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REVIEW ARTICLE

The *Ah* receptor and the mechanism of dioxin toxicity

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INTRODUCTION

Over the last several decades, dioxins have become the subject of intense public and scientific scrutiny. This is the result of not only their widespread presence in the environment but also their great toxicity. The environmental issue has been addressed through the study of the production, release and fate of dioxins and related substances, as well as the development of analytical techniques to detect and quantify these compounds in environmental matrices. The toxicology of dioxins has been addressed principally through studies of their mechanism of toxic action using animal models and is the focus of this review. In addition, the potential threat that dioxins present to human health has been addressed in a limited manner through epidemiological studies of populations known to have been exposed to dioxins.

TCDD AND RELATED CHEMICALS IN THE ENVIRONMENT

The term 'dioxin', as commonly used by the news media, is a shorthand for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Fig. 1, structure *a*). TCDD is only one member (congener) of the polychlorinated dibenzo-*p*-dioxin family, of which there are 75 possible congeners whose structures vary according to the number and location of the chlorine atoms. A source of confusion is that the term 'dioxin' is used to indicate either TCDD specifically, or the PCDD family in general. Biologically, TCDD is the most potent PCDD; most other PCDDs are less active by a factor ranging from several thousands to millions. TCDD has been studied most extensively of all the PCDD congeners, and will form the major focus of this review.

Several other aromatic hydrocarbons share biological properties with TCDD, particularly when substituted with chlorine in the lateral positions (Fig. 1). The most important of these are the polychlorinated dibenzofurans (PCDFs) and certain members of the polychlorinated biphenyl family (PCBs). The large number of possible PCDD (75), PCDF (135) and PCB (20) congeners greatly complicates environmental analysis, and complex clean-up procedures are required before such analyses can be undertaken. The environmental chemistry of PCDDs has recently been reviewed in detail by Fiedler *et al.* (1990).

TCDD came to scientific and public attention in the early 1970s in connection with the use of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) derivatives as herbicides and defoliants, notably through forest spraying programmes in the U.S. and in Viet Nam. For example, the formulation known as 'Agent Orange' was a 1:1 mixture of the *n*-butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-T. The suspicion that 2,4,5-T might cause birth defects was first publicized by Whiteside (1970) and, shortly after, confirmed experimentally in rodents (Courtney *et al.*, 1970). These observations initiated an intensive research effort to evaluate the toxicological properties of 2,4,5-T. Several independent studies revealed that teratogenicity was not caused by 2,4,5-T itself, but by TCDD (Collins *et al.*, 1971; Courtney & Moore, 1971; Sparschu *et al.*, 1971), a contaminant that forms during the commercial synthesis of 2,4,5-trichlorophenol (Milnes, 1971). TCDD toxicity in poultry was recognized at about the same time, when the disease known as 'chick oedema disease' or 'toxic fat syndrome' (from its association with recycled fats in broiler chicken feed) was shown to be caused by TCDD (Flick *et al.*, 1973).

TOXIC AND BIOLOGICAL EFFECTS OF TCDD

The molecular properties of TCDD (chemically unreactive, non-polar, lipid-soluble) make it typical of compounds that would be bioconcentrated from aqueous solution and stored in fatty tissue. In rodents, TCDD has a whole-body half-life of the order of weeks (Leung *et al.*, 1990), which is short in comparison with the 5.8 year half-life for TCDD reported for a single male human (Poiger & Schlatter, 1986). The toxic and biological effects of TCDD and related toxic halogenated aromatic hydrocarbons depend on a number of factors, such as the dose of the toxin, the route of administration, and the species, age, strain, and sex of the animals (reviewed in Poland & Knutson, 1982; Safe, 1986). An intriguing aspect of TCDD-induced toxicity is the large species-dependence in the susceptibility to this chemical. Among rodents, for example, the LD_{50} (lethal dose for 50% of the test population) varies over at least a 2500-fold range, from the highly sensitive guinea pig to the comparatively resistant hamster (Kociba & Schwetz, 1982). This large variation in TCDD sensitivity between the guinea pig and hamster is not simply the result of differences in the rate of *in vivo* metabolism

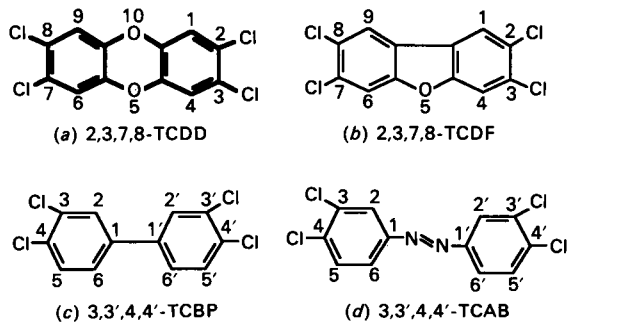

Fig. 1. Chemical structure and associated ring numbering system for the tetrachloro congeners of PCDD and several other structurally-related chemical families

TCDD, tetrachlorodibenzo-*p*-dioxin; TCDF, tetrachlorodibenzofuran; TCBP, tetrachlorobiphenyl; TCAB, tetrachloroazobenzene.

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofurans; PCB, polychlorinated biphenyl; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; AHH, aryl hydrocarbon hydroxylase; δ-ALA, δ-aminolaevulinic acid; EGF, epidermal growth factor; DRE, dioxin responsive enhancer; SRE, steroid responsive element.

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Table 1. Biological responses to TCDD

Biochemical response	References
Enzyme induction	
Glutathione *S*-transferase	Baars *et al.* (1978)
Glucuronosyl transferase	Owens, (1977), Thunberg *et al.* (1984)
DT-diaphorase	Beatty & Neal (1976)
Ornithine decarboxylase	Nebert *et al.* (1980)
δ-Aminolaevulinic acid synthetase	Poland & Glover (1973*a*,*b*)
Epidermal transglutaminase	Puhvel *et al.* (1984)
Hepatic DNA polymerase B	Kurl *et al.* (1982)
Modulation	
Steroid metabolism enzymes	Mebus *et al.* (1987)
Thyroid hormone levels	Potter *et al.* (1986), Kelling *et al.* (1987)
Receptor levels	
EFG receptor	Madhukar *et al.* (1984)
Glucocorticoid receptor	Ryan *et al.* (1987)
Oestrogen receptor	Romkes & Safe (1988), Umbreit & Gallo (1988)
Progesterone receptor	Romkes & Safe (1988)
Ah receptor	Sloop & Lucier (1987), Okey & Vella (1984), Denomme *et al.* (1986), Landers *et al.* (1990)

of TCDD, since the whole body half-life for TCDD only differs by 3-fold between these two species (Olson *et al.*, 1980; Gasiewicz & Neal, 1979*a*,*b*). A striking feature of TCDD's acute toxicity is that it does not cause death immediately. Animals suffer anorexia, and waste away over several days to weeks. The mechanism of feed refusal is unknown, but apparently does not involve a direct effect of TCDD on the brain (Stahl & Rozman, 1990).

The susceptibility of humans to TCDD is not known. Exposed subjects have reported a wide variety of lesions and symptoms. It has been difficult to attribute human deaths to TCDD with certainty, although a recent retrospective study of workers exposed occupationally to TCDD suggests an increase in soft-tissue carcinoma, with a latency period of 20 years (Fingerhut *et al.*, 1991). More work will be needed to assess the extent to which TCDD represents an environmental health threat. As noted by Silbergeld & Gasiewicz (1989), the ubiquitous occurrence of TCDD complicates the comparison between exposed and allegedly unexposed populations. It seems reasonable to conclude at this time that humans are probably less susceptible to the effects of TCDD than most laboratory rodents, and it is certainly not justifiable to present the human risk from dioxin exposure as if it were the same as in the most susceptible species, the guinea pig (LD_{50} 1 μg/kg). However, there is not sufficient evidence to determine whether, as in certain laboratory animals, TCDD will be fetotoxic and/or carcinogenic to humans (Suskind & Hertzberg, 1984; Colton, 1986; Hoar *et al.*, 1986; Hoffman *et al.*, 1986; Mocarelli *et al.*, 1986).

The toxic effects of TCDD in humans have been gauged primarily from the exposure of workers in TCP (2,4,5-trichlorophenol) and 2,4,5-T factories either during routine production or during post-accident contamination of the factory and surrounding area (May, 1973). There have been numerous incidents during the past several decades involving varying degrees of both human exposure and contamination of the surrounding area. A 1949 accident at a TCP manufacturing plant in Nitro, West Virginia, involved the exposure of a number of workers to TCP and TCDD (although the involvement of TCDD was not recognized at the time). The 228 people affected showed symptoms which included chloracne, nausea, vomiting, headaches, severe muscle ache and pain, fatigue, emotional instability and intolerance to cold (Huff *et al.*, 1980). Of the industrial accidents, that at Seveso, Italy (in 1976) has been the most closely documented. Several residents developed chloracne, the severity of which paralleled the blood levels of TCDD but, to date, no evidence for excess cancer, miscarriage, birth defect or other adverse effects have been uncovered (Mastroiacova *et al.*, 1988). The inadvertent exposure of residents in Oregon to 2,4,5-T in forest spraying programmes, and of U.S. servicemen in Viet Nam to Agent Orange (which contained 2,4,5-T), has been very controversial. Oregon mothers complained of excess incidence of miscarriage and birth defects, allegations which have been successively upheld and repudiated on statistical analysis. Similarly, Viet Nam veterans have complained of a variety of physical and psychological symptoms. Among the difficulties in attributing any of the alleged effects to TCDD is excluding those caused by exposure to 2,4,5-T, the carrier solvent, or to exposure to unrelated chemical agents. No unambiguous case for TCDD-induced damage to human health can be made from these exposures.

In addition to overt physical symptoms, PCDDs and PCDFs also cause biochemical effects in both mammals and mammalian cells in culture (Table 1). One of the most notable and characteristic responses is the induction of microsomal benzo[*a*]pyrene hydroxylase (aryl hydrocarbon hydroxylase, AHH) and several related cytochrome *P*-450-dependent mono-oxygenases (reviewed in Safe, 1986; Okey, 1990). The induction of drug-metabolizing enzymes serves the biological role of converting the inducer into a more water-soluble form so that it may be excreted. In terms of the induction of AHH and δ-ALA (δ-aminolaevulinic acid) synthetase activities, TCDD is orders of magnitude more potent than other inducers, such as 3-methylcholanthrene (MC) (Poland & Glover, 1973*a*,*b*). TCDD is the most potent member of the PCDD/PCDF families. Structure–activity trends indicate that induction (and toxicity) is associated mainly with congeners substituted in all of the 2,3,7 and 8 positions: these effects diminish by the addition of chlorine in non-lateral positions or removal of chlorine from the lateral ones. One hypothesis (Rifkind *et al.*, 1990) for the slow toxicity of TCDD is that the elevated levels of cytochrome *P*-450 induced by the toxicant increase the rate of oxidation of membrane fatty acids, and hence disrupt the integrity of cell membranes.

MECHANISM OF ACTION

A comprehensive mechanism describing the mode of TCDD action in animals would have to account, on a molecular basis, for all events from the initial exposure to the chemical through to the development of overt toxic symptoms. It must account not only for the toxic and biological effects on several organ systems (e.g. lymphoid, hepatic, reproductive, etc.), but also for interspecies effects, and for biochemical phenomena such as the alteration of the levels of drug-metabolizing enzymes (Grieg, 1972; Grieg & De Matteis, 1973).

A large body of evidence suggests that the biological and toxic responses associated with TCDD and similar compounds are not the result of direct insult of the toxin. No evidence has been presented to substantiate the TCDD-induced formation of covalent adducts with proteins or nucleic acids (Poland & Glover, 1979; Rose *et al.*, 1976; Nolan *et al.*, 1979) or direct damage to cellular DNA (Shu *et al.*, 1987). However, many of the toxic effects of TCDD have been shown to be mediated through a specific protein known as the *Ah* (aryl hydrocarbon) receptor. The sequence of events associated with a receptor-mediated mechanism can be rationalized in simple terms to involve (*a*)

entry of the toxicant into the cells, (*b*) binding of the toxicant to the *Ah* receptor, (*c*) binding of the receptor–ligand complex to DNA recognition sites, (*d*) expression of specific genes and the translation of their protein products, and (*e*) mode of action of the expressed proteins. A large gap exists in our knowledge between the initial events (*a–c*), which are understood reasonably well at the molecular level, and the final expression of overt toxicity (*d,e*).

Entry of TCDD into the cell

It is generally assumed that TCDD enters the cell by passive diffusion. Although there is no evidence for an 'active' transport mechanism, several studies indicate that passive diffusion does not account completely for the responses to this chemical. TCDD has been shown to stimulate cell growth, fatty infiltration of the liver, and hyperplastic proliferation of epidermal cells (Knutson & Poland, 1982, 1984; Gierthy & Crane, 1984). Based on these observations, Greenlee & Neal (1985) have suggested that the membrane may play a role in TCDD toxicity. Matsumura and coworkers have carried out extensive studies on the direct effects of TCDD on the plasma membranes of rat hepatocytes. Early studies demonstrated a marked decrease in plasma membrane ATPase activity (Peterson *et al.*, 1979) and subsequent evaluation of the specific protein components showed a significant reduction in both the concentrations (Brewster *et al.*, 1982) and activities (Bombick *et al.*, 1985; Brewster & Matsumura, 1984) of lipoprotein lipase and low-density-lipoprotein receptor following exposure to TCDD.

The epidermal growth factor (EGF)-like effects of TCDD have also been attributed to its ability to affect membrane components. For example, TCDD up-regulates plasma membrane EGF receptor levels (Madhukar *et al.*, 1984) and increases the activity of protein kinase C which is thought to activate EGF receptor through phosphorylation (Bombick *et al.*, 1985). More recent studies have shown that TCDD not only activates protein kinase C but also phospholipase C (Beebe & Barsotti, 1989). In terms of overt toxicity, it is conceivable that the EGF-like effects of TCDD may be related to dermal lesions such as chloracne, but this cannot be substantiated at present.

Early evidence for a receptor protein

Early studies of genetic polymorphism in mice showed that MC was capable of inducing AHH activity in the 'responsive' C57BL/6 inbred mouse strain but not in the 'nonresponsive' DBA/2 inbred mouse strain (Nebert *et al.*, 1972; Thomas *et al.*, 1972). Crossbreeding studies demonstrated that the 'responsive' phenotype segregated as a dominant trait and, similarly to other aromatic hydrocarbon-induced responses, was governed by a single autosomal gene. This 'locus', apparently controlling a variety of responses to aromatic hydrocarbons, was deduced to be regulatory in nature and perhaps to encode a protein that regulated response(s) to this family of compounds.

The ability of TCDD to induce AHH and δ-ALA synthetase led Poland & Glover (1973*a*) to postulate the existence of an 'induction receptor' which could act as the transmembrane signal, ultimately triggering the elevation of enzyme and other activities. This hypothesis provided an economical explanation for the sustained toxicity of TCDD, and several observed similarities between the toxic effects of glucocorticoids and certain PCDDs. For example, both have been shown to cause lymphoid involution (Kociba & Schewtz, 1982; Lowrence, 1984), teratogenicity in mice (Pratt *et al.*, 1984) and the induction of characteristic P-450-dependent mono-oxygenases (Poland *et al.*, 1979).

The synthesis of radiolabelled TCDD led to the identification of a C57BL mouse liver protein which specifically bound TCDD, and exhibited all of the properties of a receptor (Poland *et al.*, 1976). By analogy with the mechanism of action of certain steroid hormones, it was suggested that TCDD would passively cross the plasma membrane and bind to a cytosolic form of this receptor. This association would induce the transformation of the receptor to a form which could translocate to the nucleus, bind with high affinity to specific DNA sequences, and stimulate transcription (Fig. 2). The importance of the discovery of this receptor, known as the aryl hydrocarbon (*Ah*) receptor (or occasionally as the dioxin receptor), is in providing a link between the action of TCDD at the molecular level and observable phenomena such as overt toxicity or changes in enzyme levels. In fact, current opinion is that all known effects of TCDD are probably *Ah*-receptor-mediated (Roberts, 1991).

One of the many unresolved questions in dioxin toxicology asks why organisms should possess a receptor for TCDD at all. One suggestion is that the *Ah* receptor evolved to detoxify the products of combustion (fire) such as benzo[*a*]pyrene which, like TCDD, binds to the *Ah* receptor with high affinity and also triggers the production of several mixed function oxidases. This would imply that the binding of TCDD to the *Ah* receptor is coincidental, with TCDD assuming the role of some other exogenous ligand such as benzo[*a*]pyrene. Jakoby & Ziegler (1990) have pointed out that the enzymes responsible for detoxification of xenobiotics have evolved to handle a wide range of substrates, unlike the familiar hydrolytic and oxidative enzymes which are characterized by pronounced substrate specificity. Alternatively, TCDD may substitute for an endogenous ligand with an important, as yet undefined, physiological role. The search for the identity and function of this elusive 'natural' ligand is the topic of intense study at the present time (Perdew & Babbs, 1989).

A great deal of insight into the properties and mechanism of action of the *Ah* receptor has been obtained by comparison with the more extensively studied steroid hormone receptors. The *Ah* and the steroid hormone receptors share many properties; however, they have been shown to be distinct entities by the lack of affinity of TCDD for steroid hormone receptors (Neal *et al.*, 1979) and of steroid hormones for the *Ah* receptor (Poland *et al.*, 1976). Furthermore, monoclonal antibodies prepared against the rat liver glucocorticoid receptor do not cross-react with the *Ah* receptor from the same tissue (Poellinger *et al.*, 1983). One suggestion is that the *Ah* receptor may be some type of mutated steroid hormone receptor, but the possibility of common ancestry cannot be resolved at the present time.

Species and tissue specificity

The *Ah* receptor protein has been identified in the tissues from several mammalian and non-mammalian species. Receptor concentrations are commonly determined by labelling the receptor with radiolabelled TCDD and measuring the specifically bound radioactivity, either by sucrose gradient centrifugation (Okey *et al.*, 1979) or by the hydroxyapatite adsorption assay (Gasiewicz & Neal, 1982). Species showing relatively high concentrations of *Ah* receptor (10–100 fmol/mg of cytosolic protein) include numerous inbred strains of rodents, rabbits, ground hogs, sheep, cats, ferrets, certain birds, and primates (Denison *et al.*, 1986*a*), and trout (Lorenzen & Okey, 1990*a*). Species that have been reported not to possess detectable levels of *Ah* receptor are frogs, cows, armyworms, turkeys, pigeons, and salmon (Denison *et al.*, 1986*a*). However, it is possible that the *Ah* receptor is not detectable in some or all of these species due to limitations of the *in vitro* assay methods (e.g. unstable forms of the receptor). This is exemplified by studies on genetically responsive and unresponsive mice.

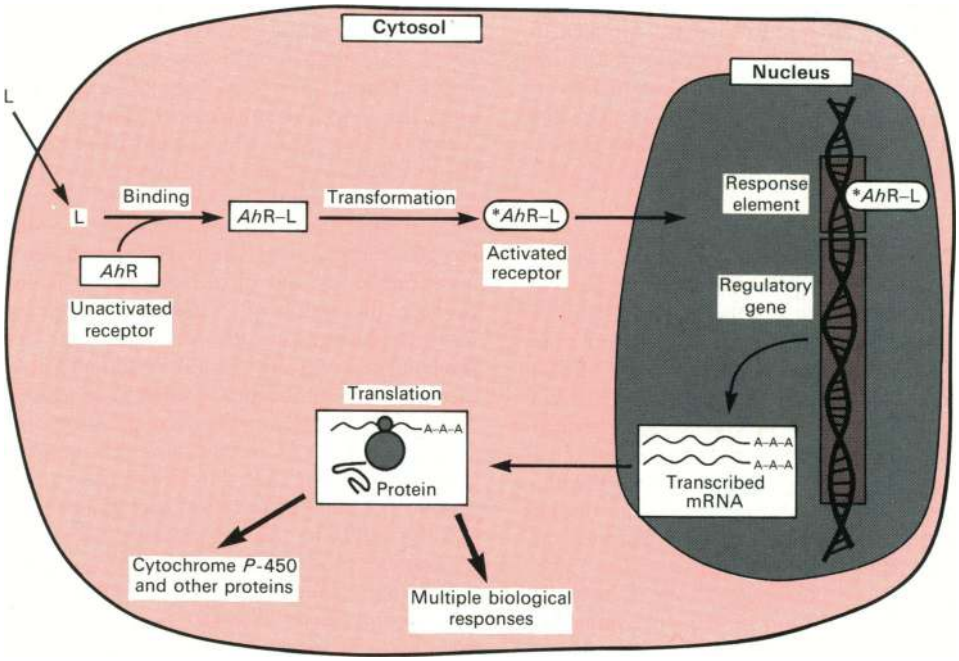

Fig. 2. Proposed mechanism of action for TCDD and structurally-related chemicals

Ligand (L) passively enters the cell where it encounters and binds to the *Ah* receptor protein (*Ah*R). The receptor-ligand complex (*Ah*R–L) 'transforms' to a DNA-binding form that can enter the nucleus (**Ah*R–L). Interaction with specific genomic sequences (regulatory elements) results in the enhanced transcription of several genes. The transcribed mRNA is translated in the cytosol, resulting in the synthesis of several cytochrome *P*-450s and a multitude of other biological responses.

The C57BL/6J mouse strain has been found to possess relatively high hepatic levels of the *Ah* receptor (40–120 fmol/mg of protein), and is very susceptible to TCDD-induced toxicity (Okey *et al.*, 1984; Carlstedt-Duke, 1979; Gasiewicz & Rucci, 1984). By contrast, early studies showed the DBA/2 strain to possess low hepatic levels of receptor (< 1 fmol of receptor/mg of cytosolic protein) and to be relatively resistant to TCDD. Poland and coworkers found that the median effective dose (ED_{50}) for AHH induction by 2,3,7,8-TCDD was approximately 20-fold higher in DBA/2 mice than in C57BL/6 mice (Poland *et al.*, 1974). More recent studies (Okey *et al.*, 1989) have shown that moderate levels of *Ah* receptor can indeed be found in DBA/2 mouse liver, but that stabilization of the receptor by molybdate is required. This is similar to the stabilizing effect of molybdate on the glucocorticoid and oestrogen receptors. In addition, TCDD binds less strongly to the DBA/2 receptor than to the C57BL/6 receptor, perhaps mirroring the lesser susceptibility of DBA mice to this toxin.

The *Ah* receptor has also been identified in several human tissues and cells in culture including lung, liver, kidney and placenta (Cook *et al.*, 1987; Greenlee & Neal, 1985; Gillner *et al.*, 1989; Roberts *et al.*, 1985; Harper *et al.*, 1988; Manchester *et al.*, 1987). Recently, *Ah* receptor has been identified in human B lymphocytes (Waithe *et al.*, 1990) and in human tonsils (Lorenzen & Okey, 1990*b*). The latter finding is consistent with the recent detection of *Ah* receptor in human thymic epithelial cells in culture (Cook & Greenlee, 1989). The *Ah* receptor from human tissue, like the receptor from the DBA/2 mouse liver, requires molybdate as a stabilizing factor.

In much the same way that steroid hormone receptors show tissue specificity, the *Ah* receptor has been detected in hepatic and several non-hepatic tissues that are susceptible to TCDD. In rats, the *Ah* receptor was detected in thymus, lung, liver, kidney, brain, testis and skeletal muscle, but not in the pancreas, adrenal glands or ventral prostate (Carlstedt-Duke, 1979).

Intracellular localization

Although the *Ah* receptor is frequently referred to as cytosolic, its location in the intact cell is unresolved. Broken-cell experiments have identified the unbound *Ah* receptor primarily in the cytosolic fraction (Okey *et al.*, 1979) and the receptor–ligand complex in the nuclear fraction (Okey *et al.*, 1980). However, these findings must be interpreted with caution, since the localization of the unbound *Ah* receptor to the cytosol or nucleus depends on the fractionation and homogenization procedure (Denison *et al.*, 1986*b*). Gudas *et al.* (1986) found the *Ah* receptor in mouse hepatoma cells in culture to be localized to the cytosol by using cytochalasin B enucleation, a technique which has proved to be successful with oestrogen receptor localization. Alternatively, the *Ah* receptor may be neither completely cytosolic or nuclear but instead localized to both compartments (Whitlock, 1987).

Knowledge of the subcellular compartmentalization of steroid hormone receptors has, unfortunately, not aided in elucidating the localization of unbound *Ah* receptor. In the case of the oestrogen receptor, immunolocalization studies showed that, in the intact cell, the unoccupied form resided in the nucleus (King & Greene, 1984). This is consistent with more recent studies by Murdoch *et al.* (1990) who have suggested that the detection of unbound oestrogen receptor in the cytosol is a consequence of the fractionation and homogenization procedure. Conversely, the unoccupied form of the glucocorticoid receptor may reside either in the cytoplasm (Antakly & Eisen, 1984), or in the nucleus, depending on the intracellular concentration of ATP (Mendel *et al.*, 1986).

The localization of the unbound receptor is important in the context of the TCDD–*Ah* receptor interaction. Most researchers

Table 2. Molecular properties of the *Ah* receptor from several species

Values are from Safe (1988).

	Sedimentation coefficient	Stokes radius (nm)	*M* (kDa)	Frictional ratio (f/f_0)	Axial ratio (a/b)
Rat hepatic cytosol					
0.1 M-KCl	8.8±0.05	7.0±0.21	257±7.7	1.7±0.03	12.4±0.69
0.4 M-KCl	5.6±0.58	5.2±0.24	121±5.0	1.6±0.05	11.3±1.00
C57BL/6 mouse hepatic cytosol					
0.1 M-KCl	9.4±0.57	7.1±0.12	277±4.8	1.7±0.02	12.2±0.04
0.4 M-KCl	9.7±0.20	6.8±0.15	274±5.3	1.6±0.02	11.0±0.40
	5.5±0.2	5.2±0.19	10.5±3.8	1.7±0.04	11.9±0.85
Mouse Hepa 1c1c7 cells	7.5–8.0	8.2–8.7	260–300	1.6–1.7	12–13
Human placenta	8.5±1.0				

have assumed a model similar to that put forward by Jensen (Jensen *et al.*, 1968) and Gorski (Gorski *et al.*, 1968) for steroid hormones. In this model, the ligand enters the cell by passive diffusion, where it encounters the cytosolic receptor. Binding within the cytosolic compartment is followed by a transformation of the receptor–ligand complex to a form which allows it to pass into the nucleus and associate with a specific recognition site(s) on the DNA. This model would be inappropriate if the unbound *Ah* receptor resided in the nucleus, and the formation of an '*Ah* receptor–ligand complex' simply enhanced the affinity of the receptor for the appropriate DNA binding sites, as suggested by Whitlock & Galeazzi (1984). Such a model has been postulated by Murdoch *et al.* (1990) for the nuclear oestrogen receptor. In this model, the steroid enters the nucleus and binds to the unactivated receptor, which is already DNA-bound, changing its conformation so as to expose regions which can bind to genomic switching sites.

Molecular properties of the *Ah* receptor

The unoccupied *Ah* receptor is readily subject to inactivation *in vitro*, which renders it incapable of binding TCDD. This is important in that the concentration of the *Ah* receptor in biological extracts is too low to permit detection by techniques such as polyacrylamide-gel electrophoresis, and thus quantification relies on the ability of the receptor to bind radiolabelled TCDD. Loss of this ability (e.g. through inactivation) results in the loss of detectability. Inactivation occurs thermally, and also in the presence of high concentrations of salts. The thermal stabilities of the unbound hepatic *Ah* receptors from several different rodent species have been compared, with mouse being the most stable, and rat the least stable (Bunce *et al.*, 1990*a*). In terms of the loss of TCDD-binding ability, the *Ah* receptor in rat hepatic cytosol has an *in vitro* half-life of approx. 30 min at room temperature. The TCDD-bound receptor is much more stable, however, and survives unchanged for several hours under these conditions (Landers & Bunce, 1990). For this reason, most studies of the molecular properties of the *Ah* receptor have been performed with the bound form which, as we shall see later, may have a substantially different structure from that of the unbound receptor.

The *Ah* receptor, like many steroid hormone receptors, is a multimeric protein complex. Sucrose density gradient separation (Okey *et al.*, 1979) has been a valuable tool for studying the *Ah* receptor complex and has been used extensively to characterize its physicochemical properties (reviewed in Safe, 1988). After radiolabelled TCDD has bound to *Ah* receptor in a tissue extract, the movement of specifically bound radioactivity through the gradient reveals information about molecular weight and shape of the protein. Table 2 summarizes the important molecular properties of the *Ah* receptor from various systems. In general, the hydrodynamic and ligand-binding properties of the receptor from various systems are very similar, but not identical. For example, Denison *et al.* (1986*c*) have found that the *Ah* receptors from C57BL/6N mice and Sprague–Dawley rats differ in size by less than 10%.

The cytosolic *Ah* receptor from rat liver is a multimeric protein complex (250±20 kDa; sedimentation coefficient of 8–9 S) in buffers containing low concentrations of salt. Higher salt concentrations dissociate the liganded receptors from rat, guinea pig, or rabbit from a form sedimenting at approx. 8–9 S to one that sediments at 5–6 S. For reasons not yet understood, the murine *Ah* receptor is more resistant to this change (Mason & Okey, 1982; Denison & Vella, 1990), just as it is more resistant to thermal inactivation (cf. Bunce *et al.*, 1990*a*). Sedimentation studies of the *Ah* receptor from mouse hepatoma cells in culture have shown that the cytosolic form has a molecular weight of approx. 270 kDa compared with approx. 180 kDa for the nuclear form (Prokipcak & Okey, 1988). The molecular sizes of the undenatured and salt-denatured forms of the *Ah* receptor are strikingly parallel to those of the glucocorticoid (Poellinger *et al.*, 1986) and androgen (Poellinger *et al.*, 1985) receptors, suggesting once again a common genetic ancestry for these various receptor proteins.

The size of the TCDD-binding subunit of the *Ah* receptor complex has been addressed through photoaffinity labelling studies. Poland & Glover (1987) found that the molecular mass of the photoaffinity-labelled TCDD-binding subunit of the *Ah* receptor from a wide variety of species varied from 95 to 120 kDa. No variation in molecular mass was found among the photoaffinity-labelled *Ah* receptors from nine different rat strains, all of which had a molecular mass of approx. 106 kDa. The TCDD-binding subunit of the cytosolic (Poland *et al.*, 1986) and nuclear (Landers *et al.*, 1989) forms of the mouse hepatic *Ah* receptor have the same apparent molecular weights of approx. 95 kDa. Okey and coworkers have further characterized the *Ah* receptor ligand-binding subunit. Trypsinization of the *Ah* receptor from mouse hepatoma cells resulted in a 16 kDa fragment that retained the bound TCDD but lost the ability to bind to DNA (Prokipcak *et al.*, 1990). This observation is similar to that of Simons *et al.* (1989) for the glucocorticoid receptor.

Almost all steroid hormone receptors have been shown to be associated with heat shock proteins. These are typically cytosolic proteins whose functions in the cell have not yet been defined (reviewed in Pratt, 1990). Recent studies have provided evidence for the association of the *Ah* receptor with heat-shock proteins. This was shown by Perdew (1988), who utilized a partially

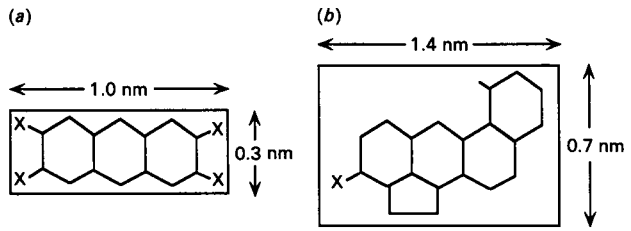

Fig. 3. Possible dimensions of the *Ah* receptor binding site

The high-affinity interaction of TCDD and structurally-related chemicals is consistent with a binding site that is planar, hydrophobic and approx. 1.0 nm × 0.3 nm in size (*a*). The high-affinity binding of other similar, but larger, compounds such 3-methylcholanthrene and benzo[*a*]pyrene suggests a larger binding site having dimensions of approx. 1.4 nm × 0.7 nm (*b*).

purified preparation of C57BL/6 mouse hepatic *Ah* receptor (22000-fold) to obtain antibodies that were not specific for the ligand binding subunit of the *Ah* receptor but instead for hsp90. These results were confirmed by Denis *et al.* (1988), who also showed that the interaction of hsp90 with the *Ah* receptor was much weaker than with the glucocorticoid receptor.

The foregoing studies have allowed simple proposals to be made concerning the oligomeric structure of the *Ah* receptor. For example, knowing that: (1) the TCDD binding subunit of both the cytosolic and nuclear murine *Ah* receptor is approx. 95 kDa, (2) hsp90 is associated with the *Ah* receptor and (3) the molecular mass of the undenatured cytosolic and nuclear forms of the *Ah* receptor are approx. 270 and 180 kDa respectively, the cytosolic form of the *Ah* receptor may be composed of three subunits (not necessarily identical) and the nuclear form of two. In the presence of salt, approximately 50 % of the TCDD-specific binding activity is lost irreversibly, perhaps representing the dissociation of one of two ligand-binding subunits from a trimeric complex (Landers & Bunce, 1990). A reasonable postulate at the present time is that the undenatured cytosolic *Ah* receptor may be composed of two ligand-binding units and one hsp90. Alternatively, the complex could consist of a single TCDD-binding subunit and some unknown number of other subunits including hsp90, that is analogous to the heteromeric structure of the glucocorticoid receptor (Denis *et al.*, 1988). Recent studies of the avian oviduct progesterone receptor highlight this possibility. Smith *et al.* (1990) have shown that progesterone receptor, upon purification by immunoaffinity chromatography, was not only associated with hsp90 and hsp70, but also with several other non-hsp subunits having molecular masses of 54, 50 and 23 kDa.

Ligand specificity

Structure–activity relationship studies for the binding of PCDDs and structurally similar halogenated aromatic compounds to the *Ah* receptor have shown that the ligand-binding site is hydrophobic, and preferentially accommodates planar non-polar ligands having molecular dimensions approximating to a 1.0 nm × 0.3 nm rectangle (Poland *et al.*, 1976; Safe, 1986). TCDD conforms most closely to the dimensions of the presumed binding site (Fig. 3*a*); it binds most strongly, and is also biologically the most potent congener of the PCDD family (Sawyer & Safe, 1982). PCDDs and PCDFs substituted with chlorine in at least three of the four lateral positions (2,3,7 and 8) bind most strongly to the *Ah* receptor (Bandiera *et al.*, 1984; Mason *et al.*, 1985, 1986, 1987; Safe, 1986, 1988; Poland & Knutson, 1982). Removal of groups from these positions or the addition of chlorine atoms at non-lateral sites diminish the binding affinities markedly. Other compounds that are structurally similar ('isosteric') to TCDD, such as 3,3′,4,4′-tetrachloroazobenzene (Fig. 1, structure b) (Poland *et al.*, 1976; Bunce *et al.*, 1989) and 3,3′,4,4′-tetrachlorostilbene (Bunce *et al.*, 1990*b*), also bind the *Ah* receptor with high affinity.

Although the majority of opinion favours a planar cavity of dimensions approximately 1.0 nm × 0.3 nm as the ligand binding site of the *Ah* receptor, high-affinity binding has been reported with substantially larger molecules such as substituted indoles (Gillner *et al.*, 1985), polynuclear aromatic hydrocarbons (Piskorska-Pliszczynska *et al.*, 1986), substituted diaryltriazines (Sweatlock & Gasiewicz, 1986) and certain dyes (Lubet *et al.*, 1983). A binding site of dimensions 0.7 nm × 1.4 nm must be postulated to accommodate the binding of these ligands (Fig. 3*b*). The significance of this information in terms of different ligand binding subunits with different binding sites, or alternatively, different isoforms of the receptor, is not yet known.

Ligand binding

The affinity of a ligand for a receptor can be discussed in terms of kinetics (the *rate* of binding) or in terms of equilibrium (the intrinsic strength of the association). The distinction between these kinds of affinity has been a source of confusion in the *Ah* receptor literature, and it is now clear that many equilibrium constants (K_d) previously reported for ligand–receptor association may not have the significance originally attributed to them.

Optimal *Ah* receptor-ligand binding *in vitro* requires an environment that is highly reduced, buffered at physiological pH and protected against calcium-dependent proteases (Poland & Glover, 1988). In addition, the binding of TCDD to the *Ah* receptor appears to be dependent on ATP (Gudas & Hankinson, 1986) and the presence of thiol groups (Denison *et al.*, 1987; Kester & Gasiewicz, 1987). Whitlock (1987) has suggested that the requirement for ATP may reflect a cyclic phosphorylation/dephosphorylation of the *Ah* receptor induced by bound TCDD.

The equilibrium dissociation constant (K_d) for the interaction of TCDD with almost all *Ah* receptors studied to date has been deduced to fall in the nM range. Weaker binding has been observed with the 'unresponsive' DBA/2 mouse and with the human receptor. These measurements have been made using Scatchard analysis, which is valid only if binding is a simple equilibration, free from side reactions (Beck & Goren, 1983). Unfortunately, the *Ah* receptors from most species are subject to rapid thermal inactivation *in vitro*, which render them unable to bind ligand (Bunce *et al.*, 1988, 1990*a*). Under these conditions, the use of an equilibrium method such as Scatchard analysis to measure binding affinity will underestimate the true strength of binding. More-recent studies indicate that binding is substantially stronger than previously supposed. For example, Bradfield *et al.* (1988) studied mouse hepatic cytosol under conditions free from thermal inactivation, while Bunce *et al.* (1988) used a kinetic approach, and evaluated K_d as the ratio of the rate constants for complex formation and complex dissociation. Both experimental approaches have led to the same conclusion, that the K_d values are not nM but in the pM range.

The rate of TCDD binding *in vitro* to the hepatic *Ah* receptor from several rodents has been studied (Bunce *et al.*, 1988; J. S. Nakai & N. J. Bunce, unpublished work). In all cases, binding is rapid (saturation half-life at room temperature < 1 h for a ligand concentration of 10^{-9} M) and is associated with a substantial enthalpy of activation, which can be associated with the reorganization of hydrogen bonds at the transition state. The enthalpy term is counterbalanced by a large positive entropy of activation, which can be explained as a hydrophobic effect, i.e., with loss of solvation of the binding site by water, and the conversion of water of solvation to bulk water. This interpretation is consistent with a substantial conformational change in

the protein concurrent with binding. This is similar to studies with the oestrogen receptor, whose unoccupied and liganded forms have been deduced to differ conformationally (Hansen & Gorski, 1985).

Quantitative structure–activity relationships (QSAR) have been used to study the relative affinities of different PCDD, PCDF, and PCB congeners for the *Ah* receptor from a number of species (Safe, 1986) including humans (Golas *et al.*, 1990). The consistent finding is that affinity correlates strongly with the lipophilicity of the ligand, again emphasizing the hydrophobic nature of receptor–ligand binding. However, the importance of other parameters such as hydrogen bonding ability and electron-donating or -withdrawing ability of the substituents differs among chemical families. Based on the data that are presently available, toxicity appears to correlate strongly with the binding affinity of the ligand for the *Ah* receptor protein. Just as the relative toxic responses of different PCDD congeners are species-dependent, so also are the relative *Ah* receptor binding affinities (Safe, 1986; Okey *et al.*, 1984). Rosengren *et al.* (1991) have recently studied the binding of six PCDD/PCDF congeners to the rat hepatic *Ah* receptor. In this series, binding was fastest and strongest for congeners recognized as more toxic (2,3,7,8-tetrachlorinated). The variation of K_d with temperature revealed that the association was, in all cases, favoured both enthalpically and entropically.

Modulation of cellular *Ah* receptor levels

The cellular levels of *Ah* receptor, at least in rodent hepatic tissue, can be modulated by pretreatment of the rodents with certain chemicals. Sloop & Lucier (1987) showed that intraperitoneal administration of TCDD to rats resulted in a 4–5-fold increase in hepatic *Ah* receptor levels; the 'additional' or 'induced' receptor appeared to be identical to the constitutive receptor, as determined by Scatchard analysis. This result supports the suggestion that intracellular *Ah* receptor levels may be controlled by a feedback mechanism similar to that which regulates the glucocorticoid receptor (Okret *et al.*, 1986). Consistent with higher receptor levels in treated animals, the whole body half-life of a radioiodinated TCDD analogue was significantly reduced when mice were pretreated with TCDD. Excretion half-lives for pretreated and 'naive' female C57BL/6J mice were 8 and 14 days respectively (Leung *et al.*, 1990).

Two lines of evidence argue against the simple feedback mechanism for regulating the intracellular level of the *Ah* receptor. First, the 'additional' *Ah* receptor protein induced by chemical pretreatment may not be identical to the constitutive *Ah* receptor. Recent studies indicate that, in the TCDD-bound form, the 'additional' receptor is much less stable than the constitutive receptor (Landers *et al.*, 1990, 1991). Second, sodium phenobarbital, which has no affinity for the *Ah* receptor (Okey *et al.*, 1979), also increases hepatic levels of this receptor (Okey & Vella, 1984). Similar observations have been made with 2,2′,4,4′,5,5′-hexachlorobiphenyl (HCBP; Denomme *et al.*, 1986), which binds to the *Ah* receptor with very low affinity (Bandiera *et al.*, 1983).

The complexity of the mechanism(s) which regulate cellular *Ah* receptor levels is further exemplified by the apparent down-regulation of *Ah* receptor by certain chemicals. *trans*-3,3′,4,4′-Tetrachlorostilbene (Bunce *et al.*, 1990*a*,*b*) and 3,3′,4,4′-tetrachloroazobenzene (Landers *et al.*, 1990) bind to the *Ah* receptor with moderately high affinity and yet depress the hepatic levels of *Ah* receptor. 2,2-Dimethyl-5-*t*-butyl-1,3-benzodioxole, which is a potent carcinogen, but probably has low affinity for the receptor, has been shown to cause a 2-fold reduction in the hepatic *Ah* receptor concentration in two strains of mice (Cook & Hodgson, 1986). This type of complex regulation also appears to exist for the steroid hormone receptors. For example, oestrogen has been shown to cause the induction of rat uterine progesterone receptor (Jordan *et al.*, 1985) while thyroxine can increase rat hepatic glucocorticoid receptor levels (Naito *et al.*, 1985). Similarly, it was shown that the 1,25-dihydroxyvitamin D receptor could be regulated by glucocorticoids (Hirst & Feldman, 1982) and that this regulation was tissue- (Hirst & Feldman, 1982) and species- (Chen *et al.*, 1983) dependent.

***Ah* receptor transformation**

In the absence of TCDD, the *Ah* receptor presumably exists as a biologically inactive complex of its constituent subunits. 'Transformation' or 'activation' is the term used to describe the conversion of the unactivated non-DNA binding form of the receptor complex to one capable of interacting specifically with DNA. According to the model proposed originally by both Jensen (Jensen *et al.*, 1968) and Gorski (Gorski *et al.*, 1968), transformation is essential to the formation of an activated receptor which, if cytosolic, can cross the nuclear membrane, associate with DNA binding sites and activate transcription. The physicochemical changes associated with *Ah* receptor transformation are unknown: conformational changes, dissociation of the oligomeric complex, and specific cleavage of the untransformed protein are all possibilities.

Similar to certain steroid hormone receptors, several lines of evidence indicate that the *Ah* receptor undergoes multi-step activation. In the case of the oestrogen (Hansen & Gorski, 1986) and glucocorticoid (Schmidt *et al.*, 1985, 1986; Smith *et al.*, 1986) receptors, several steps are thought to be involved in the transformation of unbound receptor to a biochemically functional complex. Multistep activation is supported by studies showing the existence of 'defective' receptors for oestrogens (Shyamala, 1972), androgens (Gumbach & Conte, 1985), progesterone (Boyd-Leinen *et al.*, 1982) and glucocorticoids (Gehring & Tomkins, 1974), all of which were capable of binding ligand but incapable of binding to nuclear sites. Several studies indicate that this may be the case for the *Ah* receptor since the formation of an *Ah* receptor–ligand complex is not sufficient to produce a biological response. The most convincing evidence for this conclusion comes from studies with a class of variant mouse hepatoma cells (Legraverend *et al.*, 1982; Miller *et al.*, 1983). In this cell line, the *Ah* receptor appears normal in that it binds ligand, but the receptor–ligand complex fails to interact normally with DNA binding sites. Furthermore, in response to TCDD, these variant hepatoma cells fail to induce cytochrome *P*-450 gene expression (Hankinson *et al.*, 1985; Israel & Whitlock, 1984, 1985). This may be relevant to the multiple forms of liganded receptor observed by Gasiewicz and coworkers (Gasiewicz & Bauman, 1987; Henry *et al.*, 1989).

Temperature plays a role in the transformation of the *Ah* receptor *in vitro* (Okey *et al.*, 1980; Gasiewicz & Bauman, 1987). Studies with hepatic tissue in culture show that TCDD–receptor complexes formed at 4 °C fail to bind appreciably to nuclear binding sites, while those formed above 20 °C bind avidly. This suggests that formation of a 'functional' TCDD–receptor complex requires a 'temperature-dependent' transformation step which increases the affinity of the receptor for nuclear binding sites (Okey *et al.*, 1980; Whitlock & Galeazzi, 1984). This process might involve conversion of the unoccupied receptor to a liganded form that is less negatively charged, thus enhancing its affinity for DNA. Alternatively, transformation might involve the unmasking of the DNA-binding region of the protein, either through a conformational change or through a dissociative process.

In this context, the role of hsp90 appears to be important in the transformation of the *Ah* receptor to the DNA-binding form.

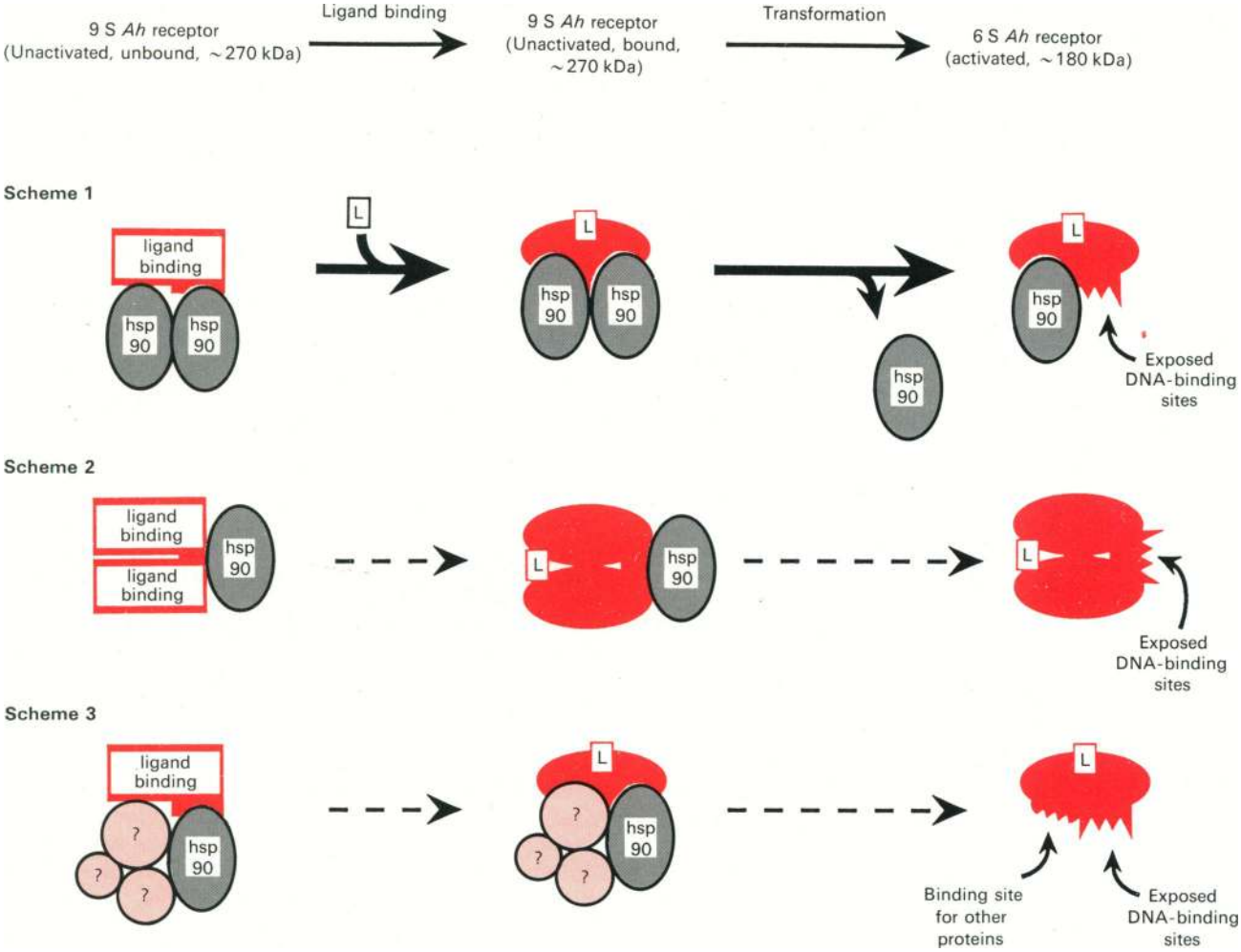

Fig. 4. Possible dissociative models for transformation of the *Ah* receptor

All scenarios depict the dissociation of a large multimeric protein complex (approx. 270 kDa) to a smaller complex (approx. 180 kDa) capable of interacting with specific DNA sites. The first two schemes assume that constituents of the untransformed receptor are present in the high-affinity DNA-binding form of the receptor. The last scheme depicts the possibility that transformation allows for the binding of the receptor to an, as yet, unidentified protein(s) that collectively form the DNA binding *Ah* receptor. **Schemes 1 and 2** depict the unactivated *Ah* receptor as a trimeric complex consisting of some unknown number of ligand binding and hsp90 subunits. Upon binding ligand, hsp90 dissociates from the complex, exposing a DNA-binding site on the receptor. **Scheme 3** depicts the unactivated *Ah* receptor as a complex containing a ligand-binding subunit, hsp90 and several unidentified nonreceptor subunits. Ligand binding induces the loss of hsp90 and other receptor-associated proteins, again exposing potential binding sites for DNA sequences as well as those for other proteins.

Hsp90 has been shown to be associated *in vitro* with several steroid hormone receptors as well as the *Ah* receptor (Perdew, 1988; Denis *et al.*, 1988). Recent studies with both the progesterone and glucocorticoid receptors suggest that a dimeric hsp90 maintains the untransformed receptor in the inactive state by interacting with the *C*-terminal portion of a single receptor molecule, thus blocking the DNA-binding region (Radanyi *et al.*, 1989). Accordingly, loss of hsp90 in the nucleus allows the receptor to associate with DNA. A current model for glucocorticoid receptor transformation involves hsp90 assisting the translocation of the ligand-binding unit from the cytosol, through nuclear pores, to nuclear docking sites, at which point the hsp90 dissociates. Recent studies *in vitro* with the *Ah* receptor suggest a similar role for hsp90 in modulating receptor transformation. Using an immunochemical approach, Wilhelmsson *et al.* (1990) found hsp90 to be associated with the unactivated (9 S) form of the receptor but not with the activated form (6 S). This provides strong support for transformation involving a dissociative process with hsp90. Some simple possibilities for a transformation-linked dissociative process(es) are depicted in Fig. 4 and take into account (*a*) the, as yet, undefined number of ligand-binding and hsp90 subunits associated with the untransformed receptor complex; (*b*) the exposure of a DNA binding site(s) following the loss of hsp90 and/or other *Ah* receptor-associated proteins, and (*c*) the possible exposure of sites for the binding of other proteins required for DNA interaction.

A completely different interpretation of transformation is provided by recent studies with the oestrogen receptor (Murdoch *et al.*, 1990). While the ligand is known to play an important role in transformation within the cell, transformation *in vitro* of the inactive oestrogen receptor to the DNA-binding form is temperature- but not ligand- dependent. This has been interpreted to imply that the oestrogen receptor is not associated with hsp90 purposefully, but instead that this association occurs when nuclear receptor is exposed to cytosolic hsp90 during the homogenization procedure. According to this hypothesis, the inactivated oestrogen receptor is always bound to DNA, and ligand binding induces the recruitment of other nuclear proteins

to form a larger complex capable of regulating gene transcription. Acceptance of this hypothesis for the *Ah* receptor system would invalidate a model whereby the receptor acts as a 'porter', carrying TCDD to the nucleus following its entry into the cell, but may be easier to reconcile with the heterodimeric nature of the nuclear form of the *Ah* receptor (Elferink *et al.*, 1990).

Receptor–DNA interactions

The interaction of the *Ah* receptor with specific nuclear binding sites has been the subject of intense study. This work has recently been reviewed in detail (Whitlock, 1987; Whitlock *et al.*, 1989; Whitlock, 1990) and therefore will only be highlighted in this review.

The 'transformed *Ah* receptor–ligand complex' possesses the structural requirements not only for translocation of the protein to the nucleus (assuming that, like the glucocorticoid receptor, the *Ah* receptor is cytosolic), but presumably also for association with DNA (Gasiewicz & Bauman, 1987; Hannah *et al.*, 1986). The *Ah* receptor–ligand complex binds to DNA and acts as a 'genomic switch' for stimulating the transcription of the cytochrome CYP1A1 gene. Israel & Whitlock (1984) have shown that this response is rapid, the rate of transcription being half maximal within minutes of the addition of TCDD. Transcription is observed even when protein synthesis is inhibited (Israel *et al.*, 1985), arguing against the participation of other newly synthesized proteins. A region of DNA flanking the 5'-end of the cytochrome CYP1A1 gene has been identified as that to which the *Ah* receptor binds. Since these domain(s) are located at least 1500 base pairs upstream from the transcription start site of the CYP1A1 gene, they have been functionally termed 'dioxin responsive enhancers' or DREs (Jones *et al.*, 1985). DREs have been identified in several species including the C57BL/6 mouse (Gonzalez & Nebert, 1985), the rat (Fujisawa-Sehara *et al.*, 1986; Sogawa *et al.*, 1986) and human (Kawajiri *et al.*, 1986), suggesting that TCDD may act by a similar mechanism in these different species.

Whitlock and coworkers have shown that the DRE contains the core sequence of 5'-TA/TGCGTG-3', which was shown to be present in several *Ah* receptor-dependent enhancers (Denison *et al.*, 1988). These results have been confirmed by Hapgood *et al.* (1989) who demonstrated the similarity of the xenobiotic responsive element (essentially equivalent to the DRE) to the glucocorticoid responsive element. Earlier analysis of the interaction of the *Ah* receptor with specific DNA domains was consistent with the binding of a single TCDD-binding subunit to the DRE (Denison *et al.*, 1989*a*) with the subsequent bending of the enhancer DNA upon doing so (Elferink & Whitlock, 1990). The most recent study by this group has shown that the transformed rat hepatic *Ah* receptor binds to the DRE as a heterodimer consisting of a 100 kDa TCDD-binding subunit and a 110 kDa protein of unknown identity (Elferink *et al.*, 1990). The identity of this protein and its cellular localization (cytosolic, nuclear, both) will be important to understanding transformation. Assuming that it is nuclear in origin would mean that the loss of hsp90 (and any other undefined accessory proteins) from the complex represents only the first step of transformation, the second involving the binding of the 110 kDa protein. In contrast, if it were part of the untransformed complex (containing hsp90), the resulting size of 300 kDa would still be consistent with the previous estimates from sedimentation studies.

Unresolved issues in the interaction of the liganded receptor with DNA include the strength of the binding in terms of a K_d value, and the DNA site occupancy necessary for initiation of transcription. Studies with the avian oviduct progesterone receptor suggest that as few as 10% of the DNA sites need to be occupied in order to activate transcription (Spelsberg, 1976).

Multiplicity of the *Ah* receptor

Although receptors have often been regarded as unique, invariable entities, the apparent heterogeneity of several receptor systems, including the steroid hormone receptors, has been established. The first intimation for *Ah* receptor multiplicity came from molecular modelling studies by McKinney *et al.* (1985), who concluded from PCB/*Ah* receptor binding studies that the data were consistent with the existence of a range of proteins capable of specifically interacting with the ligand. Direct experimental evidence has been obtained through photoaffinity labelling studies. Poland & Glover (1987) found that, unlike several other strains that were studied, 60% of Long Evans rats express two forms of the *Ah* receptor. Poland & Glover (1990) have also studied several strains of inbred and feral mice, and have identified three variants of the *Ah* receptor having different molecular masses and different thermal stabilities. The studies on induced and constitutive forms of the rat hepatic *Ah* receptor may also be interpreted in terms of different receptor isoforms. Using a kinetic approach, a second form of the hepatic *Ah* receptor appears to be induced in TCDD/HCBP-treated rats that is kinetically distinct, but physicochemically indistinguishable, from the constitutive form (Landers *et al.*, 1991).

Receptor isoforms often differ in charge states which can result from events such as phosphorylation and post-translational amino acid side chain modification. Perdew & Hollenback (1990) used two-dimensional gel electrophoresis to analyse the charge heterogeneity of the *Ah* receptor from rat thymus and several cell lines, identifying as many as three isoforms. The nature of these differences is not known. Both phosphorylated and dephosphorylated forms of most steroid hormone receptors have been identified, although their relevance to receptor function has not yet been determined. For example, phosphorylation has been suggested to explain the presence of several isoforms of the glucocorticoid receptor, although processes other than phosphorylation (e.g. acylation, prenylation) could be responsible for their charge differences (Gruol & Wolfe, 1989; Smith *et al.*, 1986). Interestingly, studies with the *Ah* receptor in crude preparations indicate that it does not rely on phosphorylation for ligand binding. A comparative study of the glucocorticoid and *Ah* receptors from rat liver showed that ligand binding to the glucocorticoid receptor was depressed following alkaline phosphatase treatment, while TCDD binding to the *Ah* receptor was unaffected (Denison *et al.*, 1989*b*).

***Ah* receptor modulation of oestrogen and its receptor**

In addition to its own receptor, TCDD has been shown to modulate several other receptors, including those for oestrogen (Gallo *et al.*, 1986; Romkes & Safe, 1988), EGF (Hudson *et al.*, 1985; Madhukar *et al.*, 1984), prolactin (Gustafsson *et al.*, 1987) and glucocorticoids (Ryan *et al.*, 1987). In general, the effect of TCDD on these receptor systems is to down-regulate their effective cellular concentrations without altering the affinity for their natural hormones. Especially interesting is the work of Gallo and coworkers, who have studied the effect of TCDD on oestrogen and its receptor (Gallo *et al.*, 1986). This approach is based largely on the observation that the toxic responses to oestrogen (immunosuppression, thymic involution, wasting syndrome) are very similar to those of TCDD.

Gallo and coworkers propose that TCDD's ability to modulate these receptor systems and their hormones could explain the observed toxicity of TCDD and additionally may provide an explanation for species-dependence of this phenomenon. They postulate that a TCDD-induced depression of cellular oestrogen receptor levels in key tissues would be compensated, in a feedback manner, by an increase in oestrogen biosynthesis. Thus, in those

tissues normally possessing high levels of oestrogen receptor, TCDD toxicity could result from antioestrogenic effects, while in those tissues not highly regulated by oestrogen, toxicity could result from the higher than normal oestrogen levels. Therefore the ability of a given species to deal with TCDD depends on its ability to synthesize oestrogen in an attempt to overcome the antioestrogenic effects of TCDD. Umbreit & Gallo (1988) suggest the following possible explanation for the difference in acute susceptibility to TCDD between hamster and guinea pig. Toxicity in the hamster can be attributed to elevated levels of oestrogen alone, suggesting that hamsters may be able to increase oestrogen biosynthesis without a substantial increase in the excretion rate of the hormone. This ability to compensate for the effect of TCDD renders the hamster relatively resistant. In contrast, the guinea pig increases its rate of oestrogen excretion as it increases the rate of oestrogen biosynthesis. As a result, the guinea pig cannot compensate for the effects of TCDD and is highly susceptible.

Antagonism between TCDD and other chloroaromatic compounds

There has been much interest in the apparent ability of certain chlorinated aromatic compounds to 'antagonize' the toxic effects of TCDD. Most of this work has focused on the teratogenic response in mice, with PCBs being the antagonists most commonly used.

The toxicology of PCBs themselves is complex, because PCB congeners themselves compete weakly with TCDD for *Ah* receptor sites (Bandiera *et al.*, 1983). Their binding affinity depends strongly on the extent of *ortho* chlorination of the PCB congener in the order no *ortho* chlorines ≫ one *ortho* chlorine ≫ more than one *ortho* chlorine. Since *ortho* chlorination decreases the planarity of a biphenyl molecule, these affinities correlate with the 'fit' of the PCB congener for the *Ah* receptor binding site.

The teratogenic response (cleft palate) to TCDD in pregnant mice can be modulated if the animals are also given high doses of PCBs. The pattern of response depends on the structure of the PCB. Birnbaum *et al.* (1985) found that 2,2′,4,4′,5,5′-hexachlorobiphenyl had little effect on TCDD-induced teratogenicity, but 2,3,3′,4,4′,5-hexachlorobiphenyl enhanced teratogenicity. Marks *et al.* (1981) had previously observed teratogenicity with the coplanar 3,3′,4,4′,5,5′-hexachlorobiphenyl in the absence of TCDD. Haake *et al.* (1987), using a commercial mixture, and Biegel *et al.* (1989), using 2,2′,4,4′,5,5′-hexachlorobiphenyl, saw striking reductions in the incidence of TCDD-induced cleft palate. Both PCBs and certain dibenzofurans, notably 6-methyl-1,3,8-trichlorodibenzofuran, have also been shown to be antagonistic to TCDD at the molecular level (Bannister *et al.*, 1989; Astroff *et al.*, 1988), by inhibiting the elevation of TCDD-inducible enzymes such as AHH and ethoxyresorufin *O*-deethylase.

These various observations can be explained by postulating that the antagonist substances occupy some of the *Ah* receptor sites essentially irreversibly, preventing their binding of TCDD (M. Brown, U. Schneider & N. J. Bunce, unpublished work). The different responses to the various hexachlorobiphenyl congeners described above provide further evidence for the concept that biological response is not determined only by whether the ligand can bind to the *Ah* receptor; the identity of the ligand also governs whether the receptor–ligand complex is biologically active.

Ah receptor-mediated mechanisms: further comparisons with steroid hormone receptors

Much of our present understanding of the *Ah* receptor, as well as the direction of future research, is based on studies of gene regulation by steroid hormone receptors (for reviews see Beato, 1989; Rories & Spelsberg, 1989; Carson-Jurica *et al.*, 1990). While there are obvious differences between the *Ah* and steroid hormone receptors, there is little doubt that, in many respects, TCDD mimics steroid hormones. For example, there is strong evidence that both steroids and TCDD bind to large multimeric protein complexes which undergo 'activation' to a form that regulates gene expression. While the interaction of both the steroids and TCDD with their respective receptors has been studied extensively, the exact details of the transformation process and receptor–DNA interaction are less clear. At present, several mechanistic models have been proposed to explain steroid regulation of gene expression and these may provide a solid basis for challenging and understanding *Ah* receptor structure and function.

From a mechanistic point of view, the challenging question is how ligand binding converts a biologically inactive receptor to one which regulates specific gene expression. The presence of receptor-associated proteins (including heat shock proteins) has been well established in several systems, although the role of these have not yet been defined. Toft and coworkers have not only shown the association of hsp90 (Schuh *et al.*, 1985) and hsp70 (Kost *et al.*, 1989) with the progesterone receptor, but have also identified several other non-hsp proteins (54, 50 and 23 kDa) associated with the unactivated receptor (Smith *et al.*, 1990). Similarly, a 59 kDa non-receptor protein has been identified as associated with the rabbit uterine progesterone receptor (Tai *et al.*, 1986). Using antibodies to the 59 kDa protein to induce a sedimentation shift, Prokipcak *et al.* (1989) showed that, as expected, a 59 kDa protein was associated with the progesterone receptor, but not with the *Ah* receptor. This does not provide unequivocal evidence for the lack of nonreceptor proteins in the *Ah* receptor complex since blockage of the antigenic sites could prevent binding of the monoclonal antibody. Alternatively, other proteins of unknown identity could be associated with the receptor.

While the role of accessory proteins in oligomeric receptor complexes is still not clear, several possibilities exist. For example, receptor phosphorylation, known to be concurrent with *in vitro* transformation in some systems (Orti *et al.*, 1989), may be due to the inherent kinase activity of one of the accessory proteins present in the complex. Another possibility may be that these proteins function to direct the activated receptor complex to specific nuclear docking sites. Putative sites have been identified for the progesterone (Rories & Spelsberg, 1989), androgen (Klyzsejko-Stefanowicz *et al.*, 1976) and oestrogen (Ross & Ruh, 1984) receptors.

In contrast, other studies on the oestrogen receptor suggest an alternative hypothesis for receptor activation (Murdoch *et al.*, 1990). Transformation of the inactive oestrogen receptor to the DNA-binding form is temperature-, but not ligand-, dependent. According to this hypothesis, the inactivated oestrogen receptor is already bound to DNA and induces the recruitment of other nuclear proteins to form a larger complex capable of activating gene transcription only upon binding the steroid. It will be interesting to determine whether the *Ah* receptor is similar to either of these systems in terms of receptor structure (i.e. the presence of accessory proteins) or mechanism of action. Recent evidence for the participation of hsp90 in *Ah* receptor transformation (Wilhelmsson *et al.*, 1990) makes it appear likely that similarities with the steroid receptors will exist.

One of the aspects of the *Ah* receptor mechanism still to be addressed is the possible involvement of other proteins in mediating the interaction of the receptor with the DRE or other regulatory elements. In the steroid hormone field, the discovery

of regulatory elements (SREs) that bind steroid hormone receptors has provided an explanation for the steroid regulation of gene transcription (Cato *et al.*, 1986; Strahle *et al.*, 1987; Waterman *et al.*, 1987; Bailly *et al.*, 1983). The regulatory sequences from several systems, typically upstream of the genes being controlled, bind receptor preferentially over nonspecific DNA in cell-free assays (Compton *et al.*, 1982; Scheidereit & Beato, 1984) and appear to be required for hormone-induced transcription *in vivo* (Dean *et al.*, 1983; Buetti & Kuhnel, 1986). Although the SREs are undoubtedly involved in steroid regulation of transfected genes, Rories & Spelsberg (1989) have questioned the function of SREs as acceptor sites for receptors *in vivo*. The issues include (*a*) uncertainty over the exact nucleotide sequences constituting the SREs (Miller *et al.*, 1984); (*b*) lack of saturable binding of the receptors to SREs; (*c*) protein:DNA ratios required for DNAase footprinting assays is consistent with lower binding affinities and specificity; and (*d*) lack of steroid dependence of receptor binding to the SREs (Murdoch *et al.*, 1990). They propose instead that nuclear proteins are required to form sites through which receptors interact with DNA (Spelsberg *et al.*, 1983; Rories & Spelsberg, 1989). The importance of the role of 'other' nonreceptor proteins and 'other non-SRE' DNA sequences in promoting the biological action of steroid hormones is becoming more widely recognized (Cordingly *et al.*, 1987; Edwards *et al.*, 1988). In the case of the progesterone receptor, a 10 kDa protein (termed 'receptor binding factor') which generates high affinity binding to intact chromatin has been identified and purified (Goldberger *et al.*, 1986, 1987; Goldberger & Spelzer, 1988).

In addition, Spelsberg *et al.* (1983) have postulated a cascade model whereby steroid receptors initially interact with, and activate the transcription of, early responsive genes, including several proto-oncogenes, whose protein products then play some undefined role in the activation of secondary genes. This model is supported by studies which show the rapid regulation of *c-myc* oncogene expression by glucocorticoids (Rories *et al.*, 1989), oestrogen (Dubik *et al.*, 1987) and progesterone (Fink *et al.*, 1988), and the comparatively late response of structural genes such as ovalbumin. Rowlands & Safe (1990) have reported that the administration of TCDD to MCF-7 human breast cancer cells treated with 17β-oestradiol caused a significant depression of *c-myc* oncogene expression in comparison with cells treated only with 17β-oestradiol. These results are consistent with the antioestrogenic effects of TCDD, and may indicate that, like the progesterone receptor, the *Ah* receptor interacts with both 'early' and 'late' regulated genes.

Non-*Ah* receptor-mediated mechanisms

Not all of the biological effects of TCDD can be explained using a mechanism of action analogous to that of steroid hormones. For example, the approach of Matsumura and coworkers in defining the direct effect of TCDD on plasma membranes (discussed earlier) may be an example of non-*Ah*-receptor-mediated processes. Studies by this group have shown that a variety of significant changes occur within the plasma membrane in response to TCDD. These include the down-regulation of several membrane protein components (ATPase, lipoprotein lipase, LDL receptor) and their activities, and the up-regulation of others (EGF receptor, protein kinase C, phospholipase C). With a completely different approach, McConkey *et al.* (1988) have shown that a TCDD-induced 'thymocyte suicide' may be mediated by aberrant levels of intracellular calcium. The toxicity of various other agents has been previously associated with fluctuations in the levels of calcium ion (Orrenius & Bellomo, 1986) and these may be intimately linked to some of the membrane effects identified by Matsumura and coworkers. These effects on other TCDD-susceptible tissues should be explored in detail since they may be relevant to understanding whether acute versus chronic responses to TCDD occur by different mechanisms.

THE POTENTIAL THREAT TO HUMAN HEALTH

An important motivating factor behind the intensive research effort focused on the mode of action of TCDD, both in terms of scientific manpower and public funding, is to estimate the possible health risk of human exposure to TCDD and structurally related compounds. The toxic responses to dioxin in both animals and humans were discussed briefly above, and have been covered in greater detail by Silbergeld & Gasiewicz (1989). Despite the research efforts to date, it is still not possible to provide any definitive answers regarding environmental exposure to PCDDs and related compounds and whether such exposure should be a cause for public concern.

One of the most intriguing aspects of TCDD toxicology is the diverse array of biological and toxic responses observed with various animal models. This has made it difficult to estimate the potential risk that dioxins pose to human health. While a direct comparison between humans and experimental animal systems would be ideal for addressing this problem, such an approach is hindered by several factors. First, there is a limited availability of human tissue for study, the most abundantly available tissue being placenta obtained *post partum*, and pathological samples such as tumours and tonsils (Lorenzen & Okey, 1990*b*). Secondly, rodent studies are typically carried out with immature animals since the *Ah* receptor levels generally decrease with age. This variable is difficult to control with human tissue specimens and the effect of age on tissue *Ah* receptor levels is unknown. Finally, the human receptor appears to be present in low levels and has a low stability *in vitro*, further complicating its study.

Recent evaluation of the individuals exposed to TCDD in the 1976 accident in Seveso, Italy, indicates that, after 10 years, there were no significant side effects other than chloracne (Mastroiacova, 1988). Topical exposure of volunteers to TCDD at levels greater than the LD_{50} in guinea pigs produced only mild chloracne. However, in comparison with rodents the apparently much longer whole-body half life of TCDD in humans (Poiger & Schlatter, 1986) is a cause for concern as is the facile transfer of PCDD/PCDF compounds to infants in mothers' milk (Noren, 1988). Although it has been estimated that people may be exposed to about 100 pg of TCDD equivalents daily, it is difficult, at this time, to assess the risk posed by this exposure with the use of rodent models. The toxicological significance of the tissue levels of TCDD discussed above has been assessed by Byard (1987). Based on exposure data (e.g. the Seveso accident) and toxicokinetic differences between rodents and man, he suggests that tissue levels of PCDDs/PCDFs in the general population are well below that warranting toxicological concern. This suggestion is supported by epidemiological studies which indicate that humans are probably at the less susceptible end of the TCDD toxicity spectrum. On the assumption of $K_d \sim 1$ nM, it has been argued that current dioxin exposure is too small to populate a significant proportion of intracellular *Ah* receptors (Roberts, 1991). Since toxicity is tied to receptor occupancy, this suggests that current environmental levels of dioxin may be too low to cause concern. Against this are uncertainties about the true values of K_d in both rodents and humans (see above), and about the proportion of receptors which must be occupied in order to evoke a toxic response.

FUTURE DIRECTIONS

From a molecular point of view, it will be important to learn

whether TCDD susceptibility among species results from differences at the receptor level (concentration, isoform, activity, specificity, subunit composition), at the DNA level (several types of DREs, flanking sequences, other regulatory elements, acceptor site proteins, transcription factors) or both. Undoubtedly, the purification and isolation of the *Ah* receptor will be fundamental in delineating the mechanism of action of TCDD. The first important steps in this direction have occurred. The receptor has been purified (Perdew & Poland, 1988) and polyclonal antibodies prepared against it (Poland & Bradfield, 1989). Anti-receptor antibodies will allow many unanswered questions regarding *Ah* receptor heterology, receptor synthesis and degradation *in vivo*, oligomeric receptor structure, intracellular localization, 'transformation' during or following the binding of a ligand, etc., to be addressed. In addition, anti-receptor antibodies could be used to clone the *Ah* receptor gene. Interestingly, Issemann & Green (1990) have recently reported the cloning of a receptor from the steroid hormone receptor superfamily which binds polyaromatic hydrocarbons. It remains to be seen whether this is the *Ah* receptor or an *Ah* receptor-like protein. Also, it is not known whether the *Ah* receptor, a DNA-binding protein, has any of the classical DNA binding motifs (zinc fingers, leucine zipper, helix–loop–helix) or whether other nuclear proteins (e.g. acceptor site proteins, transcription factors) are involved in protein–protein or protein–DNA interactions. In this respect, the recent discovery that the nuclear *Ah* receptor is a heterodimer (Elferink *et al.*, 1990) presents the challenge of identifying the 110 kDa *Ah* receptor-associated protein.

It has been suggested that the *Ah* receptor may be a member of the steroid hormone receptor superfamily (Evans, 1988) and analogies between these receptor systems have been very fruitful in mechanistic terms. Perhaps the most interesting analogy stems from the heterodimeric nature of the transcriptionally-active form of the *Ah* receptor. It has recently become clear that, as originally proposed by Spelsberg *et al.* (1983), nuclear proto-oncogene products may regulate the ability of certain steroid hormone receptors to enhance specific gene transcription. Yamamoto and coworkers have shown that the interaction of *c-jun* and *c-fos* with the glucocorticoid receptor regulates the glucocorticoid transcriptional activity selectively in a positive or negative manner (Diamond *et al.*, 1990). These results have been confirmed by another laboratory (Yang-Yen *et al.*, 1990) and also shown to be the case for the oestrogen receptor (Gaub *et al.*, 1990). These findings present the possibility that the undefined counterpart of the *Ah* receptor (110 kDa protein) regulates receptor function (or vice versa), perhaps in a fashion similar to the 'regulatory zipper' model proposed by Forman & Samuels (1990) for certain steroid hormone receptors. It will be important to determine the identity of the *Ah* receptor-associated protein. One is tempted to speculate that, like the glucocorticoid and oestrogen receptors, the *Ah* receptor-associated protein is a nuclear oncogene product. However, most of the nuclear oncoproteins are low in molecular mass typically less than 75 kDa (Cooper, 1990). The single exception to this rule is the product of the tumour-suppressing retinoblastoma (*Rb*) gene, a phosphoprotein which coincidentally has a molecular mass of 110 kDa (Hong *et al.*, 1989). The *Rb* oncoprotein may be a good candidate for the *Ah* receptor-associated protein since, like *c-jun* and *c-fos*, its DNA binding activities have been well documented (Lee *et al.*, 1987).

The information presently available about the *Ah* receptor raises several fundamental questions regarding the regulation of its function as a transcriptional regulator. Is the well-established 'pleiotypic' cellular response to TCDD governed by the ability of the *Ah* receptor to interact with several different nuclear proteins (i.e. the regulatory zipper model) which then direct the binding of the complex to specific DNA sequences? Is the observed variation in the molecular mass of the *Ah* receptor (ligand-binding subunit; Poland & Glover, 1987) reflective of the ability of the *Ah* receptor to interact (via dimerization) with a diverse array of nuclear regulatory proteins, and might such regulation explain the diverse action of TCDD in several species and in several tissues within a species? Does TCDD regulate the cellular levels of the other nuclear proteins with which it interacts similarly to that shown for the steroid receptors (Rories & Spelsberg, 1989)? Is the interaction with nuclear proteins ligand-dependent? Does the specificity of the *Ah* receptor-dependent association with different nuclear proteins govern the response to TCDD? Is the 110 kDa protein part of the untransformed complex or does it associate only following transformation? Does the *Ah* receptor, like the glucocorticoid receptor, bind to any negative regulatory elements? These and other questions may force the re-evaluation of the species-dependent differences in TCDD action, specifically because differences may not be completely at the receptor or DNA level, but both. Obviously there is a myriad of other candidate nuclear proteins for the *Ah* receptor-associated protein, but based on size, the steroid hormone receptors themselves might be considered. Such an association may explain some of the observed similarities in toxic responses and the TCDD-induced effects on certain steroids and their receptors (Umbreit & Gallo, 1988).

CONCLUSIONS

The involvement of the *Ah* receptor in the mechanism of action of TCDD and related compounds is now well established. While the details of the subunit structure of the *Ah* receptor and of the early steps in the mechanism (ligand binding, transformation, DNA binding) are slowly being deciphered, it is clear that the overall mechanism is very complex. Differences among species (and certain strains) have been documented for several of the physicochemical properties of the *Ah* receptor, including the molecular mass of the TCDD-binding subunit, ligand binding at the active site, affinity for TCDD, and thermal as well as salt stability. Unfortunately, none of these alone provides a reasonable explanation for the large species-dependency in toxicity or a basis for overt toxicity.

Despite extensive research efforts, we know little about the potential effects of TCDD on human health. From a toxicological point of view, gaps in our present knowledge must be addressed in order that we may understand the potential risk that dioxins pose. Many important clues suggest that humans are less susceptible to TCDD than are many strains of rodents. The molecular properties of the human *Ah* receptor appear to be more like those of the 'non-susceptible' strain of mice (DBA/2) than the 'susceptible' C57BL/6 strain in terms of stability and the requirement for molybdate stabilization *in vitro* (Okey *et al.*, 1989). This suggests that the DBA/2 mouse may prove to be a useful animal model for human toxicity, although even in this strain, human and murine responses to TCDD are not parallel. Perhaps the most urgent need in this area at the moment is better risk assessment; by answering the question whether or not 'dioxins' pose an environmental health hazard, society will be in a position to judge whether dioxin research should continue to be a priority from the public health perspective.

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