The Mechanism of Dioxin Toxicity: Relationship to Risk Assessment

Linda S. Birnbaum

U.S. Environmental Protection Agency, Health Effects Research Laboratory, Research Triangle Park, North Carolina

Risk characterization involves hazard identification, determination of dose-response relationships, and exposure assessment. Improvement of the risk assessment process requires inclusion of the best available science. Recent findings in the area of dioxin toxicity have led to a major effort to reassess its risk. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), commonly referred to as "dioxin," is the most toxic member of a class of related chemicals including the polyhalogenated dibenzo-p-dioxins, dibenzofurans, biphenyls, naphthalenes, azo- and azoxy-benzenes, whose toxicities can be expressed as fractional equivalencies of TCDD. These chemicals exert their effects through interaction with a specific intracellular protein, the Ah receptor. While binding to the receptor is necessary, it is not sufficient to bring about a chain of events leading to various responses including enzyme induction, immunotoxicity, reproductive and endocrine effects, developmental toxicity, chloracne, tumor promotion, etc. Some of these responses appear to be linear at low doses. Immunotoxicity and effects on the reproductive system appear to be among the most sensitive responses. The Ah receptor functions as a transcriptional enhancer, interacting with a number of other regulatory proteins (heat shock proteins, kinases, translocases, DNA binding species). Interaction with specific base sequences in the DNA appear to be modulated by the presence of other growth factors, hormones, and their receptors as well as other regulatory proteins. Thus, dioxin appears to function as a hormone, initiating a cascade of events that is dependent upon the environment of each cell and tissue. While Ah receptor variants exist, all vertebrates examined have demonstrated such a protein with similar numbers of receptors and binding affinity for TCDD. Most species respond similarly to dioxin and related compounds. While a given species may be an outlier for a given response, it will behave like other animals for other responses. For both in vivo and in vitro end points where animal and human data exist, such as enzyme induction, chloracne, immunotoxicity, developmental toxicity, and cancer, the sensitivity of humans appears similar to that of experimental animals. Current levels of environmental exposure to this class of chemicals may be resulting in subtle responses in populations at special risk such as subsistence fisherman and the developing infant, as well as in the general population. Increased understanding of the mechanism of dioxin's effects as well as elucidation of exposure-dose relationships is leading to the development of a biologically based dose-response model in the ongoing process of incorporating the best science into the risk assessment of TCDD and related compounds.— Environ Health Perspect 102(Suppl 9)157-167(1994)

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Introduction

Risk assessment is a scientific process that can be divided into several stages. These phases involve exposure assessment, hazard identification, and elucidation of dose-response relationships. Integration of these various activities results in scientific risk characterization. These assessments can then be put in the context of economic considerations, societal benefits, policy considerations, etc., when formulating a risk management decision. Thus, while risk assessment is an integral component of risk management, this scientific exercise is only part of the decision-making process.

Improvement of the risk assessment process requires the incorporation of the best and most current scientific thinking. In place of default positions which make certain assumptions, dosimetric and mechanistic information can be incorporated to reduce the uncertainties involved in risk characterization.

Recent findings in the field of dioxin toxicity have led to a major effort to improve the assessment of its risk. This was in response to a meeting of international experts held under the auspices of the Banbury Center (1) in the fall of 1990. While this was not a consensus conference, general agreement was reached on several issues: a) as far as is known at this time, all dioxin effects are mediated through the Ah receptor; b) people have sensitivity similar to animals to dioxin effects and c) compounds which are related in structure to dioxin and have the same mechanism of action need to be considered as part of the dioxin problem. Therefore, having a better understanding of the hazard and the mechanism of dioxin effects, a more biologically based risk assessment should be achievable.

There was not general agreement on what a "safe" level of dioxin would be, although there was a hypothesis suggested that if a threshold for receptor activation existed, then there would be an exposure level at which no responses could occur. Research needs were identified to address this crucial question.

The focus of this manuscript is to address the issue of the mechanism of dioxin toxicity and how this information can improve the risk assessment process.

Background

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the most toxic member of a class of planar, halogenated chemicals (Figure 1). It has no known industrial or commercial use and has been produced as an unwanted byproduct of certain industrial processes and combustion. TCDD has been produced during the production of certain chlorinated phenols and their derivatives, and as a result of high temperature pyrolysis and combustion of organic compounds containing halogens. Chlorine bleaching of paper pulp has also led to the
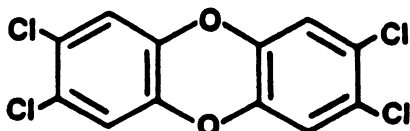
Figure 1. Structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin).

production of dioxin in paper products. Dioxin is extremely stable, both to environmental and biological breakdown, leading to its persistence in the environment and its bioaccumulation in the food chain. Because of its high lipophilicity and water insolubility, dioxin concentrates in sediments and is incorporated into the fatty tissue of fish, birds, reptiles, and mammals. Much of its presence in plants is due to atmospheric transport on particles, resulting in settling on the leafy tissues of plants. Dioxin can also be found in consumer products such as chlorinated herbicides, chlorinated phenol-containing products, and contaminated paper goods (*2*).

Biological Responses to TCDD

Dioxin causes a broad range of effects, some of which are species specific (*3–5*). Dioxin is often described as the most toxic manmade chemical because of the low doses which cause lethality in certain animal species such as the guinea pig. Dioxin causes delayed lethality, the time to death being dependent on the species in question, not on the doses (*6*). For example, the time to death varies from 1 to 2 weeks in the guinea pig, 2 to 3 weeks in the rat, 3 to 4 weeks in the mouse, and 6 to 8 weeks in the monkey. Death is usually preceded by a severe loss of body mass, called the wasting syndrome. Laboratory animals often lose from one third to one half of their body weight prior to death. This process is noticeable within days of a lethal dose. Nonlethal, but highly toxic, doses may also result in severe wasting (*7*). Atrophy of the lymphoid tissues, such as the thymus and spleen, and of the testes occurs at acutely toxic doses in adult animals.

Hyperplastic and/or metaplastic changes are also characteristic of certain epithelial tissues. The liver is a dioxin target organ in many species (*8*). Increase in liver size can occur at relatively low doses, reflecting not only enzyme induction but also changes in lipid content. Necrosis and fatty changes occur at higher doses. In the guinea pig, effects on the liver can be observed at the ultrastructural level (*9*). No effects on liver function have been observed in highly exposed humans (*10*).

Hyperplasia has also been reported in the gastric mucosa, and the bile duct and urinary bladder epithelia. Squamous metaplasia occurs in the Meibomian glands of the eyelid, resulting in blepharitis, and in the ceruminous glands of the ears, leading in both cases to waxy exudates.

Chloracne has been called the "hallmark of dioxin toxicity" (*11*). This is a severe form of cystic acne involving both hyperplastic and hyperkeratotic changes in the skin, as well as altered pigmentation. Chloracne occurs following either dermal or systemic exposure in sensitive species, which include man, monkeys, hairless mice, and rabbits. The condition is extremely persistent, in some cases lasting over 30 years following the initial exposure. Dioxin and related compounds cause a generalized ectodermal dysplasia (*12*), resulting in alterations in the teeth and nails in both humans and monkeys, as well as effects on the nails of hairless mice (*13*). Chloracne is a relatively high-dose response to dioxin, occurring in mice and monkeys at doses where effects such as thymic atrophy and some wasting are noted. In humans, it is a reliable indicator of heavy dioxin exposure (*14*).

TCDD is a developmental toxin in all species examined. However, it appears to induce major structural abnormalities following prenatal exposure only in the mouse (*15*) where it causes hydronephrosis and cleft palate at doses which are not fetally or maternally toxic. This characteristic syndrome has been used to categorize chemicals as to whether or not they are dioxinlike. Prenatal exposure of the developing mouse fetuses also has effects on the developing immune system, leading to altered differentiation of lymphocytes (*16*). Recent studies by Peterson and coworkers (*17–19*) have demonstrated that *in utero* exposure to the developing male rat pup leads to persistent demasculinization and feminization. Embryo/fetal toxicity occurs at similar maternal doses in the guinea pig, rat, and hamster (*20*).

Dioxin is highly immunotoxic in the mouse (*21*). One of the most sensitive responses is the suppression of the primary antibody response, an integrated response requiring the combined action of B-cells, T-cells, and macrophages. In addition, dioxin appears to compromise the host defenses of the mouse as shown by enhanced sensitivity to influenza virus (*22*), and mutes the response to trichinella (*148*). *In vitro* studies have suggested that mouse, monkey, and human lymphocytes are responsive to dioxin effects. Recent

studies have demonstrated, however, that the rat is relatively resistant to the immunosuppressive effects of TCDD, with doses that cause mild thymic atrophy actually resulting in enhancement of the primary antibody response (R Smialowicz, personal communication). Similar doses result in enhanced sensitivity in the rat to influenza virus (G Burleson, personal communication).

The carcinogenicity of TCDD has been examined in 17 studies in laboratory animals (*23*). All of these studies demonstrated that dioxin is a positive animal carcinogen in the rat, mouse, and hamster. It causes tumors at multiple sites in both sexes. In addition, recent studies with fish have demonstrated that dioxin is a multisite, multisex carcinogen in Medaka (*24*). While a number of inconclusive epidemiological studies have been conducted (*25*), three recent mortality studies (*26–28*) involving occupational exposure, validated by serum TCDD levels in a subset of the exposed cohort, have demonstrated an increased risk for all cancers after dioxin exposure.

Table 1. Biochemical effects of TCDD.

A. Enzyme Induction→Altered metabolism
  CYP1A1 (*3*)
  CYP1A2 (*3*)
  DT-diaphorase (*3*)
  UDP-glucuronyltransferase (*3*)
  Glutathione-*S*-transferase (*3*)
  Aldehyde dehydrogenase (*3*)
  Ornithine decarboxylase (*3*)
  Tyrosine kinase (*130,131*)
  Terminal deoxynucleotidyltransferase (*16*)
  Phosphoenolpyruvate carboxykinase (*132*)
  Plasminogen activator inhibitor-2 (*68*)

B. Modulation of hormones and receptors
  →Altered homeostasis
  Androgens (*88,133,87*)
  Estrogens (*3*)
  Estrogen receptor (*134,135,73*)
  Glucocorticoids (*3,136*)
  Glucocorticoid receptor (*73*)
  Insulin (*137,138*)
  Gastrin (*139*)
  Thyroid hormones (*140,141*)
  Melatonin (*142*)

C. Modulation of growth factors and receptors
  →altered growth and differentiation
  Vitamin A (*3*)
  EGF (*143*)
  TGFα (*31,143,144,30*)
  EGF receptor (*91,29,31*)
  TGFβ (*31,143,144*)
  TNFα (*145*)
  IL1β (*68*)
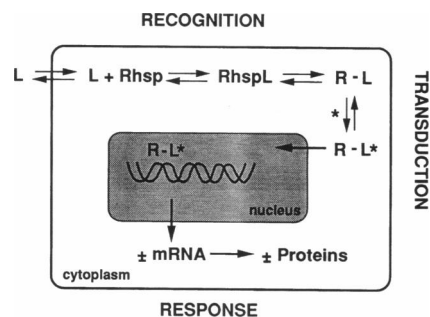 c-Ras (*146*)
  c-ErbA (*147*)

RECOGNITION

Figure 2. Cartoon for the mechanism of action of TCDD and related chemicals. Abbreviations: L, ligand; R, receptor; hsp, heat shock protein; *, activation.

The biochemical effects of TCDD may be difficult to classify as toxic or adverse responses, but they clearly represent a molecular and/or cellular response to that chemical. These effects can be grouped into three classes (Table 1): *a*) altered metabolism resulting from changes in enzyme levels; *b*) altered homeostasis resulting from changes in hormones and their receptors; and *c*) altered growth and differentiation resulting from changes in growth factors and their receptors. Not all of these effects occur in all species, and many are tissue specific. The mechanism is also not understood for many of these effects. However, changes in the drug-metabolizing enzymes involve transcriptional control. Some of the effects on hormone levels such as estrogen and thyroid hormones may involve increased metabolism of these hormones as a result of the induction of the drug-metabolizing enzymes. In contrast, the basis for the changes in the receptors for both the hormones and growth factors is not understood. The tissue and stage specificity of these effects must be emphasized. For example, while dioxin results in a decrease in the level of hepatic EGF receptor (*29*), and an increase in its ligand TGFα in human keratinocytes (*30*), the inverse is true in the developing palate (*31*). No change has been noted in the EGF receptor in hairless mouse skin undergoing a chloracenergic response (*32*).

Mechanism of Action

How does dioxin cause its biological effects? There is general agreement that all the effects of TCDD are mediated through the action of a cellular protein known as the *Ah* receptor (*33–36*). This is a high-affinity binding protein, present in low numbers per cell. It has been found in most tissues, although the number varies (*37,38*). The binding affinity appears to be similar for a large number of species, including humans (*39*). The general

scheme for the action of the *Ah* receptor is shown in Figure 2. This cartoon is analogous to that developed for several of the steroid receptors, and it has been hypothesized that the *Ah* receptor may belong to the steroid receptor superfamily (*40–42*). However, recent cloning of the *Ah* receptor (*43,44*) has revealed no sequence homology between the ligand binding subunit of the *Ah* receptor and the steroid receptor superfamily. These two groups have found that the *Ah* receptor has a basic helix-loop-helix domain that allows interaction with DNA, and, as will be discussed, interacts with DNA in the form of a heterodimer with another basic helix-loop-helix protein (*45*).

Like any kind of hormone which acts as a second messenger in the cell, TCDD action can be thought of as involving three separate steps: *a*) recognition of the signal; *b*) transduction of the signal; and *c*) response. The first step of signal recognition involves binding of the ligand, TCDD, or a related compound, to the *Ah* receptor. This interaction is highly specific; detailed structure/activity relationships have been developed for this interaction, which appears to involve not only the necessity of lateral halogenation and polarizability, but planarity and stacking interactions as well (*34,46,47*). Recent studies have shown that the form of the ligand binding subunit of the *Ah* receptor that binds to TCDD is not an isolated peptide, but part of a multimeric complex. Based on results using immunoprecipitation of the complex with either antibodies to the ligand binding subunit (*48*) or to HSP90 (*49,50*), it has been suggested that two molecules of HSP90 are involved in this ligand-binding complex. Recent studies by Perdew (*51*) have indicated that the cytosolic form of the receptor is a tetramer involving two molecules of HSP90, the ligand binding subunit, and a molecule of p50. However, the presence of two *hsp90* molecules it still tentative since recent studies using high stringency immunoprecipitation of the ligand binding subunit with monoclonal antibodies fails to bring down the stress proteins (*52*). Nevertheless, it is clear that binding of the ligand to the *Ah* receptor involves a multimeric protein complex.

Once TCDD is bound to the receptor, the other proteins dissociate. It is not clear whether this occurs prior to nuclear translocation. Perdew (*53*) has shown that the tetrameric species can be found both in the cytosol and in the nucleus. Other investigators have demonstrated that the physical behavior of the ligand-bound

receptor is different in the cytosol and the nucleus (*54*). The predominant nuclear form of the receptor appears to be a heterodimer. Using wild-type and mutant mouse hepatoma lines, Hankinson and co-workers (*55*) had demonstrated that translocation of the ligand-binding subunit into the nucleus required interaction with a protein called "arnt" (aryl hydrocarbon receptor nuclear translocating protein). More recent work by this group (*45*) has shown that the arnt protein actually dimerizes with the ligand binding subunit to form the DNA-binding species.

Activation of the receptor requires more than ligand binding, dissociation of the multimeric complex, translocation into the nucleus, and dimerization. In addition, activation of the heterodimer appears to be required. Treatment of the ligand/receptor complex with RNAse appears to block its DNA binding ability (*56*), suggesting the potential involvement of RNA in the receptor action. Phosphorylation also appears to be required for an active DNA-binding species to be formed. Phosphatase treatment blocks DNA binding (*57*), and DNA binding can be facilitated by treatment with protein kinase C (*58*), suggesting that serine/threonine phosphorylation plays an essential role in putting the receptor in a DNA binding form. However, other data suggests that dephosphorylation, possibly at another site, may also be a necessary step in the activation process since the active form of the receptor has a higher pI than the dimeric form which is unable to bind to DNA (*53*). The sequential nature of phosporylation and dephosphorylation steps in the activation of the receptor is also suggested by *in vivo* data suggesting that phorbol esters, which activate protein kinase C, can block the action of TCDD at the level of the receptor (*59*).

The activated receptor-ligand complex binds to specific sites on DNA, and appears to function as a transcriptional enhancer (*60*). This interaction has been best described for control of the *CYP1A1* gene, resulting in induction of the synthesis of this cytochrome not only in the liver, but in extraheptatic tissues. The dioxin responsive enhancer (DRE; xenobiotic responsive enhancer, XRE) is located in the 5' region upstream of the structural gene for the cytochrome. It involves a core heptanucleotide sequence, TXGCGTG, surrounded by flanking regions and occurs in multiple copies upstream of the transcription start site (*61–63*). Within the core sequence, several nucleotides are absolutely essential for binding; mutational analysis
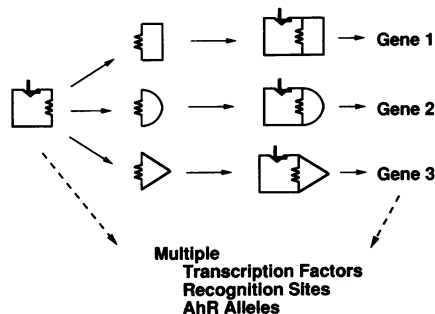
Figure 3. Possible mechanism of gene specificity.

has demonstrated that mutation of these bases eliminates binding (*64*). Use of reporter constructs has also shown that there appears to be cooperativity in the control of expression of *CYP1A1* by the DRE (*65*). Presence of two consensus sequences within the DRE results in a synergistic enhancement of transcription as compared to the presence of only one consensus sequence. It is not yet clear whether the presence of the third, or even a fourth, consensus sequence within the DRE leads to additional cooperativity. Inhibitory regions have also been suggested to be present in the regulatory region, which may block the enhancer action of the receptor (*66*).

A note of caution should be exercised when the mechanism of action of TCDD and the receptor complex is described. Almost all of the studies have involved the control of the *CYP1A1* gene in either liver or hepatoma cells. Analysis of control of *CYP1A2* has failed to reveal the presence of a consensus sequence or even the exact *CYP1A1* core in the region upstream of the *CYP1A2* gene (*67*). Sequence analysis of the regulatory region of PAI-2, which has been shown to be transcriptionally regulated in human keratinocytes (*68*), has also failed to reveal the presence of a functional *CYP1A1* DRE (C Corton, personal communication). The same caution should be exercised in the understanding of the heteromeric nature of the DNA binding form of the receptor. While the arnt protein is involved in binding to the receptor in regulating the expression of *CYP1A1*, it is possible that it is but one of a family of proteins which bind to the ligand-binding subunit (Figure 3). The presence of multiple "arnts" has been suggested by recent studies of Tukey and co-workers (*69*), indicating that a second protein may be involved in binding to the regulatory region of *CYP1A2*. It is possible that it is the interaction with this second protein that controls both the DNA binding, which is the interaction with specific and

unique genes, and the tissue specificity of dioxin's effects.

The question that is frequently asked is whether or not the *Ah* receptor controls all the effects of dioxin. The discussion above makes it clear that this is too simplistic a question. What is meant by the *Ah* receptor? Are we talking about the ligand binding subunit? If so, the answer appears to be yes. If one is talking about the tetrameric cytosolic species, which probably modulates the specificity of the ligand binding, or about the activated heteromer which is involved in interaction with DNA, the answer may be more complicated. Two lines of evidence have been used to address the role of the receptor in dioxin's effects. The first involves structure-activity studies in which there is an apparent rank order in the relationship between compounds that can bind to the receptor and their effects. Chemicals with higher affinity binding are more potent in their effects (*70*).

The second approach involves the use of mouse strains which have different alleles coding for the ligand binding subunit of the receptor (*71*). The prototypic strains are the C57BL/6, which has a high-affinity receptor, and the DBA/2, which has a low-affinity receptor. The difference in this receptor makes these two strains relatively more sensitive to TCDD and responsive to the effects of 3-methylcholanthrene, or resistant to TCDD and nonresponsive to 3MC. Poland and Glover (*71*) demonstrated that a number of the effects of TCDD—lethality, thymic atrophy, induction of cleft palate, enzyme induction—segregated with the *Ah* allele, with those animals having the responsive allele being more sensitive. Induction of chloracne and tumor promotion in hairless mice was also shown to segregate with the responsive allele (*72*). Using mice congenic at the *Ah* locus, Birnbaum and co-workers (*6*) demonstrated that effects on LD$_{50}$ values, organ weights, and clinical chemistry measures segregated with the *Ah* allele. Induction of *CYP1A1*, as measured by hepatic ethoxyresorufin-*O*-deethylase activity, was allele-dependent as was decreased binding to the estrogen receptor (*73*). The responsive congenic mice were also more sensitive to the effects of TCDD on the EGF receptor than were the nonresponsive congenic mice (*29*).

Several recent studies have suggested that binding to the *Ah* receptor may not be required for dioxin's effects. One of these studies involves the use of the two unrelated strains of mice and effects on developmental tissues from these animals (*74*). It

is well known from studies of the steroid receptors that during development, receptor function may be differentially controlled. If the ability of the ligand binding subunit to bind TCDD is controlled by its interaction with other proteins, such as HSP90 or p50, changes in these proteins during development may alter the ligand binding specificity. The second series of studies which have been interpreted to suggest that the *Ah* receptor may not be essential for dioxin's effects involve some of the immunotoxic effects of TCDD in mice (*75*). *In vitro*, the lack of structure activity relationships may be a reflection of the need for serum in the culture conditions (*76*). Dioxin's effects are easily modulated by the presence of serum and other factors in the growth media. Of greater interest is the apparent lack of difference in immunotoxic responses (specifically the response to sheep red blood cells) between C57BL/6 and DBA/2 mice when TCDD is given over a 2-week period in divided doses (*77*). Dosimetric differences between these two strains may play a role in the apparent lack of *Ah*-mediated response (*78*). While even at low divided doses the hepatic binding protein which sequesters TCDD in the liver would be induced in the responsive strain, it would not be induced in the nonresponsive strain, leaving more dioxin available to target the cells of the immune system. Until such a pharmacokinetic study is conducted it will be difficult to determine whether this immunotoxic response is really an outlier in terms of the general understanding that all the responses of dioxin require binding to the *Ah* receptor. However, it is clear that while binding to the receptor is necessary, it is not sufficient. Interaction of dioxin with the ligand binding species and subsequent interaction of the ligand-receptor complex with regulatory sequences on DNA is controlled by a host of other proteins and regulatory steps.

Species Homology

One of the major questions concerning the risk assessment of dioxin is the issue of whether or not humans are a sensitive species to the toxic effects of TCDD. The issues of species differences in sensitivity to dioxin is often discussed, but little understood. Table 2 lists the approximate oral LD$_{50}$ for a variety of animal species following a single exposure to dioxin. While there is a difference of more than three orders of magnitude in the oral dose needed to kill a guinea pig from that needed to kill a hamster, most of the laboratory species will die with a dose that is within one order of mag-

nitude of 100 mg/kg. The dose can be modulated by developmental stage and body composition (*79*). In certain species, there appears to be a sex difference in the LD_{50} dose, but there is no consistency as to whether males or females are more sensitive. While the guinea pig and hamster appear to be outliers in terms of their sensitivity to dioxin's acutely lethal effects, they differ by only an order of magnitude in respect to the sensitivity of their developing pups to TCDD-induced developmental toxicity (*20*). Using organ cultures of the developing palate from the human, rat, and mouse, Abbott and Birnbaum (*80*) demonstrated that while the sensitivity of the rat and human was the same, the developing mouse palate was approximately 1000 times more sensitive to the teratogenic effects of TCDD. This *in vitro* difference between the rat and mouse is reflected in the teratogenicity of TCDD in the mouse but not in the rat where the only time cleft palate occurs is at doses which are both maternally and fetally toxic. Thus, the mouse is uniquely sensitive to the teratogenic effects of TCDD. However, other studies have indicated that the doses which are embryo/fetotoxic in the mouse are similar to those in the guinea pig, rat, and hamster.

Chloracne has been examined as another end point for species similarity in sensitivity. Ryan et al. (*81*) have estimated the dose of dioxin-related compounds which resulted in chloracne in a population in Taiwan poisoned by contaminated rice oil. The necessary dose was very similar to that dose which causes chloracne in hairless mice, rabbit, ears, and monkeys. It should also be pointed out that the pathology of the lesion is very similar in all these species. This is most interesting given the innate human variability which is seen in the chloracne response in Seveso (*82*). While below an estimated initial body burden of <10,000 ppt no chloracne was observed, and above an estimated body burden of >60,000 ppt chloracne was always observed, there was a wide range of serum

Table 2. Acute toxicity of TCDD.[a]

Species	LD_{50}, µg/kg, po
Guinea pig	0.6–2.5
Mink	4
Rat	22–320
Monkey	<70
Rabbit	115–275
Mouse	114–280
Dog	>100–<3000
Hamster	1150–5000

[a]Data from U.S. EPA (*3*).

levels where the occurrence of chloracne was sporadic.

No studies have yet examined the issue of immunotoxic effects directly in exposed people although standard measures of immune dysfunction, such as changes in lymphocyte numbers or proportions have not revealed any effects. The most sensitive response in mice to dioxin appears to be suppression of the primary antibody response. If this were to occur in people, it could result in a low vaccination take rate in an affected population. Inuit women living along the Hudson Bay have elevated levels of dioxinlike chemicals in their breast milk, reflecting their diet of fatty sea mammals (*83,84*). The young children in this population appear to have a high rate of infections and a low rate of successful primary vaccinations (E Dewailley, personal communication). Human tonsillar lymphocytes appear to have similar sensitivity to TCDD *in vitro* as expressed by mouse splenocytes (*85*). Changes in the subpopulations of both marmoset and human lymphocytes have also been reported following exposure *in vitro* to TCDD (*86*). These data might suggest that humans have similar sensitivity in regard to their immune system as do mice and monkeys. In contrast, the rat appears to be relatively resistant to the immunotoxic effects of TCDD. This species may be an outlier for this toxic end point, as the mouse is for teratogenesis, and the guinea pig and hamster are for lethality.

Hormonal and growth factor changes have been reported in humans as well as experimental animals. A recent epidemiological study has reported that occupationally exposed workers (the same cohort in which the mortality study was conducted and that demonstrated an association of dioxin exposure and all cancers) have decreased levels of circulating testosterone (*87*). This is the same response observed in rodents following similar levels of dioxin exposure (*88*). The levels of EGF receptor decrease in the liver of mice (*89*) and humans (*90*) exposed to dioxinlike chemicals. Premature eruption of incisors was noted in children exposed prenatally to the contaminated rice oil (*12*) and in mice exposed to TCDD (*91*).

As previously mentioned, dioxin has recently been demonstrated to be a carcinogen in humans as it is in experimental animals. Comparison of the body burdens present in the exposed workers with those present in experimental animals (*92*) reveals that similar blood levels are present in the dose range where tumors occur, suggesting similar sensitivity between the species. In

fact, using the rodent data to estimate the human response would lead one to predict that if anything, the increased risk of cancer should be even less than what has been observed in the three recent studies (D Hoel, personal communication).

While developmental toxicity, immunotoxicity, dermal toxicity, and carcinogenicity are all clearly adverse effects from dioxin exposure, induction of drug-metabolizing enzymes may be adaptive, rather than toxic, responses. Nevertheless, the doses needed to bring about induction of these enzymes in the liver, and in extra hepatic tissues, are comparable. Mouse and human keratinocytes require similar concentrations to induce EROD activity (*93*). Similar levels of dioxinlike compounds are needed to result in similar AHH induction in the placenta of mice and humans (*94*). It is interesting to note that *CYP1A1* activity is only poorly induced in the liver of humans and of guinea pigs. Neither of these species responds to dioxin with liver toxicity. In contrast, in those species where *CYP1A1* is readily inducible, such as the rat and mouse, liver toxicity is an invariant correlate of dioxin's deleterious effects.

Thus, while any given species can be an outlier for any one biological response to dioxin, most species respond similarly for most effects. The existing data suggest that humans are no exception and show similar sensitivity to the toxic effects of TCDD as do other animals.

Toxic Equivalency

As mentioned earlier, dioxin is but one member of a large family of chemicals that have similar structure and activity. Other compounds that can be approximate isosteromers of TCDD include halogenated members of the dibenzo-*p*-dioxins, dibenzofurans, biphenyls, naphthalenes, azo- and azoxybenzenes (Figure 4). In general, four lateral halogens are necessary to achieve dioxinlike effects. Increasing halogenation reduces potency, in large part by decreasing binding to the *Ah* receptor, but also by limiting absorption. Some tetra-substituted congeners have limited potency *in vivo* because of rapid metabolism.

Because of the fact that dioxins and related compounds usually occur in complex mixtures, and the need to estimate the toxicity of such, the international community has come up with an approach involving toxic equivalency factors (TEFs) to address this issue (*95–97*). All of the dioxinlike compounds can be assigned a TEF which is a measure of the potency of that compound relative to TCDD. Implementation of TEFs
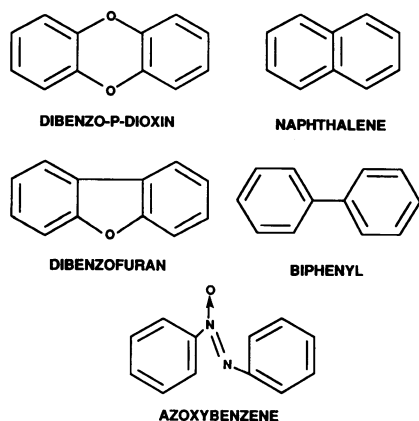
Figure 4. Basic aromatic chemical structures which can become approximate isosteromers of TCDD upon lateral halogenation.

require that compounds are structurally related and have the same mechanism of action (*98*). It is important that dose-response curves have a parallel slope in order that the relative toxicity can be assessed. Until now, this has only been done carefully for approximately ten isosteromers using the induction of cleft palate as an end point (*99*). Nevertheless, TEFs, which have been standardized for the polychlorinated dibenzofurans and dioxins, are based in large part on *in vitro* studies involving enzyme induction and receptor binding. Some *in vivo* data exist for acute responses, such as enzyme induction, thymic atrophy, and lethality. For a limited number of these chemicals, information on dermal toxicity, teratogenicity, and carcinogenicity (including tumor promotion), exists. Short-term or *in vitro* measures of TEFs fail to take into account pharmacokinetic or species differences which exist. However, the use of the TEFs for the polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) has been quite successful in estimating the toxicity of several complex mixtures.

Only limited attention has been directed toward estimating TEFs for other classes of dioxinlike chemicals. It is clearly appropriate that they be developed for the small subset of the PCBs which are dioxinlike (*98*). Safe (*70*) has compiled the existing information on the relative potency of polychlorinated biphenyls (PCBs) and suggested conservative TEFs for the dioxinlike isomers. These values have been used by regulators and risk assessors in predicting the toxicity of complex mixtures of

PCDDs, PCDFs, and PCBs. However, these values do not incorporate pharmacokinetic considerations as they are based largely on *in vitro* responses. Recent studies by Walker and Peterson (*100*) in fish and DeVito et al. (*101*) in mice indicate that Safe's suggested TEFs for PCBs are overly conservative, in some cases by several orders of magnitude.

Although little information exists on the brominated congeners, the existing data suggest that in general they are only slightly less potent than the corresponding chlorinated molecules. One exception has been in the case of 2,3,7,8-tetrabromodibenzofuran which appears to be more potent than the corresponding TCDF. This may reflect decreased metabolism of the brominated isomer (*102*). Limited data on the mixed chloro-bromo isomers suggest that they may be the most toxic (*103*).

Thus, general agreement has been reached in the dioxin community regarding three points needed to be considered in any risk assessment of TCDD. The first is that dioxin is the most potent growth dysregulator that is known and all of its responses appear to require binding to the *Ah* receptor as a necessary, but not sufficient, step. The second critical point of agreement is that people are sensitive to the effects of dioxin. The last point is that all dioxin-related molecules must be included in any assessment of the environmental risk from dioxin.

Dose-Response Relationships

Much of the concern involving human exposure to dioxin and related chemicals has concentrated on cancer. However, recent studies have indicated that cancer is a relatively high dose-response. In experimental animals, liver tumors only result at doses where acute toxicity is evident. For example in the Kociba study (*92*), at the high dose where there was a clear cut increase in liver tumors in the female rat, liver toxicity and weight loss were evident. Much of the regulatory focus has been on these liver tumors. However, it is important to remember that tumors were significantly increased at several other sites, including the nasal cavity and thyroid. In the males, lung tumors were present. It is interesting to note that the liver tumors are estrogen dependent in the female rat; ovariectomy abolished the liver tumor response (*104*). In contrast, the lung tumors appear to be blocked by estrogen as they are only present in the intact male rats (*92*) and in the ovariectomized females (*105*). In contrast, male mice appear to be more sensitive to liver tumors than are

female mice (*106*). Squamous tumors and lesions of the respiratory tract have been noted in several animal studies. The human epidemiological studies suggest that lung tumors may be increased by exposure to dioxin (*27*).

While cancer appears to require relatively high body burdens to be detected, lower doses result in other serious adverse responses. In a multigenerational study, Murray et al. (*107*) observed that the daily dose needed to result in reproductive effects (10 ng/kg/day) was an order of magnitude lower than that resulting in hepatocellular carcinomas in rats (*108*). Recent studies by Peterson and co-workers (*17–19*) have demonstrated that a single prenatal exposure of pregnant rats to 64 ng dioxin/kg bw can result in permanent effects not only in sexual behavior of the male offspring but also lead to decreased levels of androgens and spermatogenesis.

Immunotoxic responses have been detected in the marmoset and in the mouse at similar low doses. Single doses to mice of as little as 100 ng/kg caused enhanced viral mortality (*22*). A single exposure of 10 ng TCDD/kg resulted in altered patterns of lymphocyte subsets in the marmoset (*109*). However, at weekly doses of 0.3 ng/kg, no clear-cut change occurred in the total lymphocyte population (*110*). The ED^{50} for the suppression of the primary antibody response to sheep red blood cells in mice is consistently lower than that for the induction of EROD or AHH, markers for the activity of *CYP1A1* (*75, 111*). Detection of the increase in mRNA for *CYP1A1* by quantitative PCR techniques has demonstrated that increases in the message occurs at doses 100 times lower than what can be detected either enzymatically, immunochemically, or by Northern blot analysis (*112*). Recent studies have demonstrated that a significant increase in *CYP1A1* mRNA can be noted in rats following a single dose of 100 pg/kg. Given a half-life of roughly 30 days, this would be roughly equivalent to a daily dose of 1 to 3 pg/kg/day in the rat.

Enzyme induction, immunotoxicity, and reproductive effects all seem to occur at similar low doses. Since binding to the receptor is necessary before any of these effects can occur, it is clear that ligand binding occurs at very low doses. What is the shape of the dose-response curve in this region? The only responses for which good data exist are the induction of cytochromes P4501A1 and *1A2* in the liver of both mice and rats. Recent data from the laboratory of Lucier and coworkers (*113*) has shown that

the increase in the mRNA and protein for *CYP1A1/1A2* gives no evidence of nonlinearity in the low dose region. Likewise, no evidence for a threshold in the induction of these two enzymatic activities was observed in mice (*114*). Both of these studies involved repeated exposures at levels of approximately 1 to 5 ng/kg/day. Lower exposures are currently being conducted. The critical conclusion that can be drawn from these studies is that there is no evidence for a threshold in relatively simple, *Ah*-receptor mediated responses. That is not to say that other responses may not exhibit nonlinear behavior at low doses. However, it is clear that not all responses are nonlinear. Therefore, there cannot be a dose which, by definition, will have no response.

Recent studies have also demonstrated that the pharmacokinetics of TCDD and related compounds is dose-dependent. Distribution to the liver is nonlinear, with the relative concentration increasing with increasing dose (*115*). This appears to be associated with the induction of a liver-specific binding protein which has been tentatively identified as cytochrome P450 *1A2* (*116,117*). Hepatic sequestration results in decreased distribution to extrahepatic tissues with increasing dose. Many of the experimental studies have been conducted at doses where this sequestration was occurring, leading to the false assumption that humans and animals behave differently in how TCDD distributes. Physiologically-based pharmacokinetic models have been developed for both TCDD (*118*) and the related brominated congener, 2,3,7,8-tetrabromodibenzo-*p*-dioxin (TBDD) (*119*) which incorporate the induction of this binding protein and the dose-dependent distribution. These models accurately predict the effect of dose on absorption, distribution, and elimination in rats. Carrier (*120*) has used human data on the blood and adipose levels of PCDFs to develop a pharmacokinetic model to describe the behavior of these compounds which also predicts nonlinearity in disposition. These models raise some concern about the estimation of half-life in

humans assuming a one-compartment elimination (*121*).

Pharmacokinetic studies have also been conducted comparing different relevant routes of human exposure. For a number of dioxin-related compounds, both oral and dermal absorption has been shown to be dose-dependent (*122,123,124*). Relative absorption decreases as the dose is increased. At relatively low experimental doses (~1mole/kg), oral exposure results in nearly complete absorption as compared to an intravenous exposure. Intratracheal instillation, an approximation of pulmonary absorption, results in similar absorption to that observed orally. Dermal absorption is always more limited, being maximal for TCDD from an organic solvent of approximately 50% of an applied dose. Maximal dermal absorption, however, can be predicted from the octanol water partition coefficient (*125*).

Risk from Current Exposures

Where does current environmental exposure place us today? In the industrialized world, adults have approximately 6 ppt TCDD per ml serum, on a lipid adjusted basis (*126*). [In nonindustrialized areas of the Third World which have not been exposed to heavy herbicide use, body burdens are several times lower (*127*)]. If the total toxic equivalency of all the chlorinated dioxins and furans is included, the body burden is approximately 30 ppt. This value is further increased if the toxicity of the dioxinlike PCBs is included.

What are daily exposure levels? Dietary exposure accounts for the major source of the human body burden. Estimates are that daily exposure to TCDD is approximately 0.1 to 0.3 pg TCDD/kg/day, equivalent to approximately 1 to 3 pg TEQ/kg/day (*128*). If the PCBs are included in this estimate, the daily dose is 3 to 10 pg/kg/day. It is important to note that there are people within the population who have higher exposure than the average. For example, since dioxin and related chemicals are so lipophilic, they are mobilized from the adipose tissue during lactation and are elimi-

nated through the milk. Therefore, nursing infants can have daily exposures 10 to 20 times higher than the background population. Subsistence fishermen also have elevated exposure due to the presence of these compounds in fish.

From the foregoing discussion, it should be clear than exposure to high levels of dioxin and related compounds has the potential to result in a host of biological responses. At high doses, some of these are clearly adverse and have been observed in the human population (e.g., depression of circulating testosterone levels, chloracne, cancer). These overtly toxic responses have been noted at body burdens many times higher than those occurring in the general population in industrialized countries. For people with levels higher than the general populace but lower than occupationally exposed cohorts or those poisoned in industrial accidents, recent reports have indicated alterations in lipid metabolism and elevated incidence of diabetes (*129*). Exposure to a complex mixture of PCBs and PCDFs resulted in clear evidence of developmental toxicity (*12*).

The question of greater import is what is the risk of current environmental exposure to the general population? Are the subtle effects detected in experimental animals occurring in people today? If so, are these adverse? Results in enzyme induction from both rats (*113*) and mice (*114*) would suggest that at current environmental levels (~1 to 10 TEQ pg/kg/day) people may be experiencing small, but significant, increases in these markers of response. Highly exposed populations may be at special risk. Since animal studies suggest that changes in hepatic enzyme induction occur at body burdens similar to those at which immunotoxicity in mice and permanent effects on the reproductive system occur in rats, it is reasonable to hypothesize that subtle effects on these parameters may be occurring in the human population. Epidemiological studies to examine this hypothesis should be undertaken.

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