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2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN AND RELATED HALOGENATED AROMATIC HYDROCARBONS: EXAMINATION OF THE MECHANISM OF TOXICITY

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INTRODUCTION

The chlorinated dibenzo-*p*-dioxins, dibenzofurans, azo(xy)benzenes, naphthalenes, and biphenyls and the brominated biphenyls, collectively termed the halogenated aromatic hydrocarbons, have received increasing attention in the scientific literature and popular press over the past twenty-five years as toxic environmental pollutants. This group of chemicals is usually considered together, because (*a*) their chemical structures are similar, i.e. they are approximate isostereomers, (*b*) they produce a similar and characteristic pattern of toxic responses, although they vary greatly in potency, and (*c*) they are believed to act by a common mechanism. In addition these compounds share a number of other similarities: (*a*) while some are, or were, manufactured as commercial products (the chlorinated naphthalenes and biphenyls, and brominated biphenyls), and others occur only as contaminants in commercial products [the chlorinated dibenzo-*p*-dioxins, dibenzofurans, and azo(xy)benzenes], they have all become widespread in the environment; (*b*) all classes of halogenated aromatic hydrocarbons have

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produced incidents of poisoning of industrial workers, the general population, and/or farm animals; and (*c*) the chemical stability and lipophilicity of these compounds, and their resistance to degradation results in their persistence in the environment and concentration in the food chain.

Numerous reviews, monographs, and symposia have covered the chemistry, analytical detection, animal toxicology, human poisonings, environmental pollution, commercial production, and epidemiologic investigations of these compounds (1–20). The reader is referred to these sources, especially to the recent book edited by Kimbrough (20) for comprehensive coverage of various aspects of this complex subject. Our goal in this review is to examine the mechanism by which halogenated aromatic hydrocarbons produce their toxic responses. We will (*a*) survey the pattern of toxic effects produced by these compounds, (*b*) examine selected biochemical effects which they elicit, (*c*) consider the structure activity relationship for toxic and biochemical effects, and the genetic segregation of these effects in mice, and (*d*) propose a model for the mechanism of toxicity based on these observations. For brevity we will focus primarily on investigations on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which as the most thoroughly studied and most potent compound, serves as the prototype of the halogenated aromatic hydrocarbons. We wish to apologize for our omission or inadequate recognition of the contributions of many investigators in this field, especially the early pioneering studies of Kimmig & Schultz (21–23), who identified TCDD as the causative agent of chloracne, and the investigations which led to the purification and identification of the toxic fat or chick edema factor (24–28).

Commercial Production of Various Halogenated Aromatic Hydrocarbons (29)

In Figure 1 are shown representative congeners and the ring numbering systems for the various classes of halogenated aromatic hydrocarbons to which we will refer throughout the article. It should be emphasized that not all isomers are toxic. The structure-activity relationship will be developed in a subsequent section; until then, a generalization will suffice: the toxic isomers have halogen atoms in three or four of the lateral ring positions (as depicted in Figure 1) and may or may not have halogen atoms in other ring positions.

CHLORINATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS There are 75 possible chlorine-substituted dibenzo-*p*-dioxin isomers, and 135 possible chlorine-substituted dibenzofuran congeners. These compounds are not synthesized for commercial purposes, but are formed as trace contami-

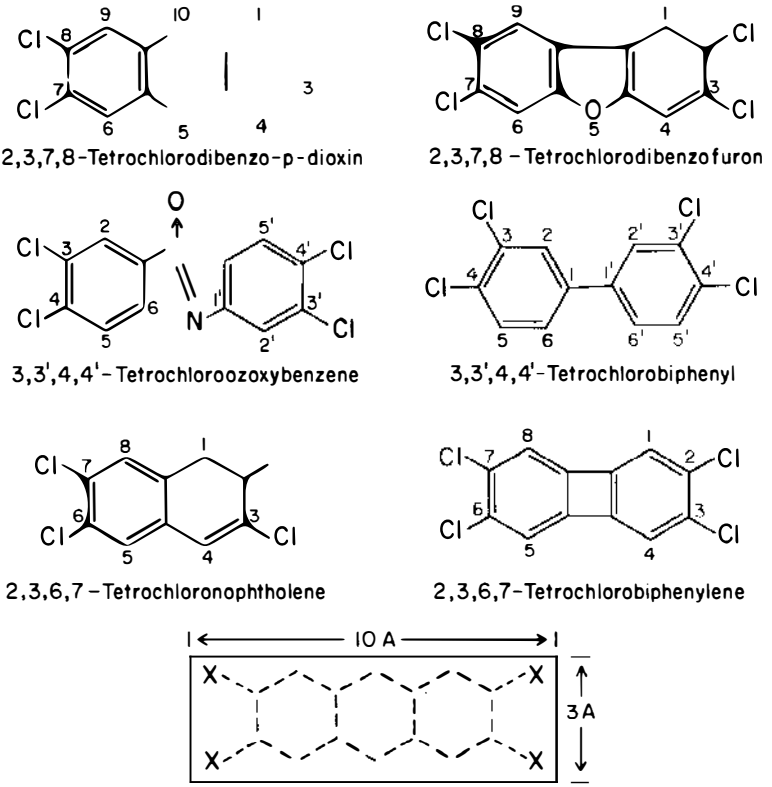

Figure 1 The isosteric tetrachloro- congeners and ring numbering systems of various halogenated aromatic hydrocarbons. The diagram at the bottom depicts the postulated recognition site on the cytosol receptor to which these isosteric compounds bind.

nants in the synthesis of several commercial products, most importantly chlorophenols. TCDD is formed as a contaminant in the synthesis of 2,4,5-trichlorophenol, which is used to manufacture 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), a broad spectrum herbicide and defoliant. Several other chlorophenols are in wide use as insecticides, fungicides, mold inhibitors, and disinfectants, with an annual production estimated at 150,000 tons. The most important of these is pentachlorophenol, used as a fungicide for slime control in the manufacture of paper pulp, the tanning of leather, and other processes. Commercial pentachlorophenol contains penta-, hexa-, hepta-, and octa- chlorodibenzo-*p*-dioxins and chlorodibenzofurans. Several chlorinated dibenzofurans have been found in commercial polychlorinated biphenyl mixtures, but no bromodibenzofurans are present in commercial polybrominated biphenyls.

CHLORINATED AZOBENZENES AND AZOXYBENZENES 3,3'4,4'-Tetrachloroazo(xy)benzene is formed as a contaminant during the commercial synthesis of chloroaniline herbicides (the acyl, carbamate, and urea derivatives of 3,4-dichloroaniline) (30, 31).

POLYCHLORINATED BIPHENYLS (PCBs) Commercial mixtures of PCBs of various degrees of chlorination have been manufactured and used world-wide, with a cumulative US production estimated at over 1 billion pounds until manufacturing was halted in 1977. The physical and chemical properties of PCB mixtures, e.g. thermal stability; resistance to acids, alkalis, oxidation, and hydrolysis; low flammability; low solubility in water; low vapor pressure; high electrical resistance and favorable dielectric constant have made them desirable for a variety of commercial uses in the past: as fluids in capacitors and transformers, hydraulic acid and heat transfer fluids, lubricants, plasticizers, and adhesives. Commercial PCB mixtures have been found to contain trace amounts of chlorinated dibenzofurans.

POLYBROMINATED BIPHENYLS (PBBs) Commercial mixtures of PBBs are used primarily as flame retardants for synthetic materials. In addition to PBB isomers, these mixtures have been reported to contain brominated naphthalenes, but not brominated dibenzo-*p*-dioxins or dibenzofurans.

POLYCHLORINATED NAPHTHALENES Commercial mixtures of chloronaphthalene isomers have many of the same properties and uses as PCBs, but production has decreased since the 1950s.

The chlorinated terphenyls and biphenyl ethers, while sometimes grouped with the above classes of halogenated aromatic hydrocarbons, will not be considered because of the limited information on their toxicity.

TOXICOLOGY OF TCDD AND RELATED HALOGENATED AROMATIC HYDROCARBONS

There is a commonality of the toxic responses produced by halogenated aromatic hydrocarbons. This is difficult to appreciate from the literature because the toxic effects observed after administration of a given compound vary with the dose, length of exposure (acute versus chronic administration), and, most importantly, the species of animal. Animal species vary greatly in their sensitivity to halogenated aromatic hydrocarbons. The acute oral LD_{50} of TCDD varies over a 5000-fold range in different species [LD_{50} (micrograms per kilogram)]: guinea pig, 1 (32); chicken embryo (injected into air cell), $<$ (26); rat (male), 22, (female), 45 (32); monkey, $<$ 70 (33); rabbit, 115 (32); mouse, 114 (34, 35); dog, $>$ 300 (32); bull frog,

> 500 (36); hamster, 5000 (37, 38). This large variation in species sensitivity to TCDD is not accounted for by an appreciable difference in the rate of metabolism of TCDD; the LD_{50} in the guinea pig and hamster differs by more than three orders of magnitude, but the whole body half-life of TCDD differs by only threefold in these species (see section on Carcinogenicity, Metabolism, and Pharmacokinetics) (39, 40). The toxic responses of animal species also vary qualitatively. Many of these responses are highly species specific and confined to one or a few species (Table 1). In general, all of the halogenated aromatic hydrocarbon congeners (*a*) show the same order of species sensitivity and (*b*) will elicit the same pattern of toxic responses within a given species. We will briefly review the toxic syndrome produced by TCDD and related compounds.

WASTING After an acute lethal dose of TCDD or congeners, all species of animals have a latent period of a week or more prior to death in which they develop a weight loss or reduced weight gain accompanied by a depletion of adipose tissue. At death, the loss in body weight may be as great as 50% (43). Reduction in body weight and adipose tissue is not entirely attributable to lack of food intake (52). The lethality of TCDD is not attributable to the wasting syndrome because TCDD-treated, parenterally

Table 1 Histopathology to TCDD or related chlorinated aromatic hydrocarbons: species differences[a]

	Monkey	Guinea pig	Cow	Rat	Mouse	Rabbit	Chicken	Hamster
Hyperplasia and/or metaplasia								
Gastric mucosa	++[b]	0	+	0	0			0
Intestinal mucosa	+							++
Urinary tract	++	++	++	0	0			
Bile duct and/or gall bladder	++		+		++			
Lung: focal alveolar				++				
Skin	++		*[c]	0	0	++		
Hypoplasia, Atrophy, or Necrosis								
Thymus	+	+	+	+	+		+	+
Bone marrow	+	+			±		+	
Testicle	+	+		+	+		+	
Other								
Liver lesions	+	0		++	+	++	+	+
Edema	+	0		0	+		++	+

[a] References: monkey (33, 41, 42); guinea pig (35, 43, 44); cow (45); rat (43, 46, 47); mouse (32, 35, 48); rabbit (21, 32, 49); chicken (32, 41, 50, 51); hamster (37, 38).

[b] Symbols: 0, lesion not observed; +, lesion observed (number of "+" denote severity); blank, no evidence reported in literature.

[c] Skin lesions in cattle are observed, but they differ from the skin lesions observed in other species (see text).

fed rats gain weight at the same rate as the control parenterally fed animals, retain their adipose tissue, but nevertheless die 13–17 days after treatment (53).

LYMPHOID INVOLUTION In all species studied, TCDD and congeners produce a loss of lymphoid tissue, especially in the thymus, but also in the spleen and lymph nodes (35, 54–56). The thymus may be reduced to 20% of normal size, with a loss of cortical lymphocytes. While this lesion is often referred to as thymic atrophy or involution, careful time-course studies reveal some necrosis of lymphocytes (43). In young animals (rats, mice, and guinea pigs), thymic loss produced by TCDD is accompanied by suppression of the immune response. Despite considerable investigation, neither the precise nature of this immune suppression, nor the target cells affected have been determined (56–61). The lack of effect of TCDD on primary lymphocytes and thymic cell lines in vitro suggests that halogenated aromatic hydrocarbons may not act directly on T-cells (56, 62), but may produce loss of thymocytes by acting indirectly (i.e. by affecting the thymic epithelium). Lethally exposed animals generally do not die from infections, nor does a germ-free environment protect them from death (63).

HEPATOTOXICITY TCDD and related compounds produce hepatomegaly in all species, even at doses well below the lethal dose (43, 47). The enlarged liver is due to hyperplasia and hypertrophy of parenchymal cells, especially a proliferation of the smooth endoplasmic reticulum (64, 65). This morphologic change is accompanied by an increase in microsomsal monooxygenase activity (see below). In the rat liver, TCDD increases DNA synthesis and the total content of DNA (53, 66).

Other liver lesions vary among species: in the rabbit there is widespread necrosis (49); in the mouse there are focal, centrilobular lesions (34); and in the guinea pig (35) the histopathologic changes are minimal. In the rat, the most studied species, there is some distortion of the hepatic lobules, parenchymal cell necrosis, development of large multinucleated cells, lipid accumulation, pigment deposition, and infiltration of inflammatory cells and fibrous proliferation in necrotic areas (47, 55, 65, 67). Slight hyperplasia and hypertrophy of the epithelial cells of the extrahepatic bile ducts and/or gall bladder are more common in monkeys, cattle, and horses (33, 45, 68).

CHLORACNE AND EPIDERMAL CHANGES The most characteristic and frequently observed lesion produced by chlorinated aromatic hydrocarbons in humans is chloracne (69, 70). This lesion consists of hyperplasia and hyperkeratosis of the interfollicular epidermis, hyperkeratosis of the hair follicule, especially at the infundibulum, and squamous metaplasia of the

sebaceous glands which form keratinaceous comedones and cysts (4). Similar epidermal changes have been produced by TCDD and related compounds in rhesus monkeys (33, 42, 71–73), the ear of the rabbit (21), and hairless mice (74). Painting the rabbit ear has become a standard bioassay to detect the presence of halogenated aromatic hydrocarbons (75). These changes have not generally been observed in other laboratory animals, such as guinea pigs, hamsters, rats, and mice.

In rhesus monkeys fed TCDD, several epidermal changes in the integument have been noted: (*a*) hair is lost from the face and chest; (*b*) finger and toenails are sloughed; (*c*) the Meibomian glands of the eyelids (which are modified sebaceous glands) become thickened, tortuous, and keratinized; and (*d*) the ceruminous glands of the ear canal fill with keratinaceous debris (33, 42, 72, 73).

The chloracne observed in humans by halogenated aromatic hydrocarbons is thought usually to arise from skin contact; however, oral administration of TCDD produces this lesion in rabbits (49), monkey (42), and hairless mice (unpublished observation, J. Knutson & A. Poland).

Cattle exposed to chlorinated naphthalenes develop "X-disease," which is characterized by different skin lesions (76, 77), especially a hard thickening of skin around the eyes, dorsal neck, and shoulders. A similar syndrome was noted with cattle exposed to a commercial mixture of polybrominated biphenyls (78), and to commercial pentachlorophenol which contained polychlorinated dibenzofurans and dibenzo-*p*-dioxins as contaminants (45, 79).

GASTRIC LESIONS Chlorinated aromatic hydrocarbons produce a hyperplastic and hypertrophic lesion of the gastric mucosa in a few species, notably the monkey and cow (45, 80), but this response has not been observed in rat, mouse, and guinea pig (35). The lesion consists of a greatly thickened hyperplastic gastric mucosa with extension of the gastric glands into the submucosa and formation of submucosal cysts. Becker et al (81) have described the pathology as an apparent arrest of differentiation of the generative cells of the isthmus and neck region of the gastric glands into parietal and zymogenic (chief) cells and the appearance of largely mucous-secreting cells. A similar lesion was observed in cows fed technical pentachlorophenol, which contained chlorinated dibenzo-*p*-dioxins and dibenzofurans, but not in cows fed analytical grade pentachlorophenol (45).

Hyperplasia of the epithelium of the intestines has been noted in monkeys and hamsters (37, 43). In the monkey, the mucous epithelium of the colon proliferates and invades the lamina propria with the formation of cysts, while in the hamster, oral administration of TCDD produces hyperplasia

of the mucosa of the small intestines and some necrosis and hemorrhaging in this mucosa.

URINARY TRACT HYPERPLASIA In the guinea pig, monkey, and cow the transitional epithelial lining of the urinary tract (renal pelvis, ureter, and urinary bladder) proliferates to two or three times its normal thickness (33, 35, 45).

OTHER LESIONS (*a*) The most characteristic toxic response produced by TCDD in chickens is edema, ascites, and hydropericardium (24, 25, 41, 82). Subcutaneous edema is also observed in mice and monkeys (33, 35, 73, 83). (*b*) TCDD and its congeners produce decreased spermatogenesis and testicular weight and degeneration of the seminiferous tubules (35, 41, 47, 80). (*c*) Severe hypocellularity of bone marrow, pancytopenia, is most apparent in moribund animals, especially monkeys (42), while varying degrees of marrow hypocellularity are observed in other animals with sublethal exposures (35). (*d*) TCDD produces fetal death and resorptions in rats (84–86), and fetal wastage, embryotoxicity, and malformations in mice. Cleft palates and kidney malformations are the primary defects in mice, with few limb or head abnormalities reported (87).

SUMMARY OF TOXICITY In Table 1, we have tried to classify the toxic responses produced by halogenated aromatic hydrocarbons in several animal species as: (*a*) involving hyperplasia and/or altered differentiation (metaplasia); (*b*) involving tissue loss, hypoplasia, atrophy, and/or necrosis; and (*c*) other responses which are difficult to categorize. Nearly all of these pathologic changes affect epithelial tissues. The histopathologic changes which are hyperplastic and/or metaplastic affect the gastric mucosa, intestinal mucosa, and the urinary tract epithelium primarily in monkey and cow, and the epidermis of monkey, cow, rabbit, and humans. In contrast, thymic involution and loss of the seminiferous tubules, both atrophic responses, are seen in most species. It is difficult to categorize some of the lesions. Both hyperplasia and necrosis are observed in the liver, and the cell types responsible for edema and the embryotoxic/teratogenic effects are unknown.

Whereas extensive hepatic necrosis in the rabbit and pancytopenia and hemorrhage in the monkey may be responsible for death in these species, it is generally difficult to specify the organ whose dysfunction is responsible for death.

Carcinogenicity, Metabolism, and Pharmacokinetics of TCDD

Only a few chlorinated dibenzo-*p*-dioxin congeners and commercial mixtures of polychlorinated biphenyls and polybrominated biphenyls have been

tested for carcinogenicity. TCDD has been the most studied compound. It is useful to summarize studies on the pharmacokinetics and metabolism of TCDD before reviewing the carcinogenicity studies.

PHARMACOKINETICS AND METABOLISM OF TCDD Rose et al (88) reported that following oral administration of ^{14}C-TCDD to rats, the radioactivity is stored primarily in the liver and to a lesser extent in the fat as the parent compound, and is eliminated in the feces and a smaller amount in the urine. The whole body half-life of TCDD was estimated to be 23.7 and 31 days in the rat (88), between 22 and 43 days in the guinea pig (40, 89), and 10.8 and 12.0 days in the hamster (39). Polar metabolites of TCDD have been identified in the urine, feces, and bile of rats and hamsters, but not in liver or adipose tissue, indicating that the metabolites are rapidly eliminated (39, 88, 90, 91). Several studies have revealed no evidence of covalent binding of radiolabeled TCDD in vivo (88, 92, 93).

CARCINOGENICITY OF TCDD Van Miller et al (94) reported that chronic dietary administration of low levels of TCDD to rats enhanced the incidence of a wide variety of neoplasms, but the group size was inadequate for statistical significance. Since such a diversity of tumors is not commonly observed with many carcinogens, the authors suggested that TCDD might be acting as a tumor promoter. Kociba et al (46) found in a chronic dietary study of Sprague Dawley rats of both sexes (receiving a daily intake of TCDD of 0, 1, 10, and 100 ng/kg), that female rats, receiving the highest dietary level, had a significant increase in hepatocellular carcinoma, stratified squamous cell carcinoma of the hard palate and nasal turbinates, and keratinizing squamous cell carcinoma of the lung. Nearly half the female mice receiving 0.1 μg TCDD/kg/day (life time dose 2.2×10^{-7} mol/kg) developed one of these three major neoplasms, indicating the extraordinary carcinogenic potency of TCDD. A preliminary report (95) of the, as yet, unreleased National Cancer Institute study on TCDD, confirmed the carcinogenicity of this compound in mice and rats. In a two-stage carcinogenesis model in mouse skin, TCDD acted neither as an initiator or promoter, but TCDD diminished the carcinogenicity of some polycyclic aromatic hydrocarbons (96–98).

The carcinogenic potency of TCDD is especially interesting in light of (*a*) inconclusive evidence that it is a mutagen (99–103), and (*b*) the failure to demonstrate that TCDD is metabolized to an electrophile or covalently binds in vivo (93). The maximum estimate of covalent binding of TCDD in vivo to rat liver DNA is less than 1 molecule of TCDD per 10^{11} nucleotides, or 4 to 6 orders of magnitude lower than that observed for most chemical carcinogens. Pitot et al (104) recently demonstrated in partially hepatectomized rats, initiated with diethylnitrosamine, that TCDD admin-

istration (equivalent to 10 and 100 ng/kg/day for 7 months) greatly enhanced the number of enzyme altered foci and hepatocellular carcinomas. Thus, in this two stage model of liver carcinogenesis TCDD acts as a tumor promoter, and this mechanism may account for all of the observations on the tumorigenicity of TCDD.

OTHER COMPOUNDS A mixture of two hexachlorodibenzo-*p*-dioxins (1,2,3,6,7,8-hexachloro- and 1,2,3,7,8,9-hexachloro- isomers) administered over 2 years was found to increase the incidence of hepatocellular carcinomas in female Osborne-Mendel rats and both sexes of $B6C3F_1$ mice (105, 106). With the same strains of animals and a similar protocol, 2,7-dichlorodibenzo-*p*-dioxin was not found to be carcinogenic in either sex of rat or female mice, but caused a marginal increase in leukemias, lymphomas, hemangiosarcomas, and hepatocellular carcinomas in $B6C3F_1$ mice (107), and dibenzo-*p*-dioxin was not carcinogenic in either sex of rats or mice (108).

Commercial mixtures of polychlorinated and polybrominated biphenyls have been shown to produce hepatocellular carcinomas in rats (109–111). Polychlorinated biphenyls act as promoters in a two stage model of rat hepatocarcinogenesis (112).

BIOCHEMICAL RESPONSES

Spectrum of Biochemical Effects

The biochemical effects produced by the halogenated aromatic hydrocarbons are as varied as the toxic responses, but, surprisingly, many important biochemical pathways are not adversely affected. In this section we will briefly survey some of these effects, in an attempt to outline the scope of the response rather than to provide a comprehensive list. The most well characterized biochemical response to these compounds, the induction of microsomal monooxygenase activity, will be examined in the next section. It has been suggested that the induction of monooxygenase activity and other coordinately expressed drug metabolizing enzymes and the consequent enhanced metabolism of steroids (113, 114) and other endogenous substances (115) may play a role in mediating the toxicity of TCDD. However, there is no direct evidence to support this hypothesis.

ENDOCRINE EFFECTS Chronic exposure to halogenated aromatic hydrocarbons impairs reproduction (116, 117). Female mated rats fed diets containing TCDD have fewer vaginal plugs, an increased interval between mating and parturition, and smaller litter sizes at birth (116). Decreased uterine size, decreased number and size of corpora lutea, and aberrant

ovarian stroma cells observed in TCDD- and PCB-exposed rats suggest that the estrus cycle may be suppressed (47, 117). Plasma progesterone and estrogen concentrations are decreased in TCDD-treated monkeys (118), and a decreased plasma progesterone concentration is observed in polychlorinated biphenyl-treated rats (117).

In rats administered TCDD and polychlorinated biphenyls, the serum thyroxine concentration is decreased to one-half the normal concentration, both the total biliary excretion of thyroxine and the fraction which is excreted as the glucuronide is increased, thyrotropin concentration is elevated, and the thyroid weight is increased (119, 120). Liver microsomes from rats fed polychlorinated biphenyls have an increased rate of glucuronidation in vitro (121). Neither the plasma concentration nor the biliary excretion of triiodothyronine are altered by TCDD exposure (119). The administration of polychlorinated biphenyls produces hyperplasia and hypertrophy of the thyroid follicular cells and ultrastructural alterations which appear to limit colloid droplet-lysosome interaction, suggesting proteolysis of thyroglobulin and release of thyroid hormones are impaired (122). It remains to be established whether the decreased plasma thyroxine level is primarily due to increased thyroxine conjugation or diminished hormone release.

The plasma concentration of corticosteroids is elevated in rats administered a lethal dose of TCDD (123). This glucocorticoid response does not appear to mediate the toxicity of TCDD, for adrenalectomized rats respond to TCDD with thymic involution (124). TCDD does not compete for the glucocorticoid receptor (123), and cortisol and synthetic glucocorticoids do not compete for the TCDD receptor (125).

VITAMIN A Some of the toxic responses produced by the halogenated aromatic hydrocarbons, especially epithelial hyperkeratosis, resemble the effects of vitamin A deficiency (4). The administration of TCDD to rats produces a dose-related decrease in the hepatic storage of retinol, a response observed with many other unrelated compounds (115, 126). However, there is no evidence that vitamin A deficiency is responsible for any toxic responses of TCDD.

EFFECTS ON LIPID METABOLISM TCDD and the related halogenated aromatic hydrocarbons produce marked fatty livers in several species (127–130). In rats, the total lipid content of the liver is increased: a sublethal dose of TCDD produces an increase in triglycerides and free fatty acids and a decrease in sterol esters, while a lethal dose increases cholesterol esters and free fatty acids (127). Poli et al (131) reported a dose-dependent increase in total and high density lipoprotein cholesterol in plasma of rats adminis-

tered sublethal concentrations of TCDD. Of the many factors contributing to a fatty liver, the effects of the halogenated aromatic hydrocarbons have been examined on only lipid synthesis and utilization. Cunningham & Williams (132) found no difference in lipid synthesis between control and TCDD-treated rats. Hinton et al (65) confirmed these results in polychlorinated biphenyl-treated rats, and also reported evidence for an increased half-life of liver lipids. Albro et al (127) suggested that ultrastructural changes in liposomes and reduced levels of ester hydrolases may indicate decreased lipid utilization.

A role for increased lipid peroxidation in the toxicity of TCDD has been postulated, based on two indirect lines of evidence. (*a*) Lipofuscin pigments, considered to be by-products of lipid peroxidation (127), are increased in the hearts of rats treated with TCDD, and (*b*) iron deficiency reduces the hepatotoxic effects of TCDD (see section on Segregation of Toxicity with the *Ah* Locus) and iron is required for in vitro lipid peroxidation (133, 134). There is no direct evidence to support this hypothesis.

PORPHYRIA Chronic, sublethal exposures to TCDD and related compounds produce an accumulation of porphyrins in liver and an increase in urinary porphyrin excretion (47, 135–140). Work by Elder (141) indicates that the primary lesion in hexachlorobenzene-induced hepatic porphyria in rats is a decrease in the activity of uroporphyrinogen decarboxylase activity. Jones & Sweeney (138) observed a decrease in this enzyme activity, as well as elevated levels of highly carboxylated porphyrins in mice treated with TCDD for several weeks. TCDD and related compounds also induce δ-aminolevulinic acid synthetase, the initial and rate-limiting step in heme synthesis, to a small extent in mice and rats (135, 136, 142), whereas a larger increase is observed in chicken embryos (137, 143).

EFFECTS ON EXCRETORY TRANSPORT The administration of TCDD to rats results in a dose-related decrease in the biliary excretion of injected ouabain, a neutral nonmetabolized compound (144, 145). This decrease in liver function correlates with a decrease in the liver membrane Mg^{++}-ATPase and Na^+, K^+-ATPase in TCDD-treated rats (146, 147). However, the two effects can be altered independently, indicating that the ATPases are not directly responsible for the reduced ouabain excretion (148).

Impaired renal function has also been observed in TCDD-treated rats (149, 150). Anaizi & Cohen (149) reported a decrease in renal glomerular filtration rate and an increase in the tubular secretion rate of the organic anion phenosulfonaphthalein.

BIOCHEMICAL PATHWAYS NOT ADVERSELY AFFECTED Several investigators report no decrease in the incorporation of radioactive precursors into DNA, protein, and lipid in the liver of TCDD-treated rats (132, 151). Neal et al (123) concluded that the synthesis of ATP is not affected by TCDD treatment in rats, for there is no alteration in the liver ATP content, the oxidation-reduction state of the liver (as expressed by NAD to NADH ratios), or in the state 3 and state 4 respiration of liver mitochondria (123, 152). No difference was reported for the metabolism of ^{14}C-glucose, ^{14}C-alanine, and ^{14}C-oleate to $^{14}CO_2$ in TCDD-treated guinea pigs, indicating that the general intermediary metabolism of the poisoned animal functions normally (123). Neal et al (123) also report no change in cyclic nucleotide metabolism or any interference with the activity of enzymes containing riboflavin coenzymes.

Enzyme Induction

We wish to examine in considerable detail the investigations on one response produced by TCDD and congeners, the induction of microsomal monooxygenase activity and other coordinately expressed enzymes. Enzyme induction is the most studied and best understood response produced by these compounds, and provides the basis for subsequent formulation of their mechanism of toxicity.

THE MICROSOMAL MONOOXYGENASE SYSTEM The following section is a brief overview of this enzyme system, and the reader unfamilar with this area is referred to more comprehensive reviews (153–157). The microsomal monooxygenase system metabolizes most foreign lipophilic chemicals which enter the body to more polar and readily excretable products. This enzyme complex, embedded in the endoplasmic reticulum, consists of a flavoprotein NADPH-cytochrome P-450 reductase, and a group of hemoproteins, collectively termed cytochromes P-450. At present at least seven distinct species of cytochrome P-450 have been identified in rat liver; four of these species have been purified to homogeneity and shown to differ in molecular weight, amino acid composition, terminal amino acid sequence, and antigenicity (158). Cytochrome(s) P-450 is the enzyme active site and determines substrate specificity. Different cytochrome P-450 species have distinct, but often overlapping, substrate specificities, and are under independent genetic control. Phenobarbital and 3-methylcholanthrene (MC) represent prototype compounds which induce different species of cytochrome P-450, and increase different monooxygenase activities (153). The microsomal monooxygenase activity most frequently assayed as a measure of the MC response is aryl hydrocarbon hydroxylase (AHH) activity [as-

sayed as the rate of 3-hydroxylation of benzo(a)pyrene]. TCDD and related halogenated aromatic hydrocarbons evoke an MC-like pattern of induction.

ENZYME INDUCTION BY TCDD AND CONGENERS The administration of TCDD produces a dose-related induction of hepatic AHH activity in chicken embryos (159). The dose of TCDD which produced one-half the maximal induction, ED_{50}, was 0.3×10^{-9} mol/kg. Among a series of 15 halogenated dibenzo-*p*-dioxins that were examined for their capacity to induce hepatic AHH activity in the chicken embryo, a clear structure-activity relationship was observed. Isomers which induce AHH activity have two common properties: (*a*) halogen atoms occupy at least three, and for maximal potency, four, of the lateral ring positions (positions 2, 3, 7, and 8); and (*b*) at least one ring position is unsubstituted, i.e. the octachloro-derivative is inactive, but both heptachloro-compounds have modest potency (Figure 1). When the same series of 15 halogenated dibenzo-*p*-dioxin congeners was examined for potency to induce hepatic δ-aminolevulinic acid synthetase activity (the initial enzyme in heme synthesis) in the chick embryo, an identical structure-activity relationship was observed (143, 159). Subsequently a much larger series of halogenated dibenzo-*p*-dioxins and dibenzofurans have been tested for their potency to induce AHH activity in chick embryo liver (125, 160) and in a rat hepatoma cell line (161, 162), and the results confirm the structure-activity relationship.

The most important observation from these studies, however, is that for chlorinated dibenzo-*p*-dioxin congeners, there is an excellent correlation between their potency to induce AHH activity and their toxic potency. Subsequent studies have noted a similar correlation for congeners from other classes of halogenated aromatic hydrocarbons. The precise correspondence of these structure-activity relationships suggested that understanding the mechanism of AHH induction would provide insight into our understanding the mechanism of toxicity. In the subsequent discussion we will examine in considerable detail investigations on the mechanism of induction of AHH activity, before returning to a consideration of toxicity.

As noted above, MC is the prototypical compound that induces cytochrome P_1-450 and AHH activity. In a comparison of MC and TCDD for their capacity to induce hepatic AHH activity in the rat, it was found that both compounds produced parallel dose-response curves, both compounds produced the same maximal enzyme induction, and simultaneous administration of maximally inducing doses of MC and TCDD produced no greater response than the administration of either compound alone (163). However TCDD was approximately 30,000 times as potent as MC, with an ED_{50} for hepatic AHH induction of 0.85×10^{-9} mol/kg. Following the administration of a single dose of TCDD (3.1×10^{-8} mol/kg) to a rat, hepatic AHH

activity and cytochrome P_1-450 remained induced for over 35 days, reflecting the prolonged biological half-life of the compound. MC and TCDD appear to evoke the same induction response, but TCDD has a greater potency and a more prolonged duration of action.

For brevity, we have confined our comparison of TCDD and MC to the induction of hepatic AHH activity. Numerous studies have shown that both compounds produce a similar induction of a number of microsomal monooxygenase activities and other coordinately expressed nonmonooxygenase enzyme activities in liver and other tissues (see section on Coordinate Gene Expression) (152, 164–168).

AH LOCUS: GENETIC EXPRESSION OF AHH ACTIVITY IN INBRED STRAINS OF MICE Nebert and colleagues (169, 170) and Thomas et al (171) have shown that randomly bred and certain inbred strains of mice, when administered MC, respond with the induction of hepatic cytochrome P_1-450 and AHH activity; however, certain other inbred strains, when challenged with MC, fail to respond, i.e. there is no increase in hepatic cytochrome P_1-450 or AHH activity. The prototype strain responsive to MC is C57BL/6 and the prototype-nonresponsive strain is DBA/2. In genetic crosses and back crosses between C57BL/6 and DBA/2 mice, the trait of responsiveness to aromatic hydrocarbons (i.e. the induction of hepatic AHH activity by MC or other polycyclic aromatic hydrocarbons) is inherited in a simple autosomal dominant mode. The genetic locus that controls this trait is designated the *Ah* locus (for *A*romatic *h*ydrocarbon) and the allele for responsiveness denoted as Ah^b (b for C57BL/6 or B6 mice) and the allele for nonresponsiveness Ah^d (d for DBA/2 mice).

The extraordinary potency of TCDD relative to MC for inducing hepatic AHH activity prompted an examination of its effects on inbred strains of mice responsive and nonresponsive to MC. TCDD (1.2×10^{-7} mol/kg) induced hepatic AHH activity in all strains of mice tested, regardless of their response to MC (172). By a number of criteria, the hepatic cytochrome P_1-450 and enzyme activity induced in C57BL/6 mice by MC or TCDD, were similar to those induced in DBA/2 mice by TCDD. This suggested that the same gene product(s) was induced by both compounds in C57BL/6 mice and only by TCDD in DBA/2 mice.

Thus, mice that are nonresponsive to MC do respond to the more potent stimulus TCDD, and therefore these mice do possess the structural and regulatory genes necessary for the expression of AHH activity. The failure of these mice to respond to MC suggested that they fail to recognize MC as a signal for induction. It was postulated that the mutation in nonresponsive mice results in a defective recognition or receptor site which has a diminished (or absent) affinity for MC. Why then is AHH activity induced

by TCDD in nonresponsive mice? There seemed to be two obvious possibilities. (*a*) TCDD acts at a site that is independent and distinct from the postulated receptor for MC. If this were the case, the mutation in nonresponsive mice (assuming it is a point mutation) should not affect the sensitivity of these mice to TCDD. (*b*) Alternatively, TCDD might act at the same receptor as MC, but because of its greater potency, and hence presumed greater receptor affinity, TCDD is able to saturate this receptor even in nonresponsive mice. This hypothesis suggested that nonresponsive mice would be less sensitive to TCDD than responsive strains, i.e. it would require a larger dose of TCDD to induce AHH activity comparably.

To distinguish between these hypotheses, the dose-response curves for TCDD induction of hepatic AHH activity were examined in responsive and nonresponsive inbred strains of mice (173). The inbred strains responsive to MC were sensitive to TCDD (ED_{50} for induction of hepatic AHH activity-1×10^{-9} mol/kg); the strains nonresponsive to MC were less sensitive to TCDD ($ED_{50} \geq 1 \times 10^{-8}$ mol/kg). Niwa et al (174) similarly found about a 10-fold difference in sensitivity to AHH induction by TCDD in cell cultures from C57BL/6 and DBA/2 fetal mice. These observations support the hypothesis that TCDD and MC act on the same putative receptor, and that the mutation in nonresponsive strains of mice results in a receptor with a diminished affinity for inducing compounds, producing a nearly absolute unresponsiveness to weak inducers like MC, and a diminished sensitivity to more potent inducers like TCDD.

Induction Receptor

The postulated receptor should be identifiable in vitro as a macromolecular species with specific binding properties that are predictable from the biology observed in vivo. One would expect this binding species to have the following properties: (*a*) reversible binding of TCDD with a high affinity (K_D) which should correspond to the in vivo potency (ED_{50}) of TCDD to induce AHH activity, (*b*) this binding moiety from responsive mice should have a higher avidity for TCDD than that from nonresponsive mice, (*c*) the rank-ordered binding affinities for other halogenated dibenzo-*p*-dioxin congeners should correspond to their potencies to induce AHH activity in vivo, and (*d*) MC and other polycyclic aromatic hydrocarbons which induce AHH activity should bind to this moiety.

A macromolecule with these properties was observed in the 105,000 xg supernatant fraction of liver from C57BL/6 mice through the use of ^{3}H-TCDD of high specific activity with a charcoal-dextran binding assay (125). The specific binding sites of ^{3}H-TCDD (the pool of high affinity, saturable sites) had a binding affinity (K_D) $= 0.27 \times 10^{-9}$m (which compares quite favorably with the in vivo potency of TCDD to induce AHH activity

in these mice, $ED_{50} = 1 \times 10^{-9}$ mol/kg), and a maximal binding capacity (n) of approximately 8.5×10^{-14} mol of TCDD/mg cytosol protein, equivalent to 8.5×10^{-12} mol/gm of liver or approximately 5×10^4 binding sites per cell. Much lower specific binding of ^{3}H-TCDD was observed in liver cytosol from DBA/2 mice at all concentrations of the radioligand, and limited aqueous solubility of ^{3}H-TCDD prevented sufficient concentrations to saturate the cytosol species and estimate the K_D in nonresponsive mice. This failure to demonstrate specific binding in nonresponsive mice is most likely attributable to a binding species with reduced affinity rather than a diminished concentration of this moiety, because the dose-response curve for induction of AHH activity by TCDD in nonresponsive mice is shifted to the right, but the maximum response is not diminished compared to responsive mice.

For 23 halogenated dibenzo-*p*-dioxin and dibenzofuran congeners, there was an excellent correlation between their binding affinities (K_D) for the cytosol species and their potencies (ED_{50}) to induce AHH activity in vivo (125). Polycyclic aromatic hydrocarbons and benzoflavones which induce AHH activity also compete for the cytosol binding species, with a surprisingly high affinity (1/3 to 1/30 that of TCDD). The high binding affinity of MC ($K_D = 1.7 \times 10^{-9}$M) and other polycyclic aromatic hydrocarbons for the cytosol species, in contrast to their weak potency in vivo ($\sim 3 \times 10^4$ less potent than TCDD), suggests their low potency may be due to rapid metabolism in vivo in contrast to TCDD and other halogenated congeners.

Phenobarbital, pregnenolone 16α-carbonitrile, and other compounds which induce a different pattern of cytochrome(s) P-450 and microsomal monooxygenase activity do not compete for this cytosol binding species. Steroid hormones and thyroxine also fail to compete with ^{3}H-TCDD specific binding, suggesting the TCDD-binding species is distinct from cytosol binding proteins previously described.

The cytosol binding species is heat labile and inactivated by trypsin, hence presumed to be a protein. Carlstedt-Duke et al (175) have characterized this protein in rat liver cytosol by gel filtration and ultracentifugal sedimentation as having a Stokes radius of 6.6 nm, a sedimentation coefficient of 5.0S, and a calculated mol wt of 136,000 with a frictional ratio of 1.79. The TCDD-cytosol protein complex, but not the cytosol protein alone, binds to DNA-cellulose. These authors have identified the TCDD-binding protein in the cytosol fraction of rat liver, lung, thymus, and kidney with much lower concentrations in testis, brain, and skeletal muscle (176, 177).

The cytosol binding protein mediates the nuclear uptake and binding of TCDD in mouse liver (178). Okey et al (179) have recently demonstrated that the TCDD-cytosol binding species translocates to the nucleus and is

extractable by high salt, analogous to the behavior of the steroid receptors. Further characterization of the cytosol binding species in liver and cell culture has recently been reported by several laboratories (180, 181).

In summary, the cytosol binding protein, by a variety of criteria, has the in vitro properties expected of the receptor for the induction of cytochrome P-450 and AHH activity. The correlation of several lines of investigation (genetic evidence, structure-activity data, dose-response curves for AHH induction, and receptor binding in vitro) strongly suggest that the *Ah* locus (in mice) is the structural gene for the cytosol receptor. The polymorphism in this locus between responsive and nonresponsive inbred strains of mice determines the sensitivity of these strains to all of the pleiotropic effects produced by TCDD and congeners.

COORDINATE GENE EXPRESSION The administration of MC or TCDD to laboratory animals stimulates not only the induction of cytochrome P-450 and an increase in associated microsomal monooxygenase activity, but also a number of other coordinately expressed enzyme activities, such as UDP-glucuronosyltransferase (182), DT-diaphorase (183), ornithine decarboxylase (184), δ-aminolevulinic acid synthetase (159), glutathione-S-transferase B (185), τ-aldehyde dehydrogenase (186), and choline kinase (187) activities. The induction of several of these enzyme activities have been shown to segregate with the *Ah* locus in inbred strains of mice (182, 184, 188). Thus the cytosol receptor appears to control not only the induction of AHH activity, but the coordinate expression [and perhaps repression, e.g. uroporphyrinogen decarboxylase activity (140)] of a number of enzymes. The battery of enzyme activities controlled by the *Ah* locus varies with the tissue and animal species. For instance, TCDD and MC induce hepatic τ-aldehyde dehydrogenase in the rat, but not the mouse, and both compounds induce hepatic DT-diaphorase in the rat and mouse, but not in the guinea pig.

ROLE OF THE RECEPTOR IN TOXICITY

Structure-Activity Relationship

The structure-activity relationship among halogenated aromatic hydrocarbon congeners to bind to the cytosol receptor and induce AHH activity corresponds with that to produce a variety of toxic responses. This observation suggests (*a*) that the parent compounds, and not metabolites, are responsible for toxicity; (*b*) that the toxic effects produced by these compounds are mediated through their binding to the cytosol receptor; and (*c*) that the capacity of a congener to elicit a single toxic response indicates the potential to produce the complete syndrome.

A substantial number of halogenated aromatic hydrocarbon congeners have been examined for their potency to induce AHH activity and their binding affinity for the cytosol receptor. To facilitate a quantitative comparison, all of the compounds have been compared in the same biological systems: the ED_{50} for induction of AHH activity was determined in the chicken embryo liver (125, 159, 160) (and also in a rat hepatoma cell line) (161), and the receptor affinity using liver cytosol from C57BL/6 mice (125). The receptor affinity for TCDD is quite similar in different species (see next section); however, the toxic potency, LD_{50}, of TCDD (and congeners) varies greatly in different species. This prevents a direct comparison of the absolute value for toxic potency and receptor affinity. For many histopathologic responses produced by these compounds, the data are reported as the presence or absence of the lesion, following the administration of a congener at a given dose. Thus, to relate the structure-activity relationship of congeners for toxicity (LD_{50}, or a particular toxic lesion) and receptor affinity, a rank-ordered comparison of congeners is usually employed.

In this section we wish to summarize briefly the structure-activity relationship for each class of halogenated aromatic hydrocarbons for receptor binding, induction of AHH activity, and toxicity. For a more comprehensive review of this subject see Goldstein (189). See Figure 1 for the ring numbering systems.

HALOGENATED DIBENZO-*p*-DIOXINS AND DIBENZOFURANS Congeners which bind to the cytosol receptor and induce AHH activity have: (*a*) halogen atoms in at least three, and for maximal potency four, of the lateral ring positions (positions 2, 3, 7, and 8); (*b*) an order of potency of substitution which is $Br > Cl > F, NO_2$; and (*c*) at least one unsubstituted ring position; the octachlorodibenzo-*p*-dioxin and dibenzofuran are inactive or very weak (125, 159, 160).

The lethal potencies (LD_{50}) of nine chlorinated dibenzo-*p*-dioxins have been determined in guinea pigs, mice, and chick embryos (35, 161). The rank orders for these compounds for lethal potency, for potency to AHH activity, and for binding affinities correspond very well (190). McConnell et al (35) further showed that, provided a toxic dose is administered, each dibenzo-*p*-dioxin isomer produced the same spectrum and severity of lesions in mice and guinea pigs. Numerous studies have established that the structure-activity relationship for halogenated dibenzo-*p*-dioxin and dibenzofuran isomers for toxic responses in other species follows the criteria necessary for binding and enzyme induction listed above (32, 44, 73, 130, 191–193).

AZO AND AZOXYBENZENE The 3,3'4,4'-tetrachloro-isomers bind to the receptor, induce AHH activity, and produce chloracne in hairless mice and

keratinization in XB cells (see section on XB), whereas the 3,3',5,5'-tetrachloro-isomers do none of these (30, 194, 195). Disparity between biological potency and binding affinity can arise, particularly with the azobenzenes, because of metabolism.

HALOGENATED BIPHENYLS In studies with a limited series of halogenated biphenyl congeners, the only compounds which induced AHH activity and bound to the receptor were the 3,3',4,4'-tetrachloro-, 3,3',4,4',5,5'-hexachloro- and the 3,3',4,4',5,5'-hexabromo- analogues (189, 196, 197). This suggests that biologically active halobiphenyls: (*a*) have halogen atoms in at least two adjacent lateral positions on each benzene ring and (*b*) lack halogen substitutions adjacent to the biphenyl bridge (the 2,2',6 or 6' positions) which lead to marked nonplanarity. These halogenated biphenyls are rather weak agonists, with receptor affinities and biological potencies at least 100-fold less than TCDD. In a series of five symmetrical hexachlorobiphenyls initially tested in chickens, only the planar (3,3',4,4',5,5'-hexachloro) isomer produced the characteristic toxic effects of edema and thymic involution (130). Subsequent studies on the toxicity of these and other isomers in a variety of species have supported the apparent requirement for planarity in a toxic isomer (72, 198–201). In support of the idea that planarity or near planarity is necessary for receptor binding, it was observed that 2,3,6,7-tetrachlorobiphenylene, a rigid planar analogue of 3,3',4,4'-tetrachlorobiphenyl, was comparable to TCDD in receptor affinity and potency to induce AHH activity (197). However, recent investigations by Aust and coworkers (202, 203) and Safe and coworkers (204–207) on a much larger series of bromobiphenyl and chlorobiphenyl isomers have modified this structure-activity relationship and demonstrated that planarity is not essential. Halobiphenyls with one and even two halogens substituted adjacent to the biphenyl bridge, producing marked nonplanarity, have been shown to bind to the receptor and induce AHH activity, albeit with biological potencies an order of magnitude lower than the planar isomers.

HALOGENATED NAPHTHALENES AND OTHER COMPOUNDS Goldstein et al (208) have shown that 2,3,6,7-tetrabromonaphthalene induces AHH activity; McConnell & McKinney (209) have reported that this compound also produces a characteristic pattern of halogenated aromatic hydrocarbon toxicity in the guinea pig. The 2,3,6,7-tetrachloro isomer, which has a smaller length, has a much lower potency to induce AHH activity (189). Whereas commercial chlorinated naphthalene mixtures produce chloracne in man (210) and X-disease in cattle (77), there has been no examination of the structure-activity relationship among pure isomers.

Steric analogues of TCDD, tricyclic compounds with four chlorine atoms in the lateral ring positions, such as anthracene, phenanthrene, phenoxathiin, *N*-acetylphenoxazine, and biphenylene, have been synthesized and shown to bind to the receptor and to induce AHH activity (211).

To generalize the structure-activity relationship for halogenated aromatic hydrocarbons, one may compare a prototype compound from each class—TCDD, 2,3,7,8-tetrachlorodibenzofuran, 3,3',4,4'-tetrachloroazoxybenzene, and 3,3',4,4'-tetrachlorobiphenyl. These compounds are all approximate isostereomers, they are planar or can assume a nearly planar configuration, and have molecular structures that roughly fit into a rectangle 3 X 10 Å with halogen atoms in the four corners of the rectangle. This model serves as a rough approximation, or mnemonic, for the generalized structure-activity relationship, but there are several exceptions that limit its universal applicability. First, 2,3,6,7-tetrabromonaphthalene is a smaller molecule; the distance separating the halogens is only 8.06 Å (189). Second, several halobiphenyl isomers with halogen-substitution adjacent to the biphenyl bridge (e.g. 2,3',4,4',5,5'-hexabromobiphenyl) which leads to marked nonplanarity of the rings are weak receptor agonists.

Perhaps the most serious limitation of this model is that it does not account for the polycyclic aromatic hydrocarbons and benzoflavones which bind to the same site. There have been a few attempts to explain the structure-activity relationship for halogenated dibenzo-*p*-dioxins (212, 213) and biphenyls (214, 215) in terms of molecular orbital parameters, which suggest that the polarizability of the carbon-halogen atoms may be important in receptor binding.

Questions Raised by the Receptor Mediated Model of Toxicity

The similarity of the structure-activity relationship for toxicity and that for receptor binding suggests that the halogenated aromatic hydrocarbons exert their toxicity through the cytosol receptor. However, many tissues *in vivo* and cell lines in culture that contain the receptor and respond to TCDD with the induction of AHH activity show no evidence of a toxic response. Thus, while the cytosol receptor may be essential for toxicity, it is not sufficient. Let us consider several questions raised by the hypothesis that toxicity is receptor-mediated.

IMPORTANCE OF THE INDUCTION OF AHH ACTIVITY, PER SE, IN TOXICITY Because of the central role of microsomal monooxygenase activity in metabolizing some foreign compounds to reactive electrophilic metabolites which covalently bind to tissue macromolecules and produce toxicity, it might be postulated that halogenated aromatic hydrocarbons, by

inducing cytochrome P_1-450 (and increasing the associated monooxygenase activities such as AHH), stimulate their own metabolism to toxic metabolites. This suggestion has numerous shortcomings: (*a*) TCDD, the prototype of this group of toxic chemicals, is slowly metabolized; the metabolites have only been identified in bile and excreta, never in tissues, and the maximal estimate of covalent binding of TCDD is so small that it approaches the limit of detection (93). (*b*) The structure-activity relationship for toxicity is for the parent compounds, and the chemical diversity of the various classes of halogenated aromatic hydrocarbons would presumably give rise to very different metabolites. (*c*) It is hard to imagine how toxic metabolites or covalent binding could produce the diverse pattern of toxic responses observed, especially the epithelial proliferation and metaplasia.

Alternatively one might argue that the stimulation of microsomal monooxygenase activity by TCDD and congeners might enhance the metabolism of some vital endogenous compound (e.g. increased steroid degradation and lower steroid hormone levels). While this may be true, it does not explain the entire spectrum of toxic responses observed. Thus, the induction of AHH activity may be viewed as a signal response, not implicated directly in the mechanism of toxicity.

CONCENTRATION AND AFFINITY OF THE RECEPTOR If we assume that TCDD (and congeners) exerts its toxic action through the cytosol receptor, variations in the affinity and/or concentration of the receptor in different tissues and different animals might explain the large quantitative differences in species sensitivity to TCDD (LD_{50}) or the qualitative differences in tissue specificity of animals. While the available data are limited, they do not support this view. The binding affinity and concentration of the cytosol receptor in liver from guinea pig, rat, responsive mouse (C57BL/6), rabbit, and hamster are very similar despite a 5000-fold difference in the LD_{50} for TCDD between guinea pig and hamster (A. Poland and E. Glover, unpublished data). One might of course, argue that since the critical target organs are unknown, comparisons of receptor concentrations in liver are irrelevant.

SEGREGATION OF TOXICITY WITH THE *AH* LOCUS The one instance where we know receptor affinity to differ is between responsive and nonresponsive strains of mice. If the toxicity of TCDD is mediated through the cytosol receptor, then one would expect that mice with a high affinity receptor which are sensitive to induction of AHH activity should be sensitive to toxicity, and conversely, nonresponsive strains, with a lower affinity receptor, should be less sensitive to toxicity. This is the case, and will be discussed below.

POLYCYCLIC AROMATIC HYDROCARBONS VS HALOGENATED AROMATIC HYDROCARBONS While both classes of compounds bind to the cytosol receptor and induce AHH activity, the characteristic spectrum of toxic responses are observed only with halogenated compounds. If toxicity is mediated through the receptor, one must explain this discrepancy. One might postulate that enzyme induction is an early event, but that toxicity (especially epithelial cell proliferation and metaplasia) is a much later event and requires persistent receptor occupation and gene expression. In most tissues the polycyclic aromatic hydrocarbons are rapidly metabolized and do not persist. In an appropriate situation, a tissue or cell type which expresses toxicity to TCDD and which has low capacity to metabolize polycyclic aromatic hydrocarbons, one would expect these latter compounds to produce a toxic response similar to TCDD.

Segregation of Toxicity with the Ah Locus

As noted above, if toxicity of TCDD is mediated through the receptor then one would expect toxicity to segregate with the *Ah* locus, the gene that determines the cytosol receptor.

THYMUS INVOLUTION TCDD produced a dose-related decrease in the thymus weight in C57BL/6, DBA/2, and hybrid B6D2F$_1$ (201). The dose-response curve for thymic weight loss in C57BL/6 (responsive mice) was about an order of magnitude more sensitive than that in DBA/2 (nonresponsive) mice, and the heterozygote B6D2F$_1$ mice (Ah^b Ah^d) showed an intermediate sensitivity. In the genetic backcross B6D2F$_1$ X DBA/2, the heterozygous responsive mice (Ah^bAh^d) were more sensitive to thymic involution than were the nonresponsive mice (Ah^dAh^d). Thus thymic involution does segregate with the *Ah* locus. The cytosol receptor is demonstrable in the thymus of C57BL/6 mice, but not in DBA/2 mice.

TCDD induces AHH activity in the thymus, but both the control and induced monooxygenase activity are very low, more than 1000-fold less than in the liver. Thus, the thymus, a tissue which develops toxic response to halogenated aromatic hydrocarbons, and which has a very low monooxygenase activity, afforded an opportunity to test whether polycyclic aromatic hydrocarbons could mimic this response. MC did produce a dose-related thymic involution, and β-naphthoflavone also produced a modest decrease in thymic weight (201, 216).

TERATOGENESIS Courtney & Moore (86) first reported that TCDD was teratogenic to mice with primarily two responses observed—cleft palate formation and hydronephrosis—and they further noted C57BL/6 mice were more sensitive than DBA/2 mice. Poland & Glover (201) found that

cleft palate formation produced by TCDD followed the strain distribution of the *Ah* locus; a dose of 30 μg/kg produced a 0–3% incidence of cleft palate formation in five nonresponsive inbred strains of mice, and 54–95% incidence of cleft palates in 4 of 5 responsive strains. The one exception, CBA mice, a responsive strain with low incidence to cleft palate formation, is also resistant to cortisone-induced cleft palates (217) and may have overriding genetic or developmental factors that make them resistant to cleft palate formation from a variety of stimuli.

HEPATIC PORPHYRIA Jones & Sweeney (138, 140) reported that (*a*) TCDD produced a decrease in uroporphyrinogen decarboxylase activity and caused hepatic porphyria with an increased urinary porphyrin excretion in mice, (*b*) that with a given dose of TCDD, these changes were greater in C57BL/6 mice than in DBA/2 mice, and (*c*) these effects segregated with the *Ah* locus in the B6D2F_1 X DBA/2 backcross. Sweeney et al (133) further observed that the histologic lesions in the liver, the hepatic porphyria, and the decrease in uroporphyrinogen decarboxylase activity produced by TCDD in C57BL/6 mice were significantly reduced by iron deficiency. But iron depletion did not prevent TCDD-produced thymic involution in C57BL/6 mice, nor epidermal hyperplasia and chloracne in HRS/J (*hr/hr,* hairless) mice (218). This suggests that while iron may be important in the expression of some of the hepatic toxicity produced by TCDD, it does not effect other characteristic toxic responses.

In summary two independent lines of evidence suggest the toxicity of halogenated aromatic hydrocarbons is mediated through their binding to the cytosol receptor: (*a*) the correlation of the structure-activity relationship for receptor binding and that for toxic potency, and (*b*) segregation of three toxic responses produced by TCDD in the mouse with the *Ah* locus.

EXPERIMENTAL MODELS OF TOXICITY

Investigation of Toxicity In Vitro

Investigation of the toxicity of halogenated aromatic hydrocarbons in an in vitro cell culture system would permit a more thorough characterization of the sequential events that comprise toxicity in a homogeneous cell population. Finding an in vitro system in which to study toxicity may not be simple, as suggested by several observations from toxicity studies in whole animals, namely: (*a*) the histologic changes produced by TCDD in a given tissue often occur in few animal species, (*b*) most histopathologic changes develop over a time period of days to weeks, and may not be seen in short term culture, and (*c*) toxicity in some tissues (thymus, seminiferous tubules, and bone marrow) may involve necrosis, atrophy, or hypoplasia, but in

other tissues, one observes hyperplasia and metaplasia. Thus, the effects of TCDD in vitro may vary with the cell type.

Over 30 cell types, primary cultures, established and transformed cell lines, from at least six animal species, and a variety of tissues (liver, kidney, lymphocytes, embryonal teratomas, and fibroblasts) have been examined for their response to TCDD [(62, 174, 219, 220); J. Knutson & A. Poland, unpublished observations]. A number of these cell types were inducible for AHH activity, indicating the presence of the cytosol receptor. Surprisingly, there have been no observations of cell toxicity, with the exception of a report by Niwa et al (174) in which several cell lines exposed to a very high dose of TCDD (5×10^{-6} M) for 24 hours showed a decreased viability as measured by trypan blue uptake. One can draw few conclusions from these studies except to speculate that (*a*) the true target cells were not cultured, (*b*) cells potentially susceptible to TCDD toxicity were cultured in an altered state of differentiation or were maintained under conditions which prevented expression of toxicity, or (*c*) toxicity may require the interaction of two different cell types, interacting directly or through some humoral factor.

XB CELLS XB, a cloned mouse teratoma cell line isolated and characterized by Rheinwald & Green (221), is the only cell line to date in which halogenated aromatic hydrocarbons produce a dose-related, characteristic "toxic" response. When XB cells are plated at low density (2×10^2 cells/60 mm plate) on a feeder layer of lethally irradiated 3T3 cells, XB cells form colonies of stratified, keratinized squamous epithelium, like that in epidermis. When XB cells are plated at high density (3×10^5 cells) on a feeder layer of irradiated 3T3 cells, they divide, but do not spontaneously differentiate. Under these conditions of high XB cell density (which prevent spontaneous differentiation), the addition of TCDD produces a keratinization of the cultures which is first detectable at 7 days and maximal by 10–13 days (195). TCDD produced a dose-related keratinization, with maximal response at 5×10^{-11} M. XB cells cultivated at high density in the absence of 3T3 cells, but in media in which 3T3 cells had been previously cultured, will undergo terminal differentiation in response to TCDD. Thus, TCDD acts directly on XB cells. XB cells contain a cytosol species which binds TCDD with a high affinity, and display induction of AHH activity in response to TCDD and other receptor agonists. For over 30 halogenated aromatic hydrocarbon congeners tested, there was an excellent correlation between the potencies of the compounds to produce keratinization and their binding affinities for the cytosol receptor. For 11 nonhalogenated aromatic hydrocarbons, e.g. the polycyclic aromatic hydrocarbons and benzoflavones, their capacity to produce keratinization in XB cells corresponded to their capacity to bind to the cytosol receptor. If XB cells are cultured in

the absence of the 3T3 feeder layer, they respond to TCDD with induction of AHH activity, but do not undergo keratinization.

The XB cell culture affords the first in vitro model in which to study halogenated aromatic hydrocarbons. The terminal differentiation observed in XB cells is very similar to the "toxic" response these compounds produce in vivo in the epidermis of some animals. We can draw several conclusions from these studies on XB cells: (*a*) TCDD and congeners act directly on this cell type, and the keratinization response is mediated through the cytosol receptor, (*b*) nonhalogenated agonists for the receptor, under appropriate conditions, can mimic the "toxic" response produced by TCDD, and (*c*) one can dissociate the induction of AHH activity from cell differentiation ("toxicity").

HRS/J Mice: An In Vivo Model of Epidermal Toxicity

Further insight into the mechanism of toxicity of halogenated aromatic hydrocarbons comes from examination of chloracne in hairless mice.

Hairless is a recessive trait (*hr/hr*) in mice controlled by a locus on chromosome 14. Inagami et al (74) first noted that administration of rice oil which was contaminated with polychlorinated biphenyls to hairless mice produced chloracne (epidermal hyperplasia, hyperkeratosis, and sebaceous gland metaplasia with the formation of keratinaceous cysts). We have routinely used this response in hairless mice to screen halogenated aromatic hydrocarbon congeners for toxic potency. HRS/J is an inbred strain of mice segregating for the hairless locus, maintained by breeding hairless (*hr/hr*) males with heterozygous, haired (*hr/+*) females. HRS/J (*hr/hr*) are genetically identical to their (*hr/+*) littermates except for one allele at the *hr* locus.

HRS/J mice are responsive at the *Ah* locus, i.e. have a high affinity receptor. For both *hr/hr* and *hr/+* mice, (*a*) the binding affinity and concentration of the cytosol receptor is very similar (measured in liver), and (*b*) skin painting with TCDD induces epidermal AHH activity with a similar time-course and dose response curve (A. Poland & E. Glover, unpublished observations). However only *hr/hr* mice respond to TCDD with an epidermal hyperplasia, hyperkeratosis, and squamous metaplasia, i.e. chloracne. The failure of shaved *hr/+* mice to develop chloracne does not appear to be due to hair (or normal hair follicles) per se, because (*a*) application of TCDD to the ear of haired mice, an area devoid of hair, does not produce chloracne and (*b*) in the rabbit, application of halogenated aromatic hydrocarbons produces epidermal hyperplasia in both the inner part of the pinna (which has minimal hair) and on the haired area of the back (49).

Thus while both genotypes *hr/hr* or *hr/+* (or mice which are wild-type (+/+) at the hairless locus) recognize TCDD and respond with a limited

pleiotropic response, e.g. the induction of epidermal AHH activity and other coordinately expressed enzymes, only *hr/hr* mice express an additional pleiotropic response which results in cell division and differentiation in the skin. Since *hr/hr* and *hr/+* mice differ at only one locus, *hr/+* mice presumably have the genes necessary for the hyperplastic-metaplastic response, but they fail to express them in response to TCDD. In Figure 2, we have diagrammed this model. In the skin of *hr/+* (or +/+) mice, TCDD or other halogenated aromatic hydrocarbons are recognized by the cytosol receptor and induce a limited pleiotropic response; however, in *hr/hr* mice, an additional battery of genes are expressed, which are normally restricted in *hr/+* (or +/+) mice.

A General Model of Toxicity

One observation in XB cell culture is analogous to the difference in response to TCDD seen in *hr/hr* and *hr/+* mice. XB cells cultured alone in fresh media respond to TCDD with induction of AHH activity. However, when XB cells are cultured at high density on a feeder layer of 3T3 cells, or in media in which 3T3 cells had previously grown, they respond to TCDD

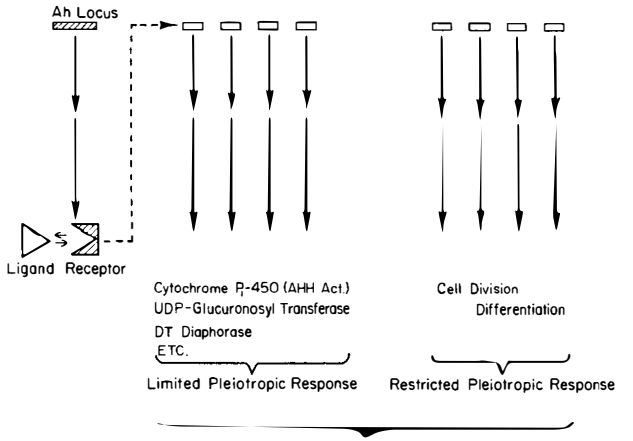

Figure 2 A proposed model of the coordinate gene expression controlled by the *Ah* locus. The *Ah* locus is the structural gene for the receptor. The cytosol receptor binds the ligand (e.g. halogenated aromatic hydrocarbon) and the receptor-ligand complex translocates to the nucleus and mediates the ensuing gene expression. In the skin of HRS/J (*hr/+*) mice, there is a limited pleiotropic response, consisting primarily of the expression of enzymes related to drug metabolism (shown on the left side of the diagram). In the epidermis of HRS/J (*hr/hr*) mice, there is a larger pleiotropic response, with the additional activation of genes involved in cell division and differentiation. These genes are restricted in (*hr/+*) mice. The same general model is proposed to distinguish cells and tissues which respond with enzyme induction (limited pleiotropic response) and those which respond with "toxicity" (the additional expression of normally restricted genes).

with induction of AHH activity and terminal differentiation. A limited response to TCDD is converted to a more extensive response involving differentiation; in HRS mice by a mutation, and in XB cells by the culture conditions.

These observations suggest a more general model in which to consider the toxicity of halogenated aromatic hydrocarbons, and which would account for the species differences in many of the specific tissue responses. One might envision that many tissues (and cell lines in vitro) respond to TCDD with a limited pleiotropic response (e.g. induction of AHH activity and other enzymes primarily related to drug metabolism), but tissues (or cells in culture) which show "toxic" responses, i.e. involution, differentiation, or proliferation, express an additional battery of genes. These additional genes under control of the cytosol receptor would presumably not be the same in all tissues. While this model may be useful in considering the toxicity of halogenated aromatic hydrocarbons, it must be emphasized that we still do not know (*a*) the target tissue, the dysfunction of which results in death, or (*b*) the nature of the ultimate biochemical lesion.

In conclusion we would like to briefly consider the induction response and its physiologic role. Classically, the induction of cytochrome P-450 and other drug metabolizing enzymes by foreign lipophilic chemicals is viewed as an adaptive physiologic response, a work load hypertrophy, the "purpose" of which is to increase the rate of metabolism of the foreign compound and hasten its elimination from the body. In this view, whether one believes the receptor recognizes only foreign compounds, or has an endogenous physiologic ligand (e.g. a hormone), that which is being regulated is the drug metabolizing enzymes. As we have seen, however, in certain tissues halogenated aromatic hydrocarbons or nonhalogenated agonists for the cytosol receptor produce a much broader response which may result in cell division, differentiation, and/or involution and atrophy. This suggests that the cytosol receptor may have arisen not just to control the drug metabolizing enzymes, but to regulate these other responses, and that there exists, or at some point in evolution there existed, a physiologic ligand. We presume that the toxicity of the halogenated aromatic hydrocarbons is due to the sustained pleiotropic response they produce, and that the physiologic ligand exerts (or did exert) a more transient effect. Because of the species-specific nature of many of the toxic responses to TCDD, one might speculate that these often restricted functions controlled by the receptor are now vestigial. Investigations on the mechanism of toxicity of halogenated aromatic hydrocarbons suggest that the present, almost Ptolemaic preoccupation with the regulation of cytochrome P-450 may be unwarranted, and may preclude our understanding of the larger regulatory response controlled by the cytosol receptor.

SUMMARY

In this review, we have examined the biochemical and toxic responses produced by halogenated aromatic hydrocarbons and have tried to develop a model for their mechanism of action. These compounds bind to a cellular receptor and evoke a sustained pleiotropic response. In many tissues this response consists of the expression of a battery of enzymes which are, for the most part, involved in drug metabolism, but in other tissues, those which develop toxicity, an additional set of genes is expressed which effects cellular involution, division, and/or differentiation. The toxicity of these compounds appears to be due to the sustained expression of a normal cellular regulatory system, of which we were previously unaware. In future investigations it is hoped that we will learn the nature and physiologic role of this regulatory system. Only then can we hope to understand the mechanism of toxicity of these compounds.

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