins or in the same protein. In either case, multi-functional binding or a multiple sequential step protein-protein interaction mechanism involving different structural components of the molecule offers an attractive hypothesis and would build in considerable structural specificity for the control process. A similar mechanism of metabolite transfer by enzyme-enzyme complex formation has already been proposed (*71*). As a working model (Fig. 4), we have suggested (*62*) that a cytosol acceptor protein may initially complex with thyroid hormone through a stacking interaction with the tyrosyl ring. This complex in turn is translocated to the nucleus where the lateral substituents on the phenolic ring serve to probe the chromatin surface for nuclear acceptor sites that are complementary with the cytosol acceptor complex and permit protein-protein interactions to occur (Fig. 5*A*). Thus, the thyroid hormone structure can be viewed as providing a steering mechanism for assembling the protein-protein-DNA complex that may be involved in initiating gene expression. This model is also compatible with recent independent work (*72*) that suggests that protein dimers involving two different proteins may be the entity that interacts with DNA and regulates gene expression. This receptor complex model for THs is similar to that proposed by others (*16*) based primarily on structure-activity considerations. TCDD can similarly fulfill this role (Fig. 6) with the dif-

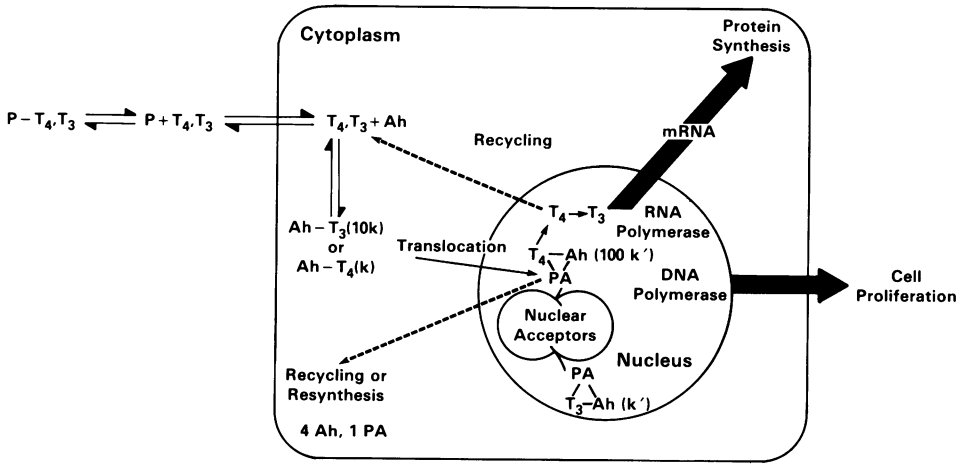

FIGURE 4. Mechanistic model for thyroid hormone action (T_3/T_4) based on cooperative protein (*Ah*/PA) binding in cytoplasm and nucleus of cell.

ference being perpendicular stacking or edge-on alignment with the cytosol acceptor site (Fig. 5*B*) as opposed to parallel stacking as in the case of thyroid hormone. The somewhat rigid planar structure in TCDD juxtaposes the important binding groups in complexation. The alignment shown in Figure 5*B* suggests that the major points of contact between the TCDD structure and the putative aromatic donor face in the cytosol acceptor site are the hydrogen atom and lateral carbon edges. This is compatible with previous theoretical work (*33*) investigating the electronic factors affecting receptor binding of dioxins to the *Ah* receptor.

The most significant relationship with observed activity was with a lowest unoccupied molecular orbital having significant areas of hole density about the hydrogen atom and lateral carbon edges. TCDD has four equivalent edges of this type and additional chlorine substitution would reduce this number. This is consistent with the reduced receptor binding and toxicity observed for the more highly chlorinated 2,3,7,8-substituted dioxins. Although the specific details of such interactions involving TCDD and related compounds are not known, there are clearly several possible modes of aromatic-aromatic interaction that could lead to favorable stabilization energies.

As discussed previously, the cytosol acceptor protein may be the *Ah* receptor, which may be a thyroid hormone binding protein. In this capacity, the *Ah* receptor can play a modulating role, perhaps in controlling access to more critical receptor sites in the nucleus of cells. Such a gating mechanism for the *Ah* receptor in modulating cellular biologic and/or toxic responses to CAHs would be consistent with the many studies supporting an essential (but not necessarily sufficient) role for *Ah* receptor binding in the mechanism of toxicity. Other indirect evidence that supports multiple protein involvement in the mechanism of toxicity of CAHs include the observations that structurally related compounds can produce both synergistic (*14*) and antagonistic (*73*) effects on biological/toxic responses of these compounds, a combination and specific ratio of thyroid hormones T_3 and T_4 are required to mimic certain toxic responses of dioxin (*61*), and no single protein binding model can account for the structure-toxicity

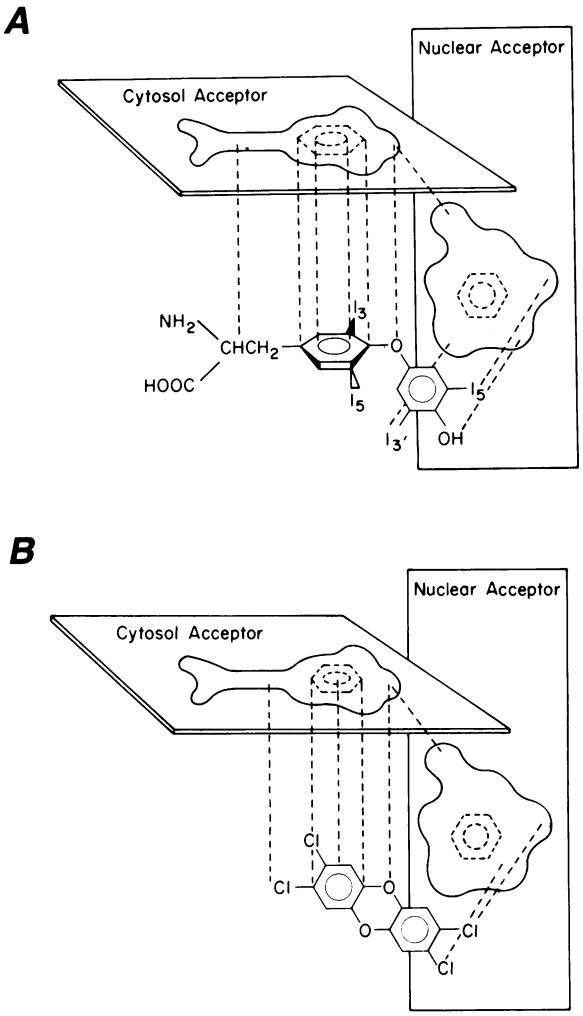

FIGURE 5. Ligand-receptor model showing multifunctional binding of (*A*) T_4 or (*B*) TCDD by cytosol (stacking) and nuclear (cleft interaction) protein binding sites facilitating protein-protein interactions.

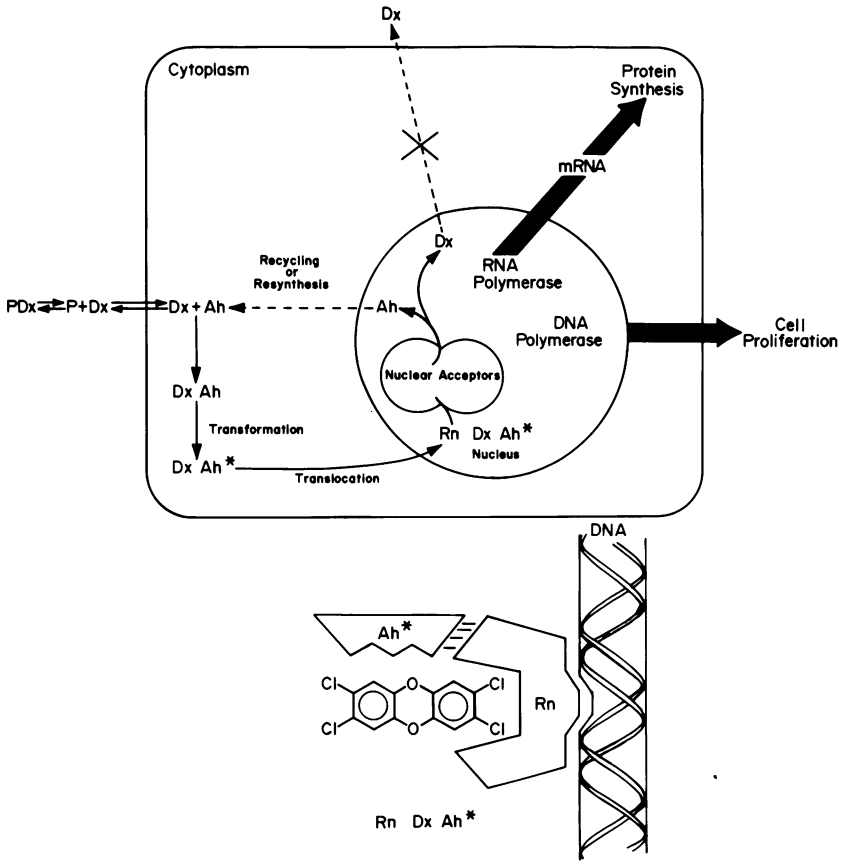

FIGURE 6. Mechanistic model for dioxin (Dx) toxicity showing multiple protein binding with *Ah* receptor and prealbuminlike, DNA-binding nuclear receptor (R_n) Reprinted with permission (*62*).

relationship for CAHs without invoking multiple or multifunctional protein binding of ligands that are both planar (or coplanar as in PCBs) and have lateral halogen substituents (*69,74*). Independent studies (*75,76*) of the thyroid hormone nuclear receptor are suggesting that it may also be a multimeric species complexed with DNA. In addition, it has been suggested (*77*) that *Ah* receptor function requires the contribution of (at least) two genes. One interpretation of these latter studies is that one gene encodes for one functional unit of the receptor complex, perhaps the cytosol acceptor, and a second gene encodes for another binding unit, perhaps nuclear in origin. The *Ah* receptor may be neither entirely cytosolic nor entirely nuclear but exists in equilibrium between the two compartments.

The molecular modeling studies with THs and CAHs better portray CAHs as T_4 agonist/antagonist rather than T_3 agonist/antagonist, especially with regard to the correspondence of lateral substituents. This presents two problems within the framework of current thinking about the mechanisms of thyroid hormone action and our multifunctional binding model. First, it is generally accepted that the active thyroid hormone is T_3, which is derived from T_4 from metabolism in tissue. However, there are several lines of evidence (*78*) which suggest that T_4 has intrinsic hormonal activity. In other words, T_3 is considered to be the major contributor to the hormonal potency of THs, but T_4 also contributes directly other than by first undergoing metabolic conversion to T_3.

But the multifunctional mechanism model offers an alternative explanation for this apparent discrepancy. It is known that the cellular ratio of T_3 to T_4 is about 4 to 1 (*79*). This ratio may be important in maintaining the concentration gradient between the cytoplasm and the nucleus in order to maximize nuclear receptor occupancy by T_4 and the associated biological response. This can result from the apparent higher capacity and affinity of cytosolic sites for T_3 but greater overall affinity (net gain of binding free energy over that for T_3) of T_4 to the cytosol-nuclear acceptor complex in the nucleus. Given externally T_3 would always appear to be more active because it is more efficient at displacing endogenous T_4 toward the nucleus (Fig. 4). T_3 through its displacing action on T_4 in cytosol is in effect potentiating the effects of T_4 in the nucleus. If TCDD is a structural surrogate for T_4 as suggested in this work, then T_3 should also potentiate the effects of TCDD as has been shown (*61*). Thus metabolism of T_4 to T_3 in tissues coupled with the concentration gradient between cytoplasm and nucleus can provide a means for regulating nuclear receptor occupancy. This brings into question the nuclear versus cytosolic origin of the T_3 receptor. In fact, recent studies (*80*) with thyroid hormone analogs are now suggesting an extranuclear site of initiation of thyroid hormone effects.

The importance of T_3 relative to T_4 in thyroid hormone action is largely attributed to its greater hormonal

potency when studied *in vivo* and its greater binding affinity to the nuclear thyroid hormone receptor in *in vitro* studies. As suggested above, the greater hormonal potency is subject to a different interpretation in the multifunctional model and may reflect the greater efficiency of T_3 (as a metabolite) in displacing cytosolic T_4 toward the nucleus. The interpretation of *in vitro* binding studies involving crude receptor preparations can also be questioned (*81*). For example, depending on experimental conditions, it may not be possible to distinguish between the binding to specific receptor sites (that may be multimeric in nature) and nonspecific binding in which acceptor or recognition sites (such as the disassociated units of the multimeric receptor complex) are involved. Consistent with this interpretation, cross-competition studies (*82*) involving ^{125}I-T_3, ^{125}I-T_4, and ^{125}I-rT_3 (reverse T_3 or 3,3′,5′-triiodothyronine) show different structural specificities indicating different binding sites in the nuclear preparations. If multifunctional binding of T_4 (Fig. 5*A*) is required for agonist activity, then rT_3 would be expected to function as a partial agonist-antagonist. This result would be expected since removal of one iodine atom from the tyrosyl ring would decrease its polarizability and simultaneously lower its rigidity both factors thus weakening (relative to T_4) any potential stacking interaction with the cytosol acceptor site while having little or no effect on the phenolic ring interaction with the nuclear site (*69*). Preferential metabolism of T_4 to rT_3 (instead of T_3) would then provide a very effective means for regulating thyroid hormone activity since it reduces the concentration of both T_4 and T_3 available to receptors while simultaneously generating a competitive antagonist. Consistent with this expected result is the recent finding (*83*) that rT_3 can antagonize thyroid hormone responses at almost equimolar (physiological) concentrations in fetal mouse liver. Similar structural arguments based on the model can be made to explain the ability of certain CAHs (*61,73*) to antagonize dioxin (or thyroid hormone) toxic effects.

At least one of the nuclear sites may correspond to the c-*erb*-A protein that has recently been identified (*84,85*) as a high affinity thyroid hormone nuclear receptor. These studies provide the first direct evidence of a causative involvement of enhancers and their binding proteins in oncogenic transformation. The c-*erb*-A protein is similar in molecular mass to the nuclear-specific binding protein in rat liver that we have shown (*69*) specifically binds both THs and CAHs isosteric with TCDD. Futhermore, workers (*86*) have recently provided evidence that TCDD causes increases in expression of c-*erb*-A and levels of protein-tyrosine kinases in selected tissues of responsive mouse strains. It has also been demonstrated (*87*) that THs can play a critical role in induction of neoplastic transformation by chemical carcinogens in tissue culture. It is perhaps not surprising in this overall context that TCDD can act as a tumor promotor (*88*). TCDD may mimic the dramatic effects of TH in possibly regulating transcriptional activity of one or more protooncogenes (*89*) (such as the *Ah* receptor) during exposure to carcinogen.

The second apparent problem with the multifunctional model is the fact that the tyrosyl ring of THs must maintain a perpendicular relationship to the plane of the TCDD molecule, for example, if the close correspondence of the lateral substituents is to be met (Fig. 7*A*). As was stated earlier, both perpendicular and parallel stacking interactions involving aromatic rings are energetically favorable processes and with other things being equal (or nearly equal) this does not represent a major structural difference from a molecular reactivity and recognition point of view. In fact, the conditions are favorable for perpendicular edge-to-edge or edge-to-face interactions to occur with TCDD and related compounds (*21-23*). It is possible that initial binding to the cytosol acceptor unit may involve parallel stacking (Fig. 2) and on forming the cytosolnuclear acceptor complex (Fig. 5*B*) perpendicular stacking or edge-on alignment is necessary to facilitate interaction with the nuclear acceptor unit. In this sequential step mechanism, a planar (or coplanar) structure that can undergo both parallel and perpendicular stacking interactions would take on added significance in determining binding affinity. PCBs that can assume a coplanar structure (*30*) can behave similarly (to TCDD) but somewhat lower binding affinity is consistent with a small energy cost in overcoming the small rotational barrier to coplanarity. In the multifunctional binding model (Fig. 5*B*), the conformationally restricted, noncoplanar (*30,32*) *ortho*-substituted PCBs would experience more difficulty (in stereoelectronic and energetic terms) in accommodating simultaneously the two different types of molecular recognition processes (Fig. 7*B*).

It has also been suggested (*90*) that certain chlorinated diphenyl ether compounds may produce some of their toxic effects through their thyromimetic properties as predicted (*62*) from our modeling studies. Active structures in addition to being conformationally restricted (contain *ortho*-substitutions in relation to the ether oxygen bridge) would also contain lateral substitutions and assume conformational structures similar to that illustrated for THs (Fig. 5*A*).

Summary

In summary, a multifunctional receptor complex offers a way of visualizing the details and possible role of both the stacking and cleft type molecular recognition factors in biological activity and provides a sense of direction for the design and conduct of further biological experimentation on the mechanism(s) of action of CAHs and THs. The multifunctional ligand-receptor model concept should also be considered in the study of structure-activity relationships and mechanisms of action for other hormonal systems such as the steroid hormones. It may be of general importance in maintaining highly structurally specific control of hormone action at effector-linked sites. Hormonal synergists and antagonists may reflect differential involvement of the various multifunctional binding groups in the receptor system.

Certain toxic CAHs possibly as a result of their high

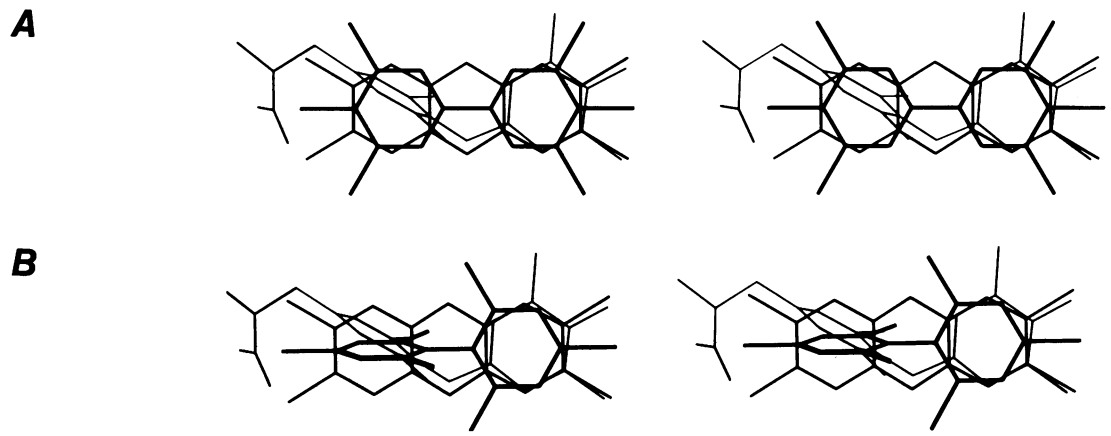

FIGURE 7. Stereopair superposition of (*A*) 3,3',4,4',5,5'-HCB (heavy line) and T_4 (light line) along with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, medium line) showing close correspondence of lateral substituents and perpendicular relationship of other phenyl rings and (*B*) 2,2',4,4',6,6'-HCB (heavy line) and T_4 (light line) and TCDD (medium line) showing lack of correspondence of lateral substituents and perpendicular relationship of phenyl rings in the HCB and inaccessibility to stacking interactions. Reprinted with permission (*50*).

affinities to both T_3 and T_4 receptors and resistance to metabolism are able to override normal control mechanisms for the expression of thyroid hormone activity. Toxic CAHs may act as potent and persistent thyroid hormone nuclear receptor agonists and produce some of their effects by altering gene expression in susceptible cells. There are a number of well-documented examples of the regulation of single and multiple clusters of genes through multiple hormonal interactions (*91*). Thyroid hormones are involved in the ultimate expression of the target genes in several of these multihormonal regulating systems. The thyroid hormone agonist property would be compatible with the ability of CAHs to alter receptor coupling or the receptor number for diverse hormones (*92*). This is supported by recent studies suggesting that THs in combination with CAHs can produce synergistic effects (*63,93*). These effects would resemble those seen in hyperthyroidism. Other nonreceptor effects of these compounds which may precede the effects on gene expression could be associated with their thyroid hormone antagonist properties, directly, e.g., by interactions with certain serum binding proteins (*94*) and metabolizing enzymes (*67*), or indirectly, e.g., by reducing the availability of T_4 as an amino acid analog. These latter effects should be expected to resemble those seen in hypothyroidism. Previous studies (*6,95*) attempting to assess the functional thyroid status of dioxin treated rats do not consider the potential complex interplay of the thyroid hormone agonist and antagonist effects of dioxin. The overall pattern of thyroid-hormone modulated responses seen in dioxin-treated animals such as effects on hepatic enzyme activities (*95*) are characteristic of effects seen in both primary hyper- and hypothyroidism.

Dioxin toxicity should not be expected to completely resemble the effects produced in experimentally induced hyper- or hypothyroidism alone. Thyroid hormone agonist and antagonist properties of CAHs are compatible with the overall toxic/biologic effects of these compounds which are in many respects characteristic of both the hyper- and hypothyroid disease states. Further biological verification of the multifunctional receptor complex and mechanistic hypothesis for CAH toxicity is necessary.

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