

Chapter 8. Dose-Response Modeling for 2,3,7,8-TCDD

Health Assessment for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and Related Compounds

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8. DOSE-RESPONSE MODELING FOR 2,3,7,8-TCDD

8.1. INTRODUCTION

The current effort to reevaluate the risk of exposure to dioxins is being termed a *Biological Basis for Risk Assessment*. The underlying premise is that this may be a special case for a nonmutagenic, receptor-mediated carcinogen. The goal of this reassessment is to consider more mechanistically based models that are sufficiently credible to the scientific community.

Most of the information presented in the Introduction is found in more extensive detail in the other background chapters. It is useful to summarize the salient features of those papers that have an impact on the development of dose-response models so that readers of this chapter will be able to evaluate the scientific foundation on which the dose-response models presented in this chapter are based.

2,3,7,8-TCDD is the most potent form of a broad family of xenobiotics that bind to an intracellular protein known as the Ah receptor. Other members of this family include halogenated hydrocarbons such as the PCBs, naphthalenes, and dibenzofurans, as well as nonhalogenated species such as 3-methylcholanthrene and β -naphthaflavone. The biological properties of dioxins have been investigated extensively in over 5,000 publications and abstracts since the identification of TCDD as a chloracneagen (Kimming and Schulz, 1957). Much of the biological activity of TCDD appears to follow the rank order binding affinity of the congeners and analogs to the Ah receptor (AhR). This rank order holds for toxic responses such as acute toxicity and teratogenicity and for changes in concentration of several hepatic proteins including the induction of cytochromes P-450IA1 and IA2 and the modulation of the estrogen receptor and ^{epidermal growth factor} (EGF) receptor. The relationship between AhR binding and carcinogenicity of TCDD is less clear. However, TCDD is a carcinogen in several strains of laboratory mice and rats, and the tumor sites include liver, thyroid, and the respiratory tract, as well as others. There is considerable evidence that TCDD does not interact directly with the DNA to cause mutations. The study most utilized for the cancer

risk assessment of TCDD is that of Kociba et al. (1978). These authors reported an increase in hepatocellular carcinomas and hepatomas, along with decreases in several endocrine tumors in female rats. Male rats were remarkably less susceptible to the hepatocarcinogenic actions of TCDD. However, there is no striking sex specificity in the hepatocarcinogenic actions of TCDD in mice.

The overall hypothesis of TCDD action, put forth by several groups, is based on the transcription of specific genes exemplified by the cytochrome P-450IA1 gene. The biological basis for this approach is outlined in ²the chapter by Whitlock. Although substantial gaps in our knowledge remain, it is accepted by most researchers that most if not all cellular responses to TCDD require interaction between TCDD and the Ah receptor. The binding of TCDD to AhR is similar, although not necessarily identical, to the interaction of many steroid hormones with their intracellular receptors, as pointed out by Gustafsson in a series of articles drawing comparisons between the AhR and the glucocorticoid receptor (Poellinger et al., 1986a, 1987; Cuthill et al., 1988). DeVito et al. (1991) have also drawn an analogy between the action of TCDD and estradiol at their respective receptor proteins.

The binding of TCDD to AhR is reversible. However, subsequent events seem to reduce the likelihood of dissociation of the ligand:receptor complex. One such event that has been recently studied is the association of the ligand:receptor complex with another macromolecule, the so-called ARNT (AhR nuclear transport) protein (Hoffman et al., 1991). There may be a family of ARNT proteins that differ by cell types, which could account, in part, for the diversity of actions of TCDD in different tissues. The association of ARNT with the ligand-bound receptor induces some physical changes in the complex, which tends to reduce dissociation of the ligand and favors the movement and/or retention of the complex into the nucleus. Overall, the relationship between TCDD concentration and nuclear AhR-TCDD concentration appears to be linear, indicating that, at low ligand concentrations, ARNT is not a rate-limiting factor. In the case of transcriptional activation of the CYP1A1 gene, the AhR-ARNT-TCDD complex (activated TCDD complex) associates with specific elements in the genome called the xenobiotic (or dioxin) responsive elements (XREs or

DREs). The association of the activated TCDD complex with the DRE is also reversible (Gasiewicz et al., 1991), and there is recent evidence that at least two DREs need to be occupied to transcriptionally activate the CYP1A1 gene (Whitlock, Chapter 2). The structure and amino acid sequence of the AhR protein ^{has been} ~~was reported by Poland and coworkers~~ (Burbach et al., 1992; Ema et al., 1992). Each AhR appears to bind one molecule of TCDD, and at low concentrations of ligand (i.e., when $[ligand] \ll [receptor]$), the binding of TCDD to AhR is likely to be linearly related to [TCDD].

Much of the sequence of events is analogous to the steroid receptors and the respective genomic response elements. This similarity helps us in proposing biological models of TCDD action and risk assessment. The steroid hormones and their receptors belong to a multigene family that includes the thyroid hormone receptors, oncogene products, glucocorticoids, mineralcorticoids, vitamin D, retinoids, androgens, estrogens, and progestins (Whitlock Chapter 2, Evans, 1988) ~~(Evans et al., 1988)~~. Biologically, these are all multipotent agents that induce a range of cellular responses in different organs, many at extremely low concentrations. They share a nuclear location for the transduction of ligand:receptor action, and their common mechanism of action is the regulation of gene expression (Jensen, 1991). Within the family of known receptors from these agents, there is considerable sequence homology and a common basic structure, consisting of a ligand-binding domain and a DNA-binding domain. The biological activity of these receptors is varyingly regulated by metals and by phosphorylation state. Some--but not all--hormone receptors may interact with chaperone-type proteins, subsequent to ligand binding, which transduce conformational changes and other events critical to nuclear translocation and DNA binding. The ARNT protein functions in this fashion (Hoffman et al., 1991). Other receptors are associated with so-called heat shock proteins or proteins that must be shed to transform the liganded receptor into a DNA-binding form, and the DNA-binding domain of some receptors contains zinc finger loops, although this does not seem to be the case for the Ah receptor.

The steroid hormone receptors, sometimes as liganded dimers, move to the nucleus and regulate gene transcription through specific DNA sequences near the target genes. These

events are complex because of interactions between the liganded receptor and nuclear proteins that function as transcription factors by binding to other DNA sites upstream of the hormone responsive elements and the regulated gene. These transcription factors may regulate the binding affinity of the steroid hormone receptor itself to DNA (Muller et al., 1991). Additional complexity is introduced by the interactions among steroid hormone receptors, at the genetic level, and by the effects of hormone upon the number, conformation, and localization of receptors. Down- and up-regulation of receptor gene transcription and receptor synthesis may also be involved in cell-level modulation of steroid hormone action. As pointed out by Muller ^{et al.} and Renkowitz (1991), every step in the signal transduction pathway of these hormones, from receptor gene transcription to ligand:receptor:DNA action, is likely to be regulated by both dependent and independent factors. Moreover, nongenomic effects of steroid hormones occur rapidly and independent of nuclear translocation of liganded hormone receptors or in the presence of protein synthesis inhibitors (Graham and McEwen, 1993). ^{Both and Munfeld, 1985}

Attempts to model the steps involved in signal transduction have examined events step-by-step as well as the overall set of reactions from entrance of hormone into the cell to cellular response. Of interest in this report is the information that may be available concerning the overall dose-response relationship for steroid hormones. The highly complex cascade of biological events that intervenes from hormone entrance to cell response may modulate hormone action in the following ways: It may amplify cell response, in the way that second messengers for membrane-associated receptors (such as neurotransmitter receptors) appear to amplify molecular signalling; it may transduce response in a manner proportional to concentration of hormone (that is, linearly); or it may introduce dampening into the response network. Amplification of signal transduction implies that, at some stage in a multistep process, more than one event is triggered as a consequence of one preceding event. Dampening implies that, at some stage, more than one event must occur before the next event is triggered. Linear transduction implies that the relationship of event to event, for all steps, is invariant.

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In considering these possible dose-response relationships, it is likely to be important to distinguish among endogenous and exogenous ligands for the same steroid hormone receptor, particularly if the two types of ligands differ in rates of turnover (degradability) or affinity for the receptor. We are hampered in our inferences for the dioxins because the endogenous ligand(s), if present, has not yet been identified, and thus we are not certain if TCDD is more or less stable than this ligand, if its affinity is higher or lower than an endogenous ligand, or if an endogenous ligand would act as an agonist or antagonist for dioxin-like effects. With respect to stability, it is unlikely that an endogenous ligand would be as stable as TCDD. Most endogenous ligands for steroid hormone and other receptors are rapidly cleared, either by compartmentation (as with neurotransmitter reuptake processes) or by enzymatic degradation, as with steroids. With respect to kinetics of binding of TCDD, its *in vivo* affinity for the receptor is extremely high, in the 10^{-11} range. If the affinity for the natural ligand is even higher, then it is likely that the overall relationships between natural ligand and receptor are even stronger than those we may explicate for TCDD; if it is lower, then it would be an unusual member of the steroid hormone family. Of course, differences in affinity, if these exist, may not influence the overall kinetics of the dose-response relationship as much as differences in the number of events required to trigger the reaction from step to step.

Evaluation of dose-response relationships for receptor-mediated events requires information on the quantitative relationships between ligand concentration, receptor occupancy, and biological response. Roth and Grunfeld in "The Textbook of Endocrinology" (1985) state:

At very low concentrations of hormone ($[H] \ll K_d$), receptor occupancy occurs but may be trivial; i.e., the curve approaches 0% occupancy of receptors. But if there are 10,000 receptors per cell (a reasonable number for most systems), the absolute number of complexes formed is respectable even at low hormone concentrations. One advantage of this arrangement is that the system is more sensitive to changes in hormone concentration; at receptor occupancy (occupied receptors/total receptors, or $[HR]/[Ro]$), below 10%,

[HR] is linearly related to [H], whereas at occupancies of 10 to 90%, [HR] is linear with $\log[H]$ —a given increase in [H] is more effective in generating HR at the lowest part of the curve than at the middle."

Figure 8-1 illustrates a situation where there is a proportional relationship between receptor occupancy and biological response. In this situation occupancy of one receptor would produce a response although it would be unlikely that this response could be detected. Moreover, the biological significance of such a response may be negligible, but this is not known and it may vary with endpoint as well as with developmental stage and cell type. It is important to note that the data in Figure 8-1 are plotted on a semilog scale. If the same data are plotted arithmetically (Figure 8-2), then the shape of the dose-response curve readily conveys the linear relationship between receptor occupancy and biological response at lower concentrations and saturation at higher concentrations.

Such a simple proportional relationship does not explain the diversity of biological responses that can be elicited by a single hormone utilizing a single receptor. For example, low concentrations of insulin produce much greater effects on fat cells than on muscle cells. These differences are due to tissue- and cell-specific factors that modulate the qualitative relationship between receptor occupancy and response. Similarly, it is expected that there are markedly different dose-response relationships for different effects of TCDD.

Coordinated biological responses, such as TCDD-mediated increases in cell proliferation, likely involve other hormone systems, which means that the dose-response relationships for relatively simple responses (i.e., CYP1A1 induction) may not accurately predict dose-response relationships for complex responses such as cancer. As we gain more understanding of the entire sequence of events responsible for TCDD-mediated toxic effects, we will enhance our ability to more accurately predict dose-response relationships. The mechanism(s) responsible for qualitative and quantitative diversity in receptor-mediated responses will be discussed in more detail in Section 8.7, Knowledge Gaps.

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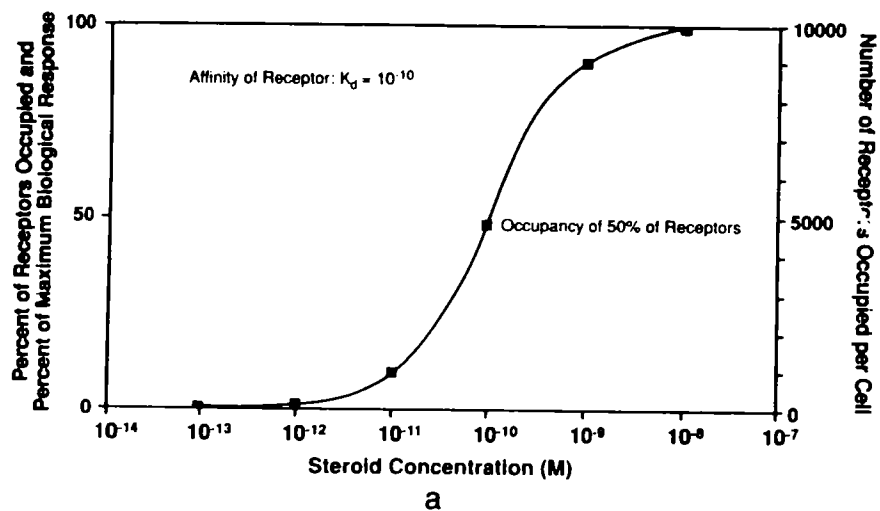


Figure 8-1. Dose/response graph showing proportional relationship between receptor occupancy and biological response (semilog scale)

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SOURCE: Roth and Grunfeld, 1985 (Figure 4-36)

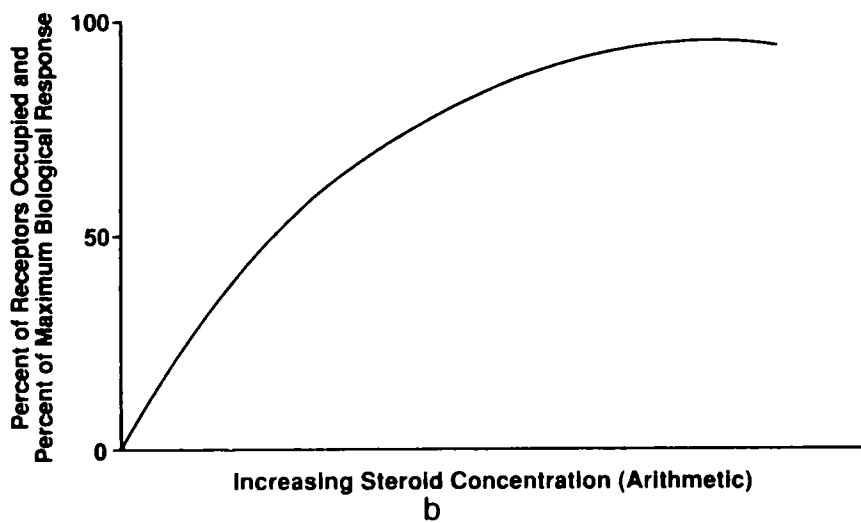


Figure 8-2. Dose/response graph showing proportional relationship between receptor occupancy and biological response (arithmetic scale)

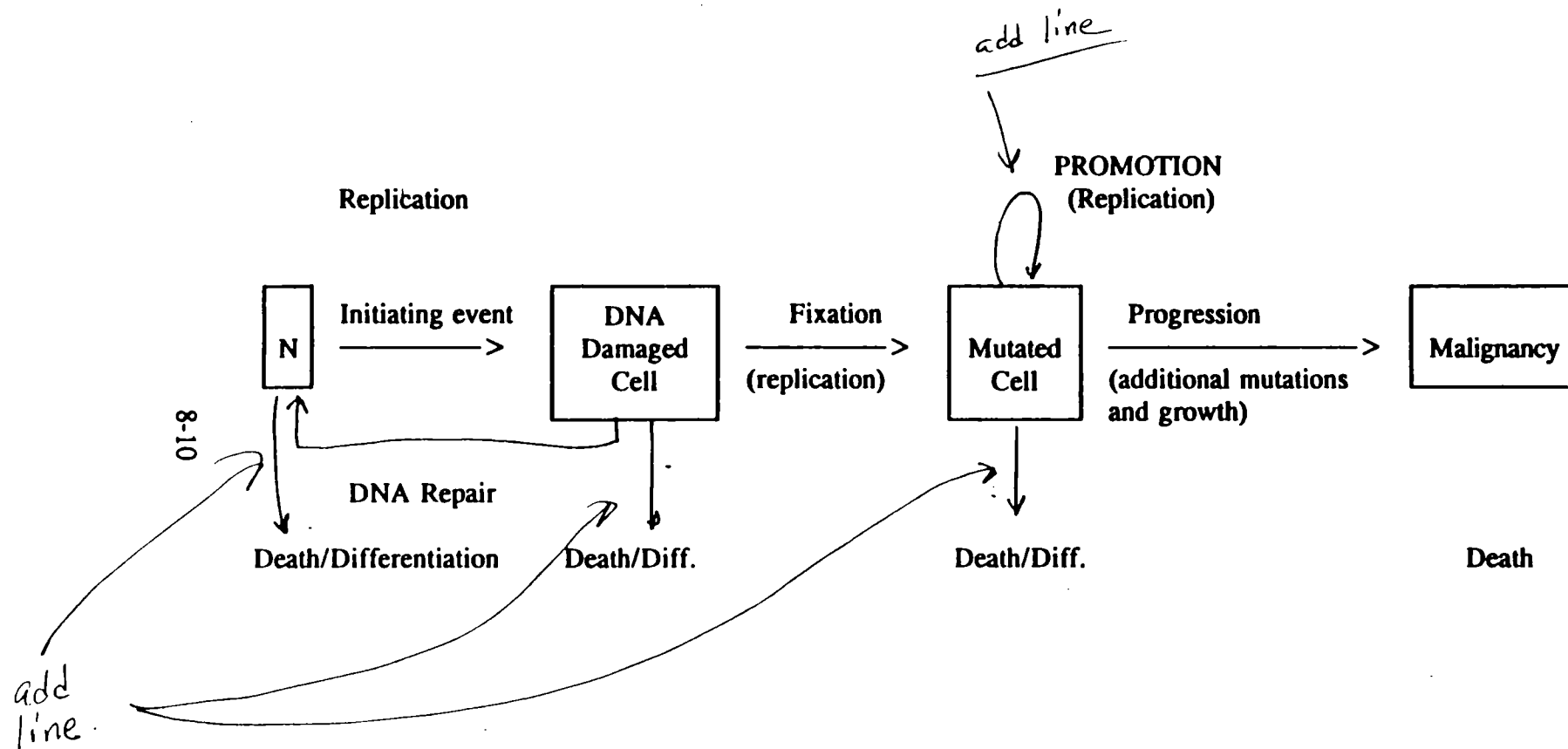
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Cancer is a multistep, multistage disease in which several operationally defined events have been described primarily on the basis of assay systems developed to detect these events. These events are initiation, fixation, promotion, and progression, as shown in Figure 8-3. Although this schematic arrays these events in a linear progression, it is important to realize that there are multiple pathways by which a cell may progress through these stages from an early alteration in gene structure or expression to the expansion of a clone into a tumor. The early alteration in gene structure or expression is often referred to as initiation. Structural damage to DNA, through alkylation or deletion, is an example of initiation. This initial event is immortalized, or stabilized, in the daughter cells of the initiated cell through the processes of fixation. Promotion is the enhanced growth of the cell population with fixed genetic damage; promotion may be supported by hormones and other modifications in cell growth and proliferation. Progression is a term used to describe additional alterations in gene structure or expression, such as second mutations ~~as described by Sugimura (1992)~~ for colon cancer, that appear to be necessary in the growth of the clone into a clinical end stage. These events are not necessarily ordered in this sequence, nor is it clear that distinctly different events--genotoxic and epigenetic--are involved in each stage. Some of these events are often defined by the test systems used to assay for their occurrence: For instance, initiation is often equated with mutation, such as the mutations that are detectable in in vitro bacterial assays of the Ames type. Promotion is often equated with a positive result in an experimental paradigm of sequential treatment of animals with a strong mutagen, followed by a putative "promoter."

Dioxin is an operational promoter, as defined in assay systems of skin and liver in mice and rats respectively ^(Pitot et al., 1987; Clark et al., 1991; Lucier et al., 1991) ~~(Pitot, Poland, etc.)~~. Since many hormones are promoters (Pitot and Dragan, 1991; Henderson et al., 1982), it is not surprising that dioxin has these properties as well. The interactions of dioxin with hormones are complex (see below), and in cancer bioassays there is evidence for interactions among dioxin and sex hormones, in that the rates of liver tumors in rodents are much higher in females than in males (NTP), and ovariectomy suppresses dioxin promotion of DEN-initiated liver tumors (Lucier et al., 1991).

Diethylnitrosamine

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Figure 8-3. Multistage carcinogenesis

SOURCE: Portier and Kopp-Schneider, 1991

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Dioxin is also a complete carcinogen; that is, it induces increased tumor yields in experimental animals not pretreated with strong mutagens (Huff et al., 1978, 1982, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025).

Dioxin is not a mutagen in in vitro systems commonly used to detect mutation through DNA damage. There is some evidence for in vivo clastogenicity of DNA (increased chromosomal breaks) in animals exposed to dioxin (Stohs et al., 1990). These data have presented challenges to the application of general models for cancer risk assessment, which are based on assumptions of mutagenesis as a fundamental mechanism for chemical- or radiation-induced cancer.

The general approach of the U.S. EPA to regulation of carcinogens is to use the Armitage-Doll model of carcinogenesis (Figure 8-4). In this model, the movement of cells from one stage to the other are assumed to be due to a sequence of mutations similar to the step of initiation/fixation discussed above. As with any mathematical model, specific forms must be chosen for the rate constants that define the process. The EPA formulation of this model assumes the mutation rates $[\mu, (d, t)]$ are a linear function of dose and are constant over time. These assumptions result in a cumulative tumor incidence rate that can be approximated by a polynomial function of dose with coefficient q_i for doseⁱ, $i=0,1,2,\dots,k$. In the low-dose region, the risk is dominated by the linear term in the polynomial (q_1). EPA generally uses a 95 percent upper-confidence limit (q_1 on the linear term of this formulation of the multistage model for cancer risk assessment. This model, using the 5 percent upper-confidence limit on the linear term is referred to as the linearized multistage (LMS) model. However, this choice has not been predicated solely on the correctness of a K-mutation process of cancer development. The linearized mathematical properties of the multistage model can be appropriate for a larger class of mechanisms: Dose-response behavior, which is linear at low dose, may have upward curvature in the intermediate range and show a downward curvature or saturation of response at high dose. In particular, arguments that a compound's action is additive to background biological processes lead to a linear response at low dose under rather general conditions (Crump, 1976). Therefore, for practical modeling purposes, it is important to address whether biological knowledge about the action

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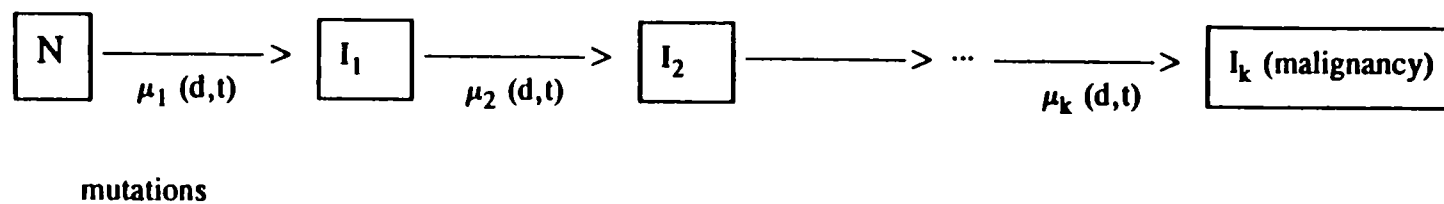


Figure 8-4. The Armitage-Doll model of carcinogenesis

of a carcinogen can fit the general dose-response shape predicted by the linearized multistage model.

Our knowledge of dioxins may challenge some of the simplifying assumptions that have been the biological basis for cancer risk assessment models. While dioxins do not possess the properties of an operationally defined "genotoxin" (using in vitro tests of mutagenicity), dioxins clearly affect gene expression (see below and also Whitlock chapter). These gene-level effects appear to involve a transcription regulator, the so-called dioxin receptor, which when liganded to dioxin and translocated to the nucleus is associated with specific recognition elements upstream of target genes whose transcription may be up-regulated or down-regulated by the dioxin receptor. It is difficult to fit these events into our current conceptual model of chemical carcinogenesis.

For other toxicological endpoints such as terata, organ toxicity, acute toxicity, etc., a threshold has often been assumed primarily as a matter of policy. For these endpoints, safety factors or uncertainty factors have been used to estimate no-effect exposure levels (NAS/NRC, 1977). This threshold approach is used by the World Health Organization to set ADIs for direct and indirect food additives. EPA now uses the term reference dose. In distinction, EPA policy assumes the dose-response curve for excess carcinogenic risk to be linear through dose zero. Several mechanisms could generally lead to this form of response, including direct mutational activity of the chemical agent and/or additivity to background rate of tumor formation (Portier, 1976). Since TCDD does not bind covalently to DNA and must exert its effects through receptor action, this default position must be carefully reexamined.

8.1.1. Introduction to Modeling for TCDD

There are several models under consideration at the present time, ranging from very simple to complex. It is obvious that the biology governing the toxicity of TCDD, beyond a few initial critical events, is not straightforward. These critical events, the first of which is binding to the Ah receptor, are generally response-independent. The response-dependent events are species-, gender-, organ-, tissue-, and perhaps cell-specific. If the binding to the

AhR is essential but not sufficient for effects to occur, then the dose-response curve for this event (as well as the rate equations) should be a better predictor of biological action than dose as long as the dose-response curves for these subsequent actions are functionally similar to the receptor binding curves. In general, the available data indicate receptor involvement is necessary for most if not all low-dose actions of TCDD. Since the AhR has been detected in virtually all cells but all cells do not respond to TCDD, there must be other factors that are necessary for TCDD action. The roles of these cell-specific factors must be elucidated before there is a complete understanding of TCDD action. However, a model may be developed for specific endpoints by using available data and reasonable assumptions.

Several important factors have been generally accepted. One, TCDD is a member of a class of xenobiotics (and probably natural products) that is nonmutagenic, binds to a cellular receptor, and alters cell growth and development. Two, a significant amount of information is available for estimating risks from exposure to this compound and the default position of directly applying the LMS model as a function of dose needs to be reevaluated. Three, the biology of receptor-mediated events should be included in any modeling exercise for TCDD. The goal of the modeling is to use as much data as possible to reduce these uncertainties and to identify the areas where data gaps exist.

One difficulty with a novel, albeit biologically based, approach is that it is replacing paradigms (safety factors and LMS models) upon which the U.S. Government's risk assessments have been based. There is no a priori reason to believe that a model based on greater experimental evidence will be more or less conservative than the LMS model. However, basing the modeling on a mechanistic understanding of the biochemistry of TCDD-induced toxicity should increase our confidence in the resultant risk estimates. As previously stated by Greenlee and collaborators (1991):

Neither the position taken by U.S. EPA or by Environment Canada (and several other countries such as Germany and the Netherlands) is based on any detailed mechanistic understanding of receptor-mediated interactions between dioxin and target tissues. Biologically-based strategies use knowledge of the

mechanistic events in the various steps in the scheme for risk assessment. Interspecies extrapolation strategies would be conducted based on how these mechanistic steps vary from species to species. There are numerous steps that can be examined mechanistically, and fairly ambitious programs have been proposed to examine the mechanistic details of many or most of these individual steps. More focused risk assessment approaches are also being proposed based on examination of individual steps believed to be critical in establishing the overall shape of the dose-response curve for the induction of tumors (or other toxic endpoints) by dioxin.

This chapter examines several endpoints and focuses on dose-response models for cancer in laboratory animals and humans. It also evaluates the use of biomarkers of TCDD action as surrogates for modeling receptor-mediated events. In addition, the chapter presents a section titled Knowledge Gaps. Critical examination of this section can lead to new experiments that will add to our knowledge of TCDD action. Increased information on key molecular, cellular, and tissue-specific events will be important to validate a new risk paradigm for TCDD and perhaps other receptor-mediated nonmutagenic toxicants. This chapter focuses on cancer risk modeling. We review some critical data on noncancer endpoints but we do not attempt to model them. These endpoints are clearly important when considering the public health risk of dioxins. However, the lack of knowledge on molecular action and molecular dosimetry limits our ability to propose mechanistically based mathematical models of noncancer endpoints at this time.

Mathematical modeling can be a powerful tool for understanding and combining information on complex biological phenomena. The development and use of mechanistically based mathematical models is illustrated by Figure 8-5. The beginning point is generally a series of experiments studying a xenobiotic agent. The experimental results (data) can indicate a mechanism, which leads to the creation of a mathematical model. The model is used to make inferences that are then validated against the existing knowledge base for the agent and effect under study. This can then lead to new experiments, which support further laps through the model development loop. On each pass through the loop, the model either

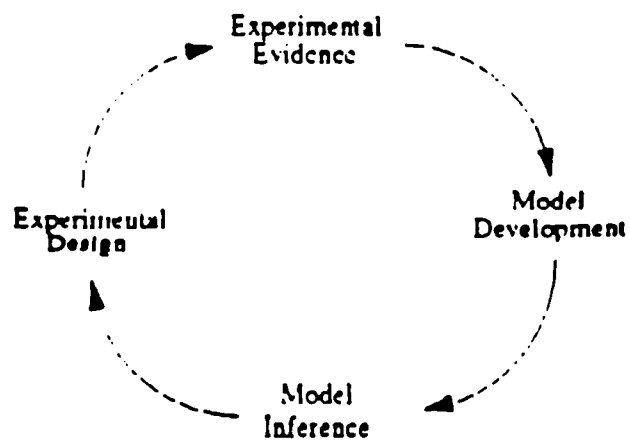


Figure 8-5. Developing a mechanistically based mathematical model

gains additional validation by predicting the new experimental results or it is modified to encompass new results without sacrificing its base in previous results. In either case, subsequent loops through the model generally increase our confidence in accepting (or rejecting) a final model (although it may be difficult or impossible to quantify this confidence).

Confidence in any one model is not only dependent on the information available for that compound but also supported by the information available on other systems that act similarly and for which models have already been developed. In the case of TCDD, the modeling of effects will be greatly enhanced by existing information on the receptor-based systems and general work in physiologically based pharmacokinetic models and in tumor incidence modeling.

There is no one model development loop for any given compound or effect. Instead, there are unusually numerous pathways leading to the development of a mechanistic model. In modeling the effects of TCDD, there are many smaller model development circles that make up the larger overall model. For example, a mechanistic approach to TCDD-induced carcinogenicity must include models of exposure, tissue distribution, tissue diffusion, cellular biochemistry, cellular action, tumor incidence, and cancer mortality. At each stage and for each model, data must be collected and understood in order for the model to be valid and acceptable as a tool for understanding the observed effects and for predicting the effects of TCDD outside of the relatively limited range of experimental or epidemiological findings.

The use of mechanistically based modeling to extrapolate risks of exposure patterns and doses outside the range of the data is in its infancy. Even though there may be high confidence in the ability of the model to predict experimental results, there could be low confidence in the ability of the model to predict outside the range of data. The use of models in risk assessment thus demands a careful scrutiny of the behavior of the model under a variety of exposure scenarios. This scrutiny has not generally been applied in science to the use of mechanistic models. It is important to note that mechanistic modeling has a role to aid in explaining and understanding experimental results, separate from its use in risk

assessment. Our confidence in the methods used in mechanistic modeling will differ with use. For a historical perspective, it is important to recognize that this is not the first loop through the cycle of mechanistic modeling for carcinogenesis. Early risk assessments based on tolerance distributions used the then current understanding of carcinogenesis to develop statistical models that could be used for risk estimation (Mantel and Bryan, 1961). Later use of the LMS model was based on an understanding of carcinogenic mechanisms. Thus, this current exercise is a part of the process of mechanistically based modeling for risk assessment purposes.

In any realistic and practical modeling exercise, the major components of the model revolve around the estimation of model parameters utilizing statistical tools. These tools range from very simple techniques, such as estimating a mean, to extremely complicated approaches, such as estimation via maximizing a statistical likelihood. The estimation of parameters is not done in a vacuum but is tied to the data available to characterize the model. The way in which data are used to estimate those model parameters is the major component in determining the confidence placed in any mathematical model. Fundamentally, sufficient data need to be available to show that the model accurately represents at least what is known of biological processes that are associated with toxic events.

In modeling biological phenomena, the data can be divided into four broad categories, as shown in Table 8-1. At the top are effects observed in the whole animal. Examples of data in this category are survival of the organism, ability to reproduce, and overall function of the organism (e.g., behavioral data). Going from whole organism to tissue/organ system responses to cellular responses down to biochemical responses in the cell, the levels of data are increasingly specific and reductionist. The data range from the bottom of Table 8-1, which is extremely mechanistic and deals with the interactions between molecules, to the data at the top of Table 8-1, which is often no more than counting bodies. All of this information is relevant and must be incorporated into a mathematical model aimed at understanding the specific biological response.

Table 8-1. Examples of levels of information available for estimating parameters in dose-response modeling

Organism	Morbidity
	Mortality
	Fertility
	Improper development/function
Tissue	Hyperplasia
	Hypertrophy
	Tumorigenesis
	Chemical distribution/disposition
Cell	Mitosis
	Cell death
	Cytoarchitectural pathology
Biochemical	Gene expression
	Protein levels
	Receptor binding
	Adduct formation

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Mathematical models that incorporate parameters that are mechanistic in nature do not automatically constitute "mechanistic models." The types of data available for the model and the method by which these data are incorporated into the model determine if a model is truly "mechanistic," that is, soundly based on the biology rather than simply a curve fit to the same data.

There are two basic ways in which biological effects can be estimated. The first and most common approach is a "top-down" approach. In the top-down approach, data on the effect of interest (e.g., carcinogenicity) are modeled directly by applying statistical tools to link the observed data (e.g., tumor incidence data from a carcinogenicity experiment) to a model (e.g., the multistage model of carcinogenesis). This approach is extremely powerful in its ability to describe the observed results and to generate hypotheses about model parameters and the potential effects of changes in these parameters. Where this modeling approach begins to lack credibility is in its ability to predict responses outside the range of the data currently being evaluated. Even when the model being applied to the data is mechanistic in the sense that the model parameters are tied to some mechanism for the toxic effect (e.g., mutation rates and molecular effects), without direct evidence concerning the value for this parameter or even evidence supporting the particular structure of the model, one is basically left with a curve fit to the data. The historical application of the LMS model in risk assessment has been in this fashion.

True mechanistic modeling must be viewed in a different fashion. In this case, the model structure and the parameters in the model are derived in a "bottom-up" fashion. The mechanistic parameters in the model are estimated directly from mechanistic data rather than from effects data or data one level higher in the hierarchy of data illustrated in Table 8-1. The goal of true mechanistic modeling is to explain all or most known results relating to the process under study in a way that is reasonable in its biology and soundly rooted in the data at hand. In this case, biological confidence in predictions from the model would be much higher than that from the "curve fitting" approach.

In practice, it is generally impossible to completely eliminate curve fitting from mechanistic modeling. At some point in the modeling process, gaps must be filled to relate the mechanistic effects to the observed toxic effects. It is generally at this point that some amount of curve fitting is necessary. Although not technically mechanistic modeling, this combined approach is preferred to simple curve fitting when inferences outside of the range of the toxic effects data are desired.

This is not intended to infer that with mechanistic modeling we can get a precise estimate of risk of a toxic effect outside the range of the data or even necessarily a more precise estimate of risk than with curve fitting alone. Without data, the statistical issue of the accuracy of a prediction cannot be easily addressed. Thus, while there may be greater biological confidence in extrapolated results, it is unlikely that an increased statistical confidence can be demonstrated. However, for each level and type of data, there are ranges of exposure beyond which it is impossible to demonstrate an effect given the practical constraints on the experimental protocols. In general, effects can be demonstrated at lower exposures for data at the bottom of Table 8-1 compared to the data at the top. If this is the case, there may be both increased biological confidence in extrapolated results and increased statistical accuracy. This is also not intended to infer that models derived through curved fitting should always be given less weight. Many of the advances in biology and science are due to the application (either formally or informally) of a model to data at a higher level in order to generate hypotheses about effects at lower levels. The major difference between the application of a "curve-fit" model in basic biology and that in risk estimation is that in basic biology one is creating hypotheses which at some point can be tested. In risk estimation, it is unlikely that one will ever be able to validate extrapolated risk estimates.

Many side issues are also related to the use of this model development loop in trying to understand a biological mechanism. One of considerable importance is experimental design. For mechanistic modeling aimed at risk assessment, we are just beginning to understand the types of experiments that may assist us. In general design situations, one would have a mechanism in mind, qualitatively describe that mechanism and form the

structure of a mechanistic model, and make educated guesses about the parameters of this model, then use the quantitative model to develop experimental designs that are optimally relevant to characterizing the mechanism. For the purpose of risk estimation, this basic outline holds. There are also some simple design rules that are not required but would aid in the extrapolation of results to doses outside the observed response range and to humans from animals. A few of these design points are listed in Table 8-2.

TCDD can be considered as a prototype for exploring and examining the ability of mechanistic modeling to improve the accuracy of quantitative risk assessment. The data base for a mechanistic modeling approach to TCDD is very extensive and contains a considerable amount of information on low-dose behavior. In addition, there is some concordance between human data and experimental evidence in animals. On the other hand, some aspects of the mechanism by which TCDD induces its effects, such as binding to the Ah receptor, have not been modeled extensively, and thus we are in only the first few loops through the model development cycle shown in Figure 8-5. Because of this, several competing mechanistic theories may agree with the existing data, adding to the uncertainty in any projected risk estimates. This outcome is inevitable for the application of the technology of mechanistic modeling to a new area. To reiterate an earlier point, mechanistic modeling can aid in explaining and understanding experimental results, beyond its proposed use in risk assessment. Our confidence in the methods used in mechanistic modeling will differ depending on its use. As we know more about the limitations of current data and current methods for the application of mechanistic models to risk estimation, we can improve experimental designs and significantly improve the process. Since this is an early attempt along these lines, we must be cautious in coming to a judgment concerning the overall utility of mechanistic modeling as an important tool for risk assessment.

In the National Academy of Sciences report "Risk Assessment in the Federal Government: Managing the Process" (National Research Council, 1983), "dose-response assessment" referred to the process of estimating the expected incidence of response for various exposure levels in animals and humans. Tissue response is not always directly

Table 8-2. Design considerations for risk assessment purposes	
Aspects of risk estimation	Design points to consider
Mechanism	Multiple times of observation
	Multiple doses
	Multiple ages at exposure
	Pharmacokinetics
Species extrapolation	Multiple species
	Both sexes
	Tissue concentrations (including blood)
	System clearance
	Essential interactions with endocrine systems
Other	Multiple endpoints for individual animals

related to exposure. This can be due to saturation and activation of metabolic pathways (Hoel et al., 1983); influence of competing pathways having different efficiencies of action for the parent compound and/or its key metabolites; and factors such as cytotoxicity, mitogenesis, or endocrine influences that can radically modify the homeostatic state of the tissue. These complex interactions can result in markedly nonlinear dose-response; nonlinearities could lead to risk estimates that may be greater or less than the risk derived from a linear model. Because of the potential for nonlinearities, it is essential to distinguish between exposure level and dose to critical tissue or cell when modeling risks from exposure to xenobiotics. It is also essential that we understand the quantitative relationship between target tissue dose and changes in gene expression. This is especially true when extrapolating to low doses and extrapolating across species.

For dioxin, the abundance of data on many levels allows one to create a collection of models that include the determination of the quantitative relationship between dioxin exposure and tissue concentration, tissue concentration and cellular action, cellular action and tissue response, and finally tissue response and host survival (Andersen and Greenlee, 1991; Portier et al., 1984). This portion of the reevaluation of dioxin risks entails the description and development of mechanistically based mathematical models of the effects of TCDD. This includes a discussion of the extrapolation of tissue dosimetry and response from high-dose exposures to those expected at much lower exposure based on empirical relationships used to derive explicit, though incomplete, biologically based mechanistic models of the events involved in the toxic action of dioxin.

Biological modeling is the process of developing mathematical descriptions of the interrelationships among the mechanistic determinants of those toxic events resulting from exposure to TCDD. Research with dioxin has focused on biological responses at the levels of organization shown in Table 8-1 (i.e., biochemical, cellular, tissue, and organism). At each level of organization, we focus on the mechanisms responsible for these observations. Mechanism refers to the critical biological factors that regulate occurrence, incidence, and severity of a particular factor and the nature of the interrelationships among these factors.

The details of the mechanisms of interaction differ markedly for the various levels of biological organization with specific determinants of the behavior at each level driving the creation on an appropriate quantitative model.

For dioxin, the mechanisms of three processes are of primary interest: (1) the dosimetry of dioxin throughout the body and specifically to target tissues; (2) the molecular interactions between dioxin and tissues, emphasizing the activation of gene transcription and increases in cellular protein concentrations of specific growth-regulatory gene products and specific cytochromes; and (3) the progressive tissue-level alterations resulting from these interactions that lead, eventually, to toxicity. The modeling process involves identification of the mechanistic determination of the dose-response continuum through experimentation and the encoding of these processes in mathematical equations. The extent to which model predictions coincide with experimental results is a test of the validity of the model structure. After validation, the model can be used for risk assessment. In addition to their use in risk assessment, these models have importance for aiding in the design of future research, both in terms of a basic understanding of dioxin toxicity and further risk estimation.

The following sections discuss the mechanistic biological modeling for dioxin with regard to dosimetry, induction of gene transcription, and tissue response, especially those associated with hepatic carcinogenesis. This modeling effort follows a natural progression related to the kind of information available at the time at which the model was developed. We will begin with a review of tissue concentration followed by modulation of protein concentrations and tissue response.

8.1.2. Dose Delivery, Tissue Modeling, and Biochemical Modeling (See Appendix for complete manuscripts by Kohn et al., 1993 and Andersen et al., 1993)

Tissue dosimetry encompasses the absorption, distribution, metabolism, and elimination of dioxin from tissues within the body. The determinants of dioxin dosimetry in the body include physicochemical properties (e.g., diffusion constants, partitioning constants, kinetic constants, and biochemical parameters for metabolism) and physiological parameters

(e.g., organ flows and volumes). The mathematical structure that describes the interconnections among these determinants constitutes a mathematical model for the tissue dosimetry of dioxin. The model used in the context of this reassessment is a PB-PK model. These types of models describe the pharmacokinetics of dioxin with a series of mass-balance differential equations. These models have been validated in the observable response range for numerous compounds in both animals and humans making them extremely useful for risk assessment, especially for cross-species extrapolation. In addition, they aid in extrapolation from one chemical to other structurally related chemicals since many of the components of the model can be deduced for structurally related compounds. The development of PB-PK models ~~for general use is discussed in Clewells and Jain (1983) and for use in risk assessment by Clewell and Andersen (1985).~~ *for general use Clewells and Jain, 1990 and for use in risk assessment by Clewell and Andersen (1985).*

In brief, a PB-PK model consists of a series of compartments that are based on the anatomy and physiology of the test animals; hence, the term PB-PK ~~(ref. original use of term)~~. The time course of behavior in each compartment is defined by an equation containing terms for input and loss of chemical. For example, if C_t represents the concentration of compound in a tissue (t) and C_B the concentration of compound in blood (B), one of the simplest relationships one might use is:

$$\frac{\partial C_t}{\partial t} = r_{Bt} C_B - r_{tB} C_t - r_m C_t \quad [1]$$

where $\partial C_t / \partial t$ represents the change in the concentration in the tissue over time (t), r_{Bt} is the rate (per unit concentration) of the movement of the compound from blood to tissue, r_{tB} the rate from tissue to blood, and r_m the rate of metabolism in the tissue. Equations of this form will be used in mass-balance modeling of the pharmacokinetic processing of TCDD.

Several PB-PK models have been developed for dioxin and related chemicals (see Chapter 1 for a brief overview). PCBs have been extensively studied (Lutz et al., 1977,

1984; Matthews and Dedrick, 1984). King et al. (1983) modeled the kinetics of 2,3 7,8-TCDF in several species, and Kissel and ^{Leung}Rambarge (1988) proposed a human PB-PK model. 

The development of PB-PK models for TCDD began with work by Leung et al. (1988) in mice. This model was extended to Sprague-Dawley rats by Leung et al. (1990a) and to 2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin in mice (Leung et al., 1990b). Since many of the regulatory standards for dioxin have been based on a finding of hepatocarcinogenicity in female Sprague-Dawley rats, we will focus on the model by Leung et al. (1990a) in this strain and species.

The Leung et al. (1990a) PB-PK model contains five tissue compartments including blood, liver, fat, slowly perfused tissue, and richly perfused tissue. This early model is blood flow limited, a condition that is appropriate when membrane diffusion is much more rapid than blood flow to the tissue. Thus, in this PB-PK model, the tissue/tissue blood compartments are lumped together as a single compartment in which the effluent venous blood concentration of TCDD is equilibrated with the tissue concentration. The model includes a TCDD-binding component in blood described by a linear process with an effective equilibrium between the bound and free TCDD given by a binding constant, K_{AB} . It also includes binding of TCDD to two classes of protein in the liver: one corresponding to the high-affinity, low-capacity Ah receptor and the other to a lower affinity, higher capacity microsomal protein (CYP1A2), which is inducible by TCDD. In the formulation of this PB-PK model by Leung et al. (1990a), both types of binding proteins are explicitly defined using an instantaneous correspondence between occupancy and induction using separate binding capacities and dissociation constants for each protein. These binding reactions are modeled via Michaelis-Menten equations.

In the Leung et al. (1990a) model, the tissue storage capacity depends on the partition coefficients (assumed to be linear with concentration) and the specific protein binding. Dioxin is very lipophilic and is found in higher concentrations in liver than would be expected based on partition coefficients. The incorporation of a ~~term~~ for specific binding of

term

dioxin to a liver protein used by Leung et al. (1988) is a modification over earlier models for these lipophilic compounds.

In various studies, dioxin has been administered by intravenous administration, intraperitoneal injection, oral feeding or intubation (gavage), or subcutaneous (sc) injection. In the PB-PK modeling framework, intravenous injection can be described by starting the integration with an initial mass equal to the dose in the blood compartment. Oral intubation and sc injection can be modeled as if they adhere to first-order uptake kinetics with dioxin appearing in the liver blood after oral administration and in the mixed venous blood after sc injection. Feeding was modeled by Leung et al. as a zero-order input on days that dioxin was included in the diet. These descriptions of the routes of uptake are clearly not defined in specific physiological terms; they are empirical attempts to estimate an overall rate of uptake of TCDD into the PB-PK model. This is one area in which additional research could improve dose-response modeling for TCDD. Efforts to provide more biological details concerning the physiological basis of absorption across these various membranes, including intact skin, would prove valuable for exposure assessments with dioxin. With the iodinated analog, 2-iodo,3,7,8-trichlorodibenzo-*p*-dioxin, the estimated rate constant for oral absorption was considerably larger in induced (0.15/hour) than in naive animals (0.4/hour). The physiological basis of this change is unknown.

With many volatile organic chemicals there are convenient in vitro methods for estimating partition coefficients (Dato and Nakajima, 1978; Gargas et al., 1989). For TCDD and other highly lipophilic, essentially nonvolatile compounds, there are no reliable in vitro methods and these constants have to be estimated from measurements of tissue and blood concentrations in exposed animals. This leads to a difficulty in differentiating between specific-tissue binding and the partitioning to the tissue. Leung et al. (1990a) overcame this problem by assuming binding occurred only in the liver and that the liver partition coefficient was the same as the kidney. This permitted estimation of the relative binding capacities and affinities of specific hepatic proteins. The predictions from this modeling exercise prompted

a series of experiments to examine the nature of these binding proteins in mice (Poland et al., 1989a, b).

Metabolic clearance was modeled as a first-order process. In the mouse with the iodo-derivative, dioxin pretreatment at maximally inducible levels caused a threefold increase in the rate of metabolism. There is no evidence to suggest an increase of metabolism in the rat for TCDD; however, ~~there are data supporting small increases in metabolic clearance at high doses for the tetrabrominated analog (Kociba et al., 1991).~~ The chapter by Olsen (5) discusses pathways for TCDD metabolism.

Finally, Leung et al. (1990a) kept all physiological parameters (e.g., flow rates, tissue weights) constant over the lifetime of the animal.

Dioxin and dioxin analogs have dose- and time-dependent kinetics in both rodents (Kociba et al., 1976; Poland et al., 1989a; Abraham et al., 1988; Rose et al., 1976; Tritscher et al., 1992) and humans (Wolf, 1992; Carrier, 1991). For single- and short-duration exposures, as the exposure level increases, the proportion of total dose found in the liver increases. For chronic exposures, there appears to be a linear relationship between dose and tissue concentration in the gavage study of Tritscher et al. (1992), but this may be simply an inability to observe nonlinearity in liver in intermediate dose ranges. The Leung et al. (1990a) model adequately predicts the tissue concentrations observed by Rose et al. (1976) but did considerably worse at predicting the results observed by Kociba et al. (1976), underpredicting concentrations at the lowest dose by a factor of 3.2 and overpredicting concentrations at the highest dose by a factor of 2. The data of Abraham et al. (1988) and Tritscher et al. (1992) were not available at the time this model was developed, but at least for the data of Tritscher et al. (1992), this model has been shown to overpredict the tissue concentrations (Kohn et al., 1992).

As mentioned earlier, the default position of the EPA in estimating risks from exposure to xenobiotics involves the use of a model that produces risk that is proportionate to dose for low doses (low-dose linearity). Thus, in discussing the models and submodels that form a basis for a mechanistic model for TCDD, we will focus on aspects of the model that

could lead to nonproportional response for low environmental doses. The model of Leung et al. (1990a) predicts slight nonlinearity between administered dose and tissue concentration in the experimental dose range. In the low-dose range, the model predicts a linear relationship between dose and concentration. (They argue, however, that tissue dose alone should not be used for risk assessment for TCDD due to the large species specificity in the ability of TCDD to elicit toxicity [Andersen, ^{et al., 1987}1987]). They instead suggest that use of time-weighted receptor occupancy linked with a two-stage model of carcinogenesis as a better approach to risk estimation. The time-weighted receptor occupancy predictions derived from the Leung et al. (1990a) model are linear in the low-dose region, reaching saturation in the range of high doses used to assess the toxicity of TCDD. Comparison of this proposed effective dose with the two-stage model of carcinogenesis is presented later in this chapter.

Looking at one aspect of modeling TCDD's effects, Portier et al. (1992) examined the relationship between tissue concentration and the modulation of the three liver proteins by TCDD in intact female Sprague-Dawley rats. The proteins studied included the induction of two hepatic cytochrome P-450 isozymes, CYP1A1 and CYP1A2, and the reduction in maximal binding to the EGF receptor in the hepatic plasma membrane. The modulation of these proteins is believed to be mediated through TCDD binding to the Ah receptor. Then, as described in earlier chapters, through a series of alterations in the receptor-dioxin complex, transport to the nucleus, binding to transcriptionally active recognition sites on DNA, activation of gene transcription, and alterations in gene mRNA products, CYP1A1 and CYP1A2 are induced. Reduction in maximal binding to the EGF receptor requires additional protein interactions.

General empirical models have been developed for the regulation of gene expression (Hargrove et al., 1990). This modeling approach includes mRNA production by a zero-order process and first-order degradation. Activation alters one or both of these rates. The production of protein is assumed to be directly related to mRNA concentration. A more specific pharmacodynamic model has been described to account for the induction of tyrosine aminotransferase (TAT) activity by the corticosteroid prednisolone (Nichols et al., 1989). In

this induction model, the input prednisolone concentration is specified by the measured time course of prednisolone in plasma. Prednisolone binding to receptor is specified by association and dissociation rate constants. The prednisolone receptor binds DNA with a specified association rate constant, and the bound receptor recycles to cytosol with a transport time, T (effective compartment transport times are included to account for delays between interaction with DNA and the appearance of TAT activity). A power function can describe a nonlinear relationship between the concentration of prednisolone receptor and the production rate of protein. The actions of prednisolone and maintenance of its tissue concentration are much more short-lived than those of dioxin, and the modeling period of interest is only on the order of several hours to a day instead of days, weeks, or months as with dioxin.

The important relationships presented here are the association of dioxin with the Ah receptor and the association of the dioxin receptor complex with DNA. As described above, Leung et al. (1988) modelled the induction of CYP1A2 as being due to a basal amount of protein plus an additional amount of protein resulting from binding of TCDD to the Ah receptor. The extent of induction was calculated as instantaneously related to percent occupancy of the Ah receptor via a Michaelis-Menten type relationship. Changes in CYP1A1 and EGF receptor binding were not modeled by Leung et al. (1990a).

Portier et al. (1992) modeled the rate-limiting step in the induction of CYP1A1 and CYP1A2 following exposure to TCDD using a Hill equation. Hill equations are commonly used for modeling ligand-receptor binding data. This equation allows for both linear and nonlinear response below the maximal induction range. A complete discussion of Hill kinetics and other models for ligand-receptor binding is given by Boeynaems and Dumont (1980); examples of the use of Hill kinetics for ligand-receptor binding include the muscarinic acetylcholine receptors (Hulme et al., 1981), nicotinic acetylcholine receptors (Colquhoun, 1979), opiate receptors (Blume, 1981), the Ah receptor (Gasiewicz, 1984), estrogen receptors (Notides et al., 1985), and glucocorticoid receptor (Sunahara et al., 1989). As a direct comparison to what was done by Leung et al. (1990a), it is interesting to note

that the Hill model can be thought of as a very general kinetic model that includes standard Michaelis-Menten kinetics when the Hill exponent is 1. Portier et al. (1992) modeled the reduction in maximal binding to the EGF receptor also following Hill kinetics, but with TCDD reducing the binding from the maximum level from control values. For all three proteins, proteolysis was assumed to follow Michaelis-Menten kinetics. The proposed models fit the data in the observable response range.

The major purpose of the paper by Portier et al. (1992) was to emphasize the importance of endogenous protein expression on the shape of the tissue concentration/response curve. For each protein, they considered two separate models. In the first, the additional expression of protein induced by TCDD is independent of the basal level expression. Such a mechanism is similar to that used by Leung et al. Under this model protein expression is given by the equation:

$$\frac{\partial P}{\partial t} = B_p + \frac{V_m C^n}{K_d + C^n} - \frac{V_p P}{K_p + P} \quad [2]$$

where P is the concentration of protein in the liver, B_p is the basal rate of production of protein, V_m is the maximal level of induction of protein by TCDD, K_d is the apparent dissociation constant for binding (in the rate-limiting step), C is the concentration of TCDD in the tissue, V_p is the maximal rate of proteolysis, K_p is the proteolysis rate constant, and n is the Hill exponent. When the Hill exponent is estimated to be an integer, the estimate of n can be interpreted as corresponding to the effective number of binding sites that must be occupied for the effect of the binding reaction to be expressed. When the Hill exponent is not an integer, other molecular meanings apply. These are discussed in detail in Boeynaems and Dumont (1980).

The second model Portier et al. considered was one in which the basal expression of these proteins was due to a ligand that competed with TCDD for binding sites. This leads to equations of the form:

$$\frac{\partial P}{\partial t} = \frac{V_m (C+E)^n}{K_d + (C+E)^n} - \frac{V_p P}{K_p + P} \quad [3]$$

where E refers to the concentration of this ligand in units of TCDD binding-affinity equivalents. Under steady-state conditions, equations (2) and (3) are simplified (Portier et al., 1992).

Using these simpler formulas, they see virtually no difference between the independent and additive models in the observable response range, even to the point of getting almost equal Hill coefficients in the two models for all three proteins. In the low-dose range where risk extrapolation would occur, the models differed depending on the value of the Hill coefficient.

In all cases, the additive model resulted in low-dose linearity. This is expected, since, under the additive model, each additional molecule of TCDD adds more ligand to the pool available for binding and thus increases the concentration of protein. Similar observations have been made with regard to statistical (Hoel, 1970) and mechanistic (Portier, 1987) models for tumor incidence. For CYP1A1, the Hill exponent was estimated to be ~2. When the Hill exponent is > 1, the independent model yields a nonlinear dose-response that is concave (threshold looking). For CYP1A2, the Hill exponent was estimated to be ~0.5. When the Hill exponent is estimated to be < 1, dose-response is again nonlinear, but in this case it is convex, indicating greater than linear increases in response for low doses. Finally, for the EGF receptor, the Hill exponent was approximately 1, in which case the two models are identical.

Thus, even though these two basic models show almost identical response in the observable response region, their low-dose behavior is remarkably different. If either CYP1A1 or CYP1A2 levels had been used as dose surrogates for low-dose risk estimation, the choice of the independent or additive model would make a difference of several orders of magnitude in the risk estimates for humans. Using CYP1A1 as a dose surrogate, the

independent model would predict much lower risk estimates than the additive model. For CYP1A2, the opposite occurs. For EGF receptor, there would be no difference.

Andersen et al. (1992) modified the model of Leung et al. (1990a) to include Hill kinetics in the induction of CYP1A1 and CYP1A2 and to use a diffusion limited approach to the development of a PB-PK model as compared to the blood flow limited approach used by Leung et al. Diffusion limited modeling is preferred when diffusion into a tissue is less rapid than blood flow to a tissue. In the model used by Andersen et al. (1992), each tissue has two subcompartments, the tissue blood compartment and the tissue itself. Free TCDD flows into the tissue blood compartment and, from there, diffuses into the tissue. There is no direct relationship between effluent venous concentrations and tissue concentration in this diffusion limited model. For TCDD, the diffusion limited approach is preferred due to the compound's potentially slow diffusion into the liver from blood (Kohn et al., 1992).

Binding of TCDD to the Ah receptor was modeled in a fashion identical to that used by Leung et al. (1990a). The concentration of CYP1A2 was modeled as before using a steady-state model, in this case, with Hill kinetics instead of a Michaelis-Menten model. The resulting equation is identical to that used by Portier et al. (1992) for the independent induction of CYP1A2 except that they related this to the concentration of Ah receptor/TCDD complex rather than the concentration of TCDD in the liver. Since they assume binding of TCDD to the Ah receptor follows Hill kinetics with a Hill coefficient of 1 (Michaelis-Menten kinetics), the model of Andersen et al. (1992) approaches the independent induction model of Portier et al. (1992) for low doses.

The induction of CYP1A1 was modeled as a time-dependent process as in equation (2), again utilizing TCDD bound to the Ah receptor rather than tissue concentration of TCDD. Most of the physiological constants and many of the pharmacological and biochemical constants used in the Leung et al. (1990a) model were changed for the Andersen et al. (1992) model to correspond to Wistar rats. The parameters in the model were optimized to reproduce tissue distribution and CYP1A1-dependent enzyme activity in a study by Abraham et al. (1988) and liver and fat concentrations in a study by Krowke et al.

(1989). For the longer exposure regimens and observation periods, changes in total body weight and the proportion of weight as fat compartment volume were included via piecewise constant values (changes occurred at 840 hours and 1,340 hours).

Andersen et al. (1992) noted that the liver/fat concentration ratio changes as dose changes due to an increase in the amount of binding protein in the liver. For high doses in chronic exposure studies, this introduces a nonlinearity into the concentration of TCDD in the liver. In the low-dose region, because the Hill coefficient for CYP1A2 and for the Ah receptor are equal to 1, the liver concentration as a function of dose is still effectively linear (i.e., small doses of TCDD will bind to the Ah receptor increasing the amount of Ah receptor/TCDD complex, which then induces additional production of CYP1A2, which can bind to free dioxin). In the observable response range, there is a slight nonlinearity in the concentration of TCDD in the liver as a function of dose under chronic exposure (Andersen et al., 1992). This nonlinearity in the dose region of 1 to 100 ng/kg/day does not agree with the findings of Kociba et al. (1976) and Tritscher et al. (1992) for chronic exposure in Sprague-Dawley rats. The plateau in total liver concentration predicted by the model of Andersen et al. (1992) does occur in the data of Kociba et al. (1976) and Tritscher et al. (1992), in the range of 100 ng/kg/day consistent with the 87 ng/kg/day predicted by Andersen et al. (1992). However, the changes in liver/fat ratio observed by Andersen et al. (1972) and supported by human evidence (Carrier, 1991) are a necessary part of the modeling for TCDD.

Finally, with regard to risk estimation, Andersen et al. (1992³) compared the induction of CYP1A1 and CYP1A2, the concentration of free TCDD in the liver, and the total concentration of TCDD in the liver to tumor incidence (Kociba et al., 1976) and the volume of altered hepatic foci (Pitot et al., 1987). Using a biweekly dosing regimen, TCDD was injected intramuscularly in female rats for 6 months. The concentration of TCDD in the liver and the concentration of induced protein were integrated over time to get summary measures of internal exposure. They concluded that tumor promotion correlated more closely with predicted induction of CYP1A1 than the other integrated quantities. No formal

measure was used to support this observation. Thus, without a formal measure of agreement, it is difficult to know if CYP1A2 induction can be rejected as correlating with tumor response or focal lesion volume. This is discussed later in this chapter. Finally, the choice of an independent induction model for CYP1A1 and a Hill coefficient > 1 leads to nonlinear low-dose behavior. If the promotional effects of TCDD follow a similar mechanism, the risk from exposure at low doses will be negligible. For risk assessment, it is important to know if an additive model also fits these data and agrees with the promotional effects of TCDD since such a model will have different low-dose behavior than the independent model.

Kohn et al. (1992) expanded upon the model of Leung et al. (1990a) to include Hill kinetics, a restricted flow-limited PB-PK formulation, and an extensive model of the biochemistry of TCDD in the liver. The goal of the model was to explain TCDD-mediated alterations in hepatic proteins in the rat, specifically considering CYP1A1, CYP1A2, Ah receptor, EGF receptor, and estrogen receptor over a wide dose range. In addition, the model describes the distribution of TCDD to the various tissues, accounting for both time and dose effects observed by other researchers. The PB-PK models developed by Leung et al. (1990a) and Andersen et al. (1992) relied on several single-dose data sets (Rose et al., 1976; Abraham et al., 1988) and were validated against dosimetry results from longer term subchronic and chronic dosing regimens (Kociba et al., 1976, 1978; Krowke et al., 1989). These and more recent studies in which female Sprague-Dawley rats received TCDD (Tritscher et al., 1992; Sewall et al., 1992³) were used by Kohn et al. (1992) to model the pharmacokinetics and induction of gene products in this sex and species. Among the data reported by Tritscher et al. (1992) and Sewall et al. (1992³) were concentrations of TCDD in blood and liver, ^{and 2)} concentrations of hepatic CYP1A1 and CYP1A2 and EGF receptor in the hepatocyte plasma membrane. Kohn et al. (1992) refer to their model as the NIEHS model. The tissue dosimetry for the NIEHS model was validated against the single dose and chronic dosing regimen experiments employed by Leung et al. (1990a) and Andersen et al. (1992).

In the biochemical effects portion of the NIEHS model, the binding of TCDD to the Ah receptor is modeled using explicit rate constants instead of dissociation equilibrium constants (equation 2 with $B_p = 0$). However, larger dissociation rates (k_d , k_i) were used leading to a formulation of the amount of TCDD-Ah-receptor complex similar to that used by Leung et al. (1990a) and Andersen et al. (1992). Many of the other binding reactions in the model were handled similarly (e.g., TCDD binding to CYP1A2 and TCDD bound to blood). This is simply a numerical trick to avoid the necessity of solving for the concentration of TCDD in the liver using the mass conservation relationship described in Leung et al. (1990a).

The physiology described in the NIEHS model is dependent on the body weight of the animal. Body weight changes as a function of dose and age were recorded by Tritscher et al. (1992) and directly incorporated into the model via a smooth function. Tissue volumes and flows were calculated as an allometric formula based on recent work by Delp et al. (1991). To allow the model to fit the data of both Rose et al. (1976) and Tritscher et al. (1992), the NIEHS model includes loss of TCDD from the liver by lysis of dead cells where the rate of cell death (and the resulting lysis) was assumed to increase as a hyperbolic function of the cumulative exposure in the liver to unbound TCDD. No information regarding the rate of TCDD from lysed cells is available; therefore, this feature of the NIEHS model predicts a net contribution of TCDD clearance by TCDD-induced cell death.

In the biochemical effects portion of the NIEHS model, the Ah-receptor/TCDD complex up-regulates four proteins; CYP1A1, CYP1A2, the Ah receptor and transforming growth factor- α (see Figure 1 in Appendix A). For all four proteins, synthesis and degradation rates are defined explicitly. Changes in CYP1A1, CYP1A2 and the Ah receptor are compared to data on these concentrations. The induction of EGF-like peptides is deduced from observations on human keratinocytes (Choi et al., 1991; Gaido et al., 1992) and is quantified based on an assumed interaction with the EGF receptor. However, TCDD-mediated induction of TGF- α has not been clearly demonstrated in liver (see Appendix A). Constitutive rates of expression for CYP1A2, Ah receptor, and EGF receptor are assumed

independent (equation 2) at the induced expression. This has no effect on low-dose rate extrapolation since the Hill coefficients for the induction of these proteins by the Ah receptor/TCDD complex were estimated to be 1. Induction of CYP1A1 was assumed to be based on additive induction (equation 3), but again the Hill exponent was estimated to be 1, leading to low-dose linearity under either model equation (2 or 3). Thus, the NIEHS model found that the induction of all gene products appears to be a hyperbolic function of dose without any apparent cooperativity (i.e., the value of the Hill exponent, n , in equation 2, is estimated to be 1). The discrepancy in the estimates of the Hill exponents between this model and the other models discussed (Portier et al., 1992; Andersen et al., 1992; Kedderis et al., 1992) is probably related to the inclusion of induction of the Ah receptor in the NIEHS model.

In the NIEHS model, the Ah-receptor/TCDD complex down-regulates the estrogen receptor. It is assumed that the estrogen receptor-estrogen complex synergistically reacts with the Ah receptor/TCDD complex to transcriptionally activate gene(s) that regulate synthesis of an EGF-like peptide. This term was introduced to partially account for the observation of reduced TCDD tumor-promoting potency in ovariectomized females as compared to intact female rats (Lucier et al., 1991). This mechanism, although supported by some data (Clark et al., 1991; Sunahara et al., 1989) is speculative (Kohn et al. (1993) (see Appendix A).

There are basically three levels of complexity of PB-PK models for the effects of TCDD. First is the traditional PB-PK model by Leung et al. (1988) with the added complexity of protein binding in the liver. The next level of complexity is the model by Andersen et al. (1992) using diffusion limited modeling and more detailed modulation of liver proteins. Finally, there is the model of Kohn et al. (1992) with extensive liver biochemistry. All three models have biological structure and encode hypotheses about the modulation of liver proteins by TCDD. However, for gene expression, all three models fall in between curve fitting and mechanistic modeling. In their derivation, the parameters were estimated using dose-time-response data for protein concentrations and enzyme activity,

which are a direct consequence of gene expression. This constitutes curve fitting at this level. However, the structure of the models is derived from qualitative information on the effects of TCDD; the PB-PK model, and even the biochemical model of Kohn et al. (1992), predict protein concentrations using data sets that were not included in the original derivation of the model and that were derived from designs other than those used to characterize the original model. This constitutes a mechanistic validation of the original models and places these exercises in the realm of partial curve fitting and partial mechanistic modeling.

In terms of low-dose risk estimation, all three models have limitations. The Leung et al. (1988) model fails to reproduce the tissue concentration data from Kociba et al. (1976) and Tritscher et al. (1992). This is probably due to the high concentration of liver-binding protein (CYP1A2) predicted by this model.

11-7 P¹¹⁻⁷ m-v
The Andersen et al. (1992) and Kohn et al. (1992) models use Hill kinetics to describe at least some of the binding reactions. Hill's equations are based on a molecular scheme of interaction in which it is assumed that there are n binding sites for the ligand and that the reaction is stable in only two states: either completely unoccupied or fully occupied. This implies that the association process is a $(n+1)$ - molecular reaction. In the application of this model to experimental concentration curves, very little molecular meaning can be attributed to the resulting model due to the flexibility of this function with regard to dose-response shape. In addition, there is no unequivocal relationship between an estimated value of n and the existence of molecular interactions between binding sites. For example, two binding sites may exist, but binding to one site produces a small effect, while binding to both sites produces a much greater effect. This would lead to a noninteger value for n when curve fitting. Considering the importance of the Hill coefficient in terms of low-dose extrapolation (Portier et al., 1993) and considering its limitations in terms of biological understanding of the sequence of molecular events involved in induction (Andersen et al., 1992), caution must be used when extrapolating to tissue dose regions outside of those examined directly in the experiment.

Some of the mechanistic assumptions in these models are speculative. Many of the binding and induction equations related to the Ah receptor/TCDD complex are encoded in equations, but their exact nature and level of control at the molecular level are unknown. This is true of CYP1A1, CYP1A2, the Ah-receptor, the estrogen receptor, and EGF-like peptides. Also, the reduction in EGF receptor by internalization described in the model by Kohn et al. (1992) represents just one mechanism for its depletion. It is also possible that the synthesis or degradation of this protein may be under direct control of the Ah receptor, although TCDD does not alter mRNA levels for the EGF receptor in either human keratinocytes (Osborne et al., 1988) or mouse liver (Lin et al., 1991), and EGF receptor does seem to move from the plasma membrane to the cell interior following TCDD exposure in female rats (Sewall et al., 1993).

It should be noted that the mechanistic models have the advantage of suggesting experimental strategies for pursuing the hypothesis of action of TCDD. These models propose specific mechanisms, which can be tested in the laboratory as a means of validation of this model. For the purposes of risk estimation, one must be careful to recognize that these models do not necessarily impart added confidence in low-dose risk estimates because the mechanistic links between TCDD-mediated changes in gene expression and toxic responses are not known.

8.2. TOXIC EFFECTS

8.2.1. Modeling Liver Tumor Response for TCDD

In this section, we have decided to model liver tumor response for TCDD because of the availability of quantitative data on this endpoint. It should be remembered that dioxin induces increased tumor yield at several sites other than liver (Chapter 6), and in humans dioxin exposure is associated with increased mortality due to lymphomas, soft tissue sarcomas, and lung and stomach cancer (see Chapter 7). We do not imply therefore by our selection of liver tumors that this is the exclusive site of dioxin carcinogenicity. It may be

that similar events occur in other sites or that the events discussed below--such as estrogen interaction--are unique to ^{not} liver.

Long-term carcinogenicity studies in rodents have shown that TCDD is a potent, complete carcinogen (Huff et al., 1991). The highest increase in yield of tumors in TCDD-treated animals as compared to controls was in female rodents. As discussed above, there is no evidence for conventional mutagenicity or DNA binding by TCDD. While TCDD clearly alters gene expression, it appears to act through a hormone-like receptor that functions as a transcription regulator of specific genes. Moreover, TCDD ~~clearly~~ interacts with several endogenous hormones at the molecular and organ level. In liver, there is clearly an interaction between TCDD and estrogen. As noted above, the most sensitive animal for cancer response is the female rodent. Ovariectomy also reduced the ability of TCDD to "promote" or produce tumors in ~~animals~~ ^{female rats} pretreated with the mutagen DEN (Lucier et al., 1991). These results are complex and, as noted above, may be relevant to liver only.

Our overall approach models the tumor response observed in rodents and comparing that modeled response to some of the biomarkers of exposure discussed in the section on the biochemistry of TCDD. This approach will deviate from the pure mechanistic modeling outline discussed in the introduction. Due to limitations in the data available for characterizing the models we will employ, some of the parameters used in this modeling exercise had to be obtained directly from the tumor incidence data. These parameters are then compared to the relative changes we would have expected using the biomarkers of effect and exposure that seem reasonable for this endpoint. Thus, this exercise falls in between curve fitting and pure mechanistic modeling. Two basic types of data will be used to find parameter estimates for the two-stage model of carcinogenesis: (1) data on the number and size of focal lesions in the liver and (2) data on the incidence of liver tumors in a 2-year feeding study.

The carcinogenicity data we will use are from a 2-year feeding study in male and female Sprague-Dawley rats (Kociba et al., 1978). For female rats, the study used 86 animals in the control group and 50 animals per group in the three treated groups given doses

of 1, 10, and 100 ng/kg/day. The original pathology of the study recorded significant, dose-related increases in tumor incidence in the lung, nasal turbinates, hard palate, and liver. The original liver pathology has been reviewed several times, most recently by a group convened by Sauer (1990). The data we will concentrate on in this analysis is the incidence of liver adenomas and carcinomas (combined) based on the most recent pathology review. A summary of these data are presented in Table 8-3.

There was a substantial reduction in survival in all experimental groups (including controls) during the course of the study. Other studies have shown that correcting for this drop can result in as much as a twofold change in the low-dose risk estimates (Portier et al., 1984). A simple correction for survival differences (Portier and Bailer, 19??) was applied to these data to present the risk summaries given in Table 8-3. In the analysis that follows, a more rigorous statistical approach was employed.

The data on the number and size of premalignant lesions is from an initiation-promotion study in female Sprague-Dawley rats (Tristcher et al., 1992). This study utilized 91 animals distributed into 10 groups, 5 of which received initiation doses of 175 mg DEN per kg body weight and 5 of which received 1 μ L saline per gram body weight as a control. One saline group and one DEN group received no TCDD as a control for the remaining groups. Animals were exposed to TCDD by gavage once every 2 weeks in doses that averaged out to 3.5, 10.7, 35.7, and 125 ng/kg/day, respectively. Exposure continued for 30 weeks, after which the animals were sacrificed. Serial sections of the liver were stained for placental glutathione-s-transferase (PGST). PGST^{positive} foci were counted and their areas recorded. Table C-1 in Appendix C summarizes these data. More detailed discussions of the experimental design and focal lesion data are given in Tristcher et al. (1992) and Maronpot et al. (1993).

8.2.2. Tumor Incidence

In recent years, there has been a resurgence in interest in refining the mechanistic representation of mathematical models of carcinogenesis. With few exceptions, the

Table 8-3. Administered dose, tumor response, and number at risk of hepatocellular neoplasms in male Sprague-Dawley rats from carcinogenicity experiments of Kociba et al. (1978) using the pathology review of Sauer (Sauer ~~et al.~~ 1990)

Administered dose (ng/kg/day)	0.0	1	10	100
Number with neoplasm	2	1	9	18 ^a
Number on study	86	50	50	50
Survival-adjusted number at risk	57	34	27	31
Lifetime tumor risk	0.035	0.029	0.333	0.581

^aAll neoplasms in all groups were hepatocellular adenoma with the exception of four carcinoma in this high-dose group.

^bUsing the "poly-3" survival adjustment suggested by Portier and Bailer (19??).

mathematic modeling of carcinogenesis at the cellular level has on the use of the multistage model. Theoretical discussions on these models began in the mid-20th century (Arley and Iverson, 1952; Fisher and Holloman, 1951; Nordling, 1953). The first practical application of models from this class was done by Armitage and Doll (1954). One major failure of the Armitage-Doll model is a lack of growth kinetics of the cell populations (Armitage and Doll, 1957; Neyman and Scott, 1967; Moolgavkar and Venzon, 1979). Several researchers proposed a second model, the two-stage model, which is illustrated in Figure 8-6.

The two-stage model assumes that carcinogenesis is the result of two separate mutations, the first resulting in an intermediate cell population and the second resulting in malignancy. Cells in the normal and intermediate populations are allowed to expand in number via replication or reduce in number due to death or differentiation. There are different mathematical approaches to this model since several groups have proposed the same model but used different mathematical developments to predict tumor incidence from this model (Armitage and Doll, 1957; Neyman and Scott, 1967; Moolgavkar and Venzon, 1979; Greenfield et al., 1984??). In the application of the two-stage model that follows, the mathematical development of this model by Moolgavkar and Venzon (1979) and subsequent development of this model will be used.

The two-stage model (Figure 8-6) has six basic rates that must be estimated. These are:

1. β_N = birth rate for cells in the normal state.
2. δ_N = death/differentiation rate for cells in the normal state.
3. μ_{N-I} = rate at which mutations occur adding cells to the intermediate state.
4. β_I = birth rate for cells in the intermediate state.
5. δ_I = death rate for cells in the intermediate state.
6. μ_{I-M} = rate at which mutations occur adding cells to the malignant state.

new 8-6.

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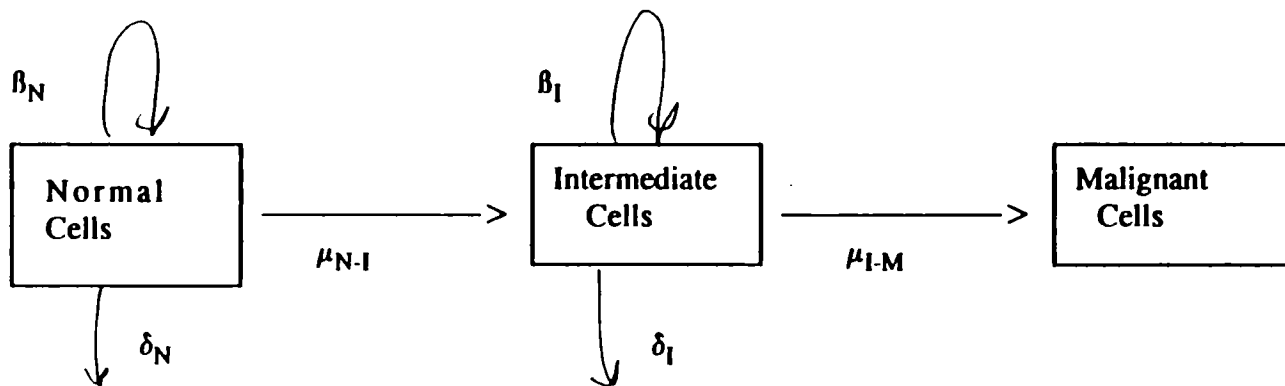


Figure 8-6. A two-stage model of carcinogenesis

To apply this model to dioxin, or any other chemical carcinogen, requires estimates of these rates as they change over dose and time. A mechanistic approach to this would be to incorporate some of the relative changes in proteins seen in the NIEHS model directly into the two-stage model as rate changes in these parameters. Considering the complexity and novelty of this approach, it is left as a research topic. Instead, we will apply this model directly to tumor incidence data, focal lesion data, and cell-labeling data comparing the resulting parameter estimates to predicted dose-surrogates from the models of Leung et al. (1988), Andersen et al. (1992), and Kohn et al. (1992).

This is not the first application of TCDD data to the two-stage model. An application of this model to TCDD was presented by Thorslund (1987). Thorslund treated the effects of TCDD as a direct promoter having an effect only on the birth rate of intermediate cells (β_I) in the two-stage model. The number of normal cells was assumed constant (this is equivalent to setting $\beta_N = \delta_N = 0$ in the model in Figure 8-6). Two parametric models of the change in β_I as a function of dose were used, one model having a single parameter (a first-order kinetic or exponential model) and the second based upon two parameters (a log-logistic model). The parameters in the exponential two-stage model were estimated from the tumor incidence data of Kociba et al. (1978) and validated by goodness-of-fit, cell-labeling data, and species/sex/strain extrapolations. The slope parameter in the log-logistic two-stage model was chosen to be 1, 2, or 3 based on slopes observed in other biological systems. The remaining parameters in this model were estimated from the Kociba et al. (1978) data.

The liver tumor response from the Kociba et al. (1978) study are given in Table 8-3 using the most recent pathology review of the liver sections (Sauer, 1991). Shown are the number of animals with tumor (row 2), number of animals placed on study (row 3), a survival-adjusted number of animals at risk (row 4), and the survival-adjusted lifetime tumor probability (row 5 which equals the entry in row 2 divided by the entry in row 4).

There are a variety of mathematical formulations that could be used to derive a tumor incidence rate under the two-stage model. As has been assumed by other authors (Portier and Kopp-Schneider, 1991; Moolgavkar and Luebeck, 1992), we assume:

↑
(correct)

1. All cells act independently of all other cells;
2. The rates in the two-stage model (Figure 8-6) are constant over the lifespan of the animal;
3. The tumor incidence rate corresponds to the rate of appearance of the first malignant cells; and
4. $\beta_N = \delta_N = 0$ (no change in the number of normal cells).

All four of these assumptions are likely to be violated for most chemicals. In most tissues, there is a homeostatic feedback system to control the number of cells in the tissue. No such system can be assumed here since it results in a mathematic formulation that is either intractable or has yet to be developed. For the large pool of normal cells in the liver, this is unlikely to have an effect, but for the small number of intermediate cells (at least for early times), this could have a small effect on tumor incidence. This issue cannot be resolved without further research. Assumption (2) is clearly violated based on the behavior of the PB-PK models presented earlier. Time-dependent changes in tumor incidence could be incorporated into the modeling and will be at a later time. Finally, the kinetics of cell growth for malignant clones have been studied (Moolgavkar and Lubeck, 1992), and assumption (3) was found to have a moderate impact on the tumor incidence rates. This will be investigated further at a later time.

The exact tumor incidence rate was used to avoid potential bias from the routinely used approximation (Kopp and Portier, 1989). Thorslund (1987) used this approximation in his analysis. The methods outlined by Portier and Kopp-Schneider (1991) employing the Kolmogorov backwards equations were used to derive the exact tumor incidence rate.

It has been suggested that the cells in PGST-+ lesions correspond to the initiated mutated cells in the two-stage model of carcinogenesis. In the following analyses, we have assumed this to be the case, but it is important to keep in mind that the number of enzyme-altered cells may not correspond to mutation rate. If this is true, it is possible to apply the

methods of DeWanji et al. (1990) to data from Lucier and colleagues (Tristcher et al., 1992) to analyze the growth characteristics of these cells (Moolgavkar et al., 1991). This can be combined with the tumor incidence data to produce a combined model that explains both sets of data. This is done below. Analyses of the tumor incidence data alone and the PGST ~~for~~ ^{positive} focal lesion data alone are given in Appendix C.

The most parsimonious two-stage model that agrees with the tumor incidence data and the focal lesion data (this is based on a combination of likelihood ratio testing and plots of the model versus the data) is a hyperbolic function in dose for the effect of TCDD on the rate of transformation from the normal state to the intermediate state, is hyperbolic in dose for the birth rate of intermediate cells, is constant in the ratio between the death rate and the birth rate for intermediate cells, and uses a power function in dose on the rate of mutation from the intermediate state to the malignant state. The resulting model is given as:

$$\mu_{N-I}(\text{dose}) = \mu_0 + \frac{\mu_V \text{dose}}{\mu_k + \text{dose}} \quad [4]$$

$$\beta_I(\text{dose}) = \beta_0 + \frac{\beta_V \text{dose}}{\beta_k + \text{dose}} \quad [5]$$

$$\delta_I(\text{dose}) = \rho \beta(\text{dose}) \quad [6]$$

$$\mu_{I-M}(\text{dose}) = \mu_2 + \mu_3 \text{dose}^{\mu_4} \quad [7]$$

Power of dose

where $\mu_0 = 3.216 \times 10^{-4}$, $\mu_V = 6.856 \times 10^{-4}$, $\mu_k = 19.75$, $\beta_0 = 3.029 \times 10^{-2}$, $\beta_V = 4.296 \times 10^{-3}$, $\beta_k = 2.75$, $\rho = 0.00302$, $\mu_2 = 1.496 \times 10^{-7}$, $\mu_3 = 3.082 \times 10^{-9}$, $\mu_4 = 2.56$, and dose is

expressed in ng/kg/day. This analysis also assumes that the average rodent liver has 6×10^8 normal hepatocytes. The fit of this model to the number and size of focal lesions from the studies of Lucier et al. are illustrated in Figures 8-7 and 8-8. It is clear from these plots that this fit is adequate; this is supported by analyses based on the magnitude of the likelihood (see Appendix C). The fit of this model to the tumor incidence data of Kociba et al. (PW6, PW6, 1990) is shown in Figure 8-9.

It is possible to compare the parameters estimated for the two-stage model to predictions from the PB-PK models to try to locate a reasonable mechanistic link between the two classes of models, to aid in species extrapolation and to help guide us in choosing the most appropriate curvature for low-dose extrapolation. An overall comparison of the dose-surrogates is given in Appendix D. What is illustrated in Appendix D is that most reasonable dose surrogates correlate well with the dose-response relationships given above and in Appendix C. For the purposes of finding a most reasonable model for low-dose risk assessment, we will use two specific dose surrogates from Appendix C.

In the NIEHS PB-PK model (Appendix A), it is hypothesized that induction of CYP1A2 could lead to an increase in the metabolism of estrogens to catechol estrogens (Graham et al., 1988) and that further activation of these catechol estrogens can lead to cell damage (for example, via free oxygen radicals) and eventually to mutations (Metzler, 1984). Thus, the area under the time-concentration curve for induction of CYP1A2 could serve as a useful dose-surrogate for the mutational effects of TCDD expressed as equation (2). Note that this relationship is speculative and that the application of dose surrogates to low-dose risk estimation is subject to a number of untestable assumptions. This is discussed in detail in Appendix D.

In this case, the shape of the response for the area under the time concentration curve for the induction of CYP1A2 parallels the hyperbolic effect given above. A better approach to this analysis would be to use the instantaneous concentration of CYP1A2 as it changes over time to directly modify the mutation rate. However, at this time, this approach is impractical due to inadequate mathematical development and a lack of computer algorithms

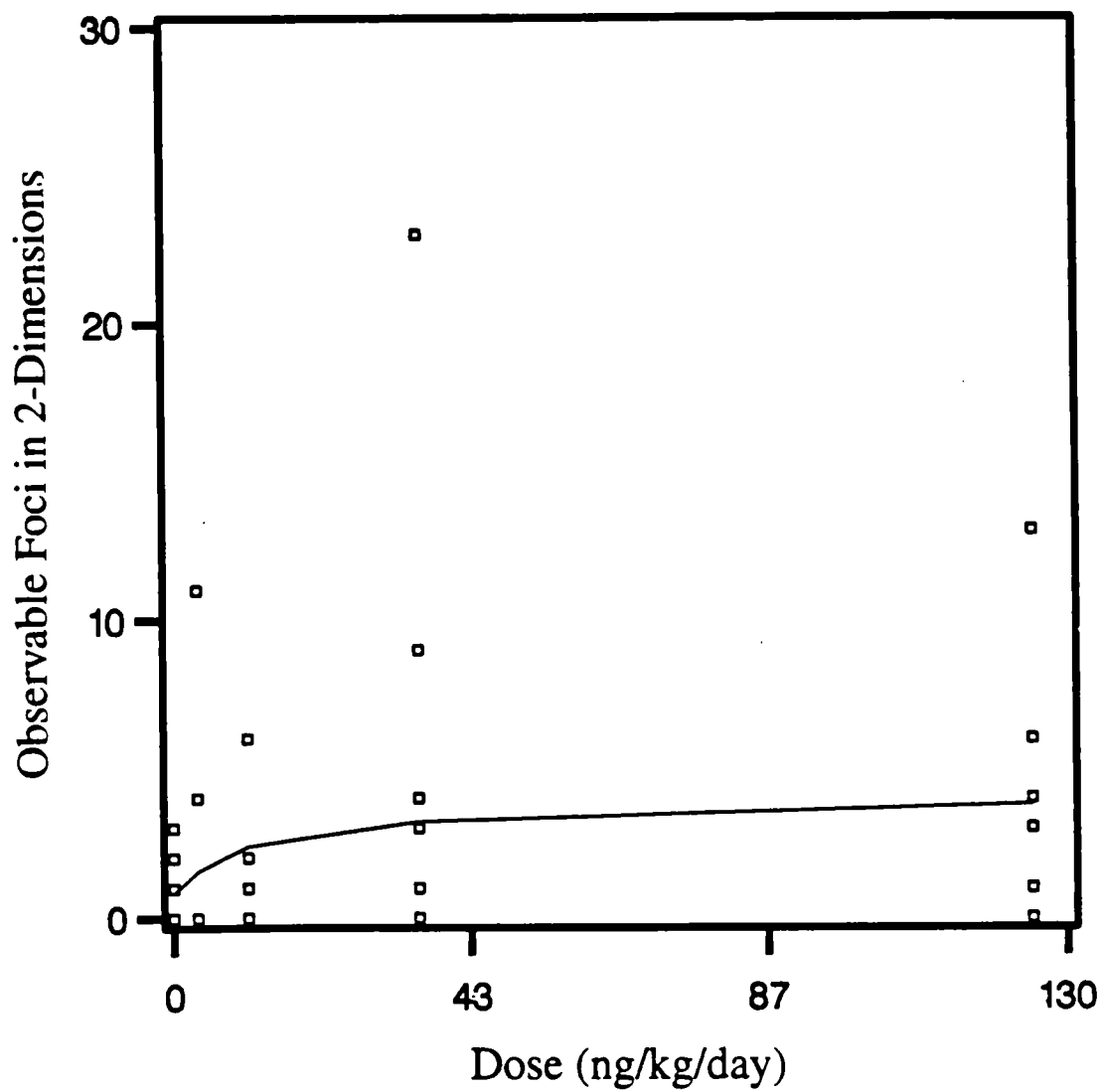


Figure 8-7. Fit of two stage model to the number of focal lesions
from the data of ~~Lucier et al.~~ Maronpot et al., 1993.

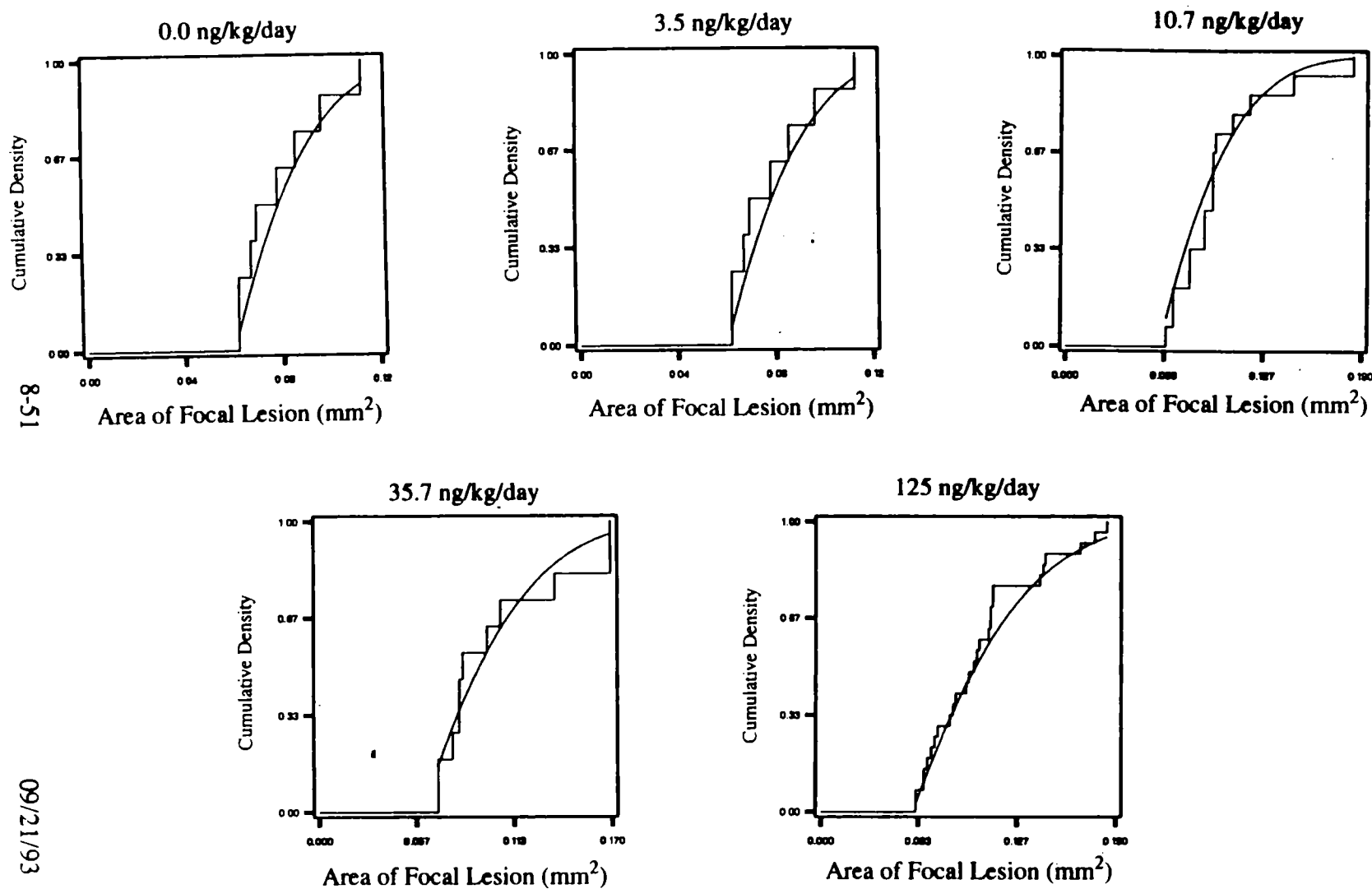


Figure 8-8. Fit of the two-stage model to the size distribution for focal lesions from the data of Lucier et al., where the smooth line is derived from the model and the step function results from the data.

Maronpot et al, 1993

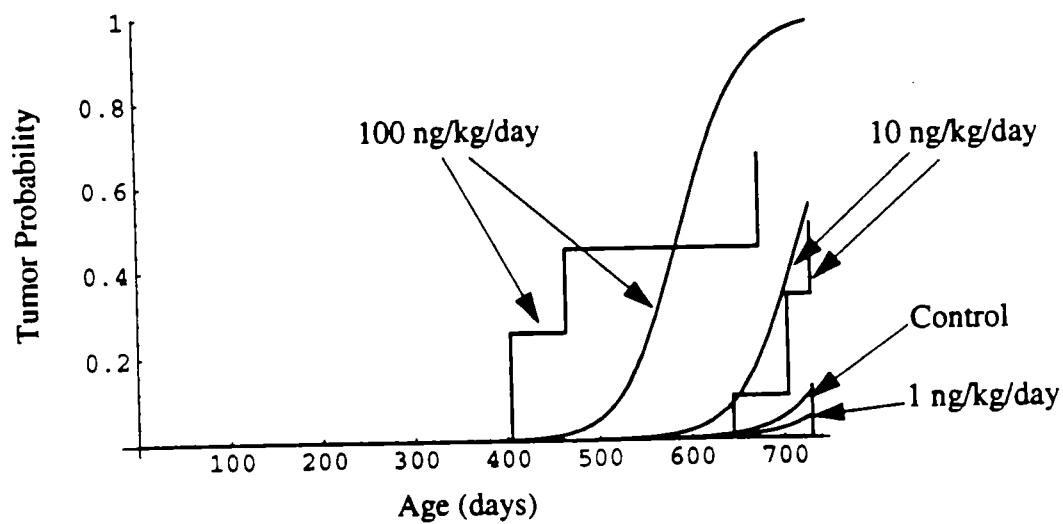


Figure 8-9. Fit of the TS model to the data of Kociba et al. using parameters for $\mu_{N-I}(d)$, $\beta_I(d)$, and $\delta_I(d)$ estimated from the focal lesion data.

and programs for addressing the problem. However, the parallel curves for CYP1A2 and the mutation rate indicate that dramatic changes in low-dose response by going to the time-varying dose surrogate is unlikely.

Kohn et al.¹⁹⁷⁸ also provided a potential mechanism for the proliferative effects of TCDD on the cells in the intermediate state. For this process, they propose a mechanism based on the incorporation of the EGF receptor in an activated state ^{in the cell interior} ~~on the internal of the cell~~ rather than on the cellular membrane. However, the relationship between the area under the time concentration curve for the internalized EGF receptor does not correlate well with the hyperbolic dose-response seen in the growth characteristics of the intermediate cells in the two-stage model given by equation (2) above. This could be due to a number of factors; incorrect two-stage model specification, incorrect specification in the NIEHS model, or a complex relationship between internalized EGF receptor and mitosis of the intermediate cells. Either way, the simple dose surrogates expressed in Appendix D are inappropriate for the parameterization of the two-stage model given above. Note that for both the mutation rate and the mitotic rate, dose-response behavior is adequately described by a linear function in the low-dose region.

The details of a complete analysis of the two-stage model and the Kociba et al. data ¹⁹⁷⁸ is given in Appendix C. The fit of the model given by ⁷ (2) above to the data by Kociba et al. ¹⁹⁷⁸ is shown in Figure 8-9. The fit is adequate and is not statistically significantly different from the best fitting two-stage model which is nonparametric for $\mu_{1-M}(d)$ and uses the parameters derived from the focal lesion analysis. It is significantly different from the best-fitting two-stage model in which all parameters are derived from the tumor incidence data alone (see Appendix C).

Because $\mu_4 > 1$, the relationship between dose and the mutation rate between the intermediate state and the malignant state is sublinear (similar to threshold behavior). As discussed in Appendix D, none of the dose-surrogates for the NIEHS model exhibit this type of behavior. In Andersen et al. (1992), induced CYP1A1 has this type of behavior (this is also seen in Portier et al., 1992). The difference between the Andersen et al. findings and

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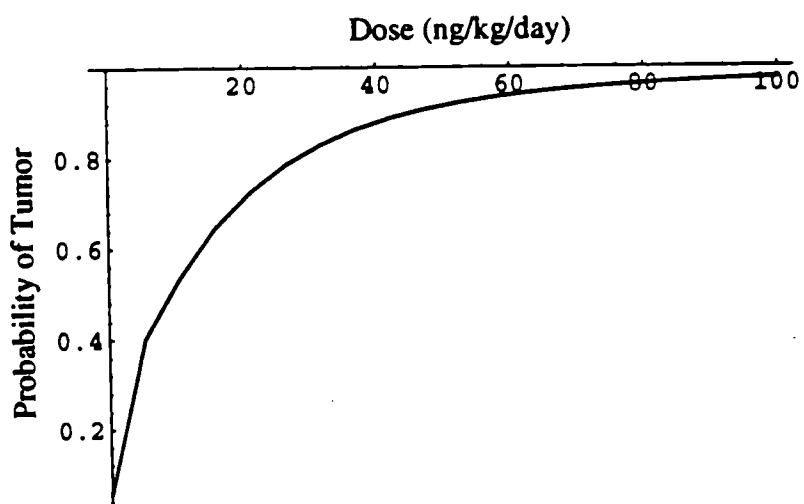
those of Kohn et al. are discussed in Appendix D and will not be repeated here. Also, as explained in Appendix D, these correlations provide very little verification of an actual mechanistic link between the dose-surrogate and the tumor incidence rate.

Even with this nonlinearity in $\mu_{I-M}(d)$, the dose-response for lifetime tumor incidence is linear in the low-dose region (Figure 8-10). This is due to the linear effect of dose on both $\mu_{N-I}(d)$ and $\beta_I(d)$ in the low-dose region. In general, with these two-stage models, if any rate in the process is linear and all other rates or non-decreasing, the low-dose behavior of the model will be linear. Extrapolation to low doses can be accomplished directly using the parameters described above or through the PB-PK models described in the previous section. In order to utilize the PB-PK models, a dose-surrogate must be chosen for a particular rate (parameter) in the two-stage model. To do this, one would proceed as follows. Let s denote the level for a dose surrogate and define $f(d)$ to be the functional relationship between dose (d) and the dose surrogate (s). If f is 1-1 and onto, then the inverse equation, f^{-1} , exists and $d=f^{-1}(s)$ can be used in the two-stage model above. For example, suppose a reasonable dose surrogate for the effect of dose on μ_{N-I} is the induction of CYP1A2. Let $c_A(d)$ denote the relationship between dose and induction of CYP1A2 in the test animal. Instead of using $\mu_{N-I}(d)$ for extrapolation and modeling, one could use $\mu_{N-I}(s)$ having first converted all administered doses into the dose surrogate, s , using $C_A(d)$. Extrapolation would then be done in units of CYP1A2 induction, and extrapolation would result in a predicted induction of CYP1A2, say ϵ_A . To estimate exposure doses in humans would then require knowledge of the induction of CYP1A2 by TCDD in humans, say $s_H=C_H(d)$. The predicted human exposure dose (presence of induction) would be $d_H=C_H^{-1}(\epsilon_A)$. It is important to reemphasize here that enzyme induction ¹⁵ should not ~~be~~ equated to toxic response¹⁶.

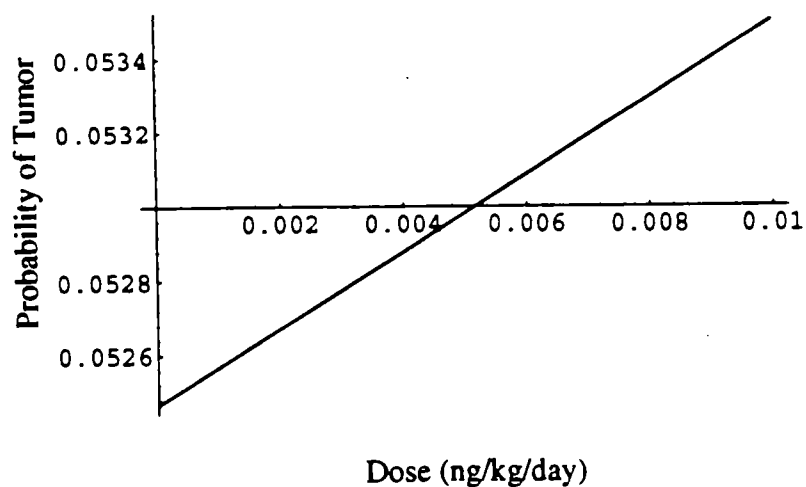
When multiple dose surrogates are used, the equations become more difficult due to the multivariate nature of the relationship. For example, if we were using CYP1A2 induction as a dose surrogate for $\mu_{N-I}(d)$ and removal of EGF receptor from the plasma membrane as a dose surrogate for $\beta_I(d)$, our functional forms would have to be concerned with the changes in both of these proteins as a function of dose. However, the basic idea

Figure 8-10. Dose-response for tumor incidence using a two-stage model of carcinogenesis.

Part A: Observable response region



Part B: Low-dose region



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remains the same, and inversion of the formulae is used to estimate safe exposure levels. Since there are, as yet, no comprehensive PB-PK models for humans, this approach could not be used here. Instead, the results of the PB-PK model are used to support dose-response relationships seen for the tumor incidence data and the focal lesion data.

The final model resulting from the combined analysis of the tumor incidence data and the focal lesion data has several shortcomings. One serious problem concerns the size of the intermediate cell population after 2 years of exposure to TCDD. The model does an excellent job of describing the size and number of focal lesions in the liver at 31 weeks; but, by 2 years, since the same rates seen in the first 31 weeks are assumed constant for 2 years, the model predicts impossibly large foci in the liver (the model predicts that the mean number of intermediate cells is two times larger than a normal rodent liver in the high-dose group). The data available for this analysis consist of single time point data so there is no data-driven way in which to estimate changes in the model parameters (mutation, birth and death rates) over time. Arbitrary changes could be used; however, these are unlikely to change the shape of the dose-response curve for $\mu_{I-M}(d)$ as long as the changes are applied proportionately across all treatment groups.

Additional data will be required to support any improvements in this model. Additional focal lesion data (size and number) at a time point other than 31 weeks would be very useful in terms of looking at time-dependent changes in the rates in this model. Considering the size and number of saline-treated animals with foci at 31 weeks, it would probably be best to have information on focal lesions at a later time. Because the proliferation rates from the focal lesion data match the observed labeling index data from Maronpot et al., (1993) the simplest change that will lead to reasonable numbers of initiated cells is a change in the ratio of the death rate to the birth rate for initiated cells. As the animal ages, this rate will have to converge to 1 or less for the expected number of initiated cells to have a reasonable size at 2 years. If the change over time is proportionately the same over time in all dose groups, it should have little or no effect on the shape of $\mu_{I-M}(d)$; the only effect should be on the magnitude of this mutation rate (increasing it). Any

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dose-dependent changes in this ratio may alter the dose-response shape of $\mu_{I-M}(d)$ and hence also alter the low-dose estimates of risk.

One final note on the final version of the two-stage model fit to the tumor incidence data and the focal lesion data. In the low-dose region, the dose-response curve for added risk from exposure to TCDD can be approximated by the formula $\text{risk} = 0.024 \text{ dose}$ where dose is in units of $\text{ng/kg} \times \text{bw/day}$. The formula can be inverted to estimate a safe exposure dose from these data. For an added risk of 1 in 1 million in the animals, the calculated exposure dose is $41.7 \text{ fg/kg} \times \text{bw/day}$. This is a point estimate of ^{exposure} ~~estimated~~ dose, confidence intervals would reduce this value.

We have described modeling as a continuing process. One must remember that, as a continuing process, modeling allows us to test certain hypotheses developed from a particular model in hopes of designing experiments that can either support or refute the model. Modeling also allows us to determine how well a particular hypothesis, derived from biological evidence, can explain the observed phenomena. One of the models presented here has analyzed two data sets and has presented a hypothesis that dioxin increases, in a dose-dependent manner, the rate at which normal cells become initiated cells, thereby increasing the number of initiated cells. If the model is correct, then dioxins are acting to increase the mutation rate in hepatocytes, thus increasing the number of initiated cells. In essence, dioxins are acting as ^{indirect} ~~mutagens~~ mutagens.

The impetus in developing a biologically based dose-response model for dioxin was that there is no evidence that dioxins are direct mutagens and that the carcinogenic actions of dioxins were due to their actions as receptor-mediated tumor promoters. In theory, tumor promoters do not alter the mutation rate and act only on previously initiated cells. In addition, the actions of a tumor promoter are reversible. If we compare this theory to that of the model's prediction of dioxin's actions, we are left with several contradictions. Either the structure of the model has led us astray or the actions of dioxins increase the mutation rate through indirect mechanisms and that these effects are not detectable in short-term assays.

There is consensus that dioxins are not directly mutagenic, and there are very few data that demonstrate that dioxins damage DNA directly and/or are mutagenic in short-term assays. (See Chapter 6 for detailed discussion.) However, a recent study suggests that the short-term assays may not be sensitive to the potential indirect mutagenic effects of dioxins. Yang et al. (1992) have reported that exposing nontumorigenic immortalized human keratinocytes to TCDD for 2 weeks transforms these cells into tumorigenic cells. Exposures for less than 2 weeks do not result in transformation of this cell line. These experiments indicate that exposure to dioxins can result in permanent changes in the phenotype of human cells. While the mechanism by which this occurs is undetermined, it is clear that these permanent alterations require prolonged exposures in order to be detected. These permanent changes are contradictory to the hypothesis that dioxins act solely on the promoter stage in the process of carcinogenesis.

If dioxins were to increase mutation rates through indirect mechanisms, what actions of dioxins would increase the likelihood of a mutational event? One possibility is that by increasing CYP1A1 and CYP1A2 activity, dioxins increase the production of reactive intermediates. Evidence to support this hypothesis is that CYP1A2 metabolizes estradiol to the catechol estrogen 2-hydroxyestradiol. Catechol estrogen formation may lead to DNA damage and may be responsible, in part, for the carcinogenic effects of estrogens.

Dioxins may also have indirect mutagenic actions through increased hepatocyte proliferation. Cells are constantly exposed to reactive intermediates that may cause DNA damage. Inappropriate stimulation of cell proliferation, by dioxins, may produce an apparent increase in the number of initiated cells in a tumor promotion study by forcing cells with nonlethal DNA damage to replicate prior to the repair of the damage. This could lead to fixed mutations in the genome and clonal expansion of the transformed cells resulting in an increase in preneoplastic foci.

Increased transcription of a gene can also lead to potential mutagenic events. Glucocorticoids can produce a variety of genetic rearrangements through epigenetic mechanisms. If cells transfected with a plasmid containing a glucocorticoid response element

and a recorder gene are exposed to dexamethasone, a variety of mutagenic events such as inversions, deletions, and insertions in the plasmid occur. In addition, antisense mRNA can also be detected following exposure to dexamethasone. This study demonstrates that increased transcription of the recorder gene by dexamethasone increased the likelihood of a mutational event in the recorder gene through epigenetic mechanisms. Dioxins act in a similar manner to dexamethasone and other steroid hormones. Both classes of chemicals bind to intracellular receptors, and the activated receptor binds to response elements on DNA resulting in an increase in gene transcription. It is possible that continued exposure to dioxins will increase the likelihood of a mutagenic event at a gene that is responsive to dioxins due to the increased rate of transcription. Since dioxins alter growth properties of a variety of different cell types such as hepatocytes (Lucier et al., 1991), skin (Stohs et al., 1990), and embryonic palate (Abbott and Birnbaum, 1991), it is possible that some of the genes that can be affected by this mechanism would be involved in the regulation of cellular proliferation.

Chemical carcinogenesis is often divided into stages of initiation, promotion, and more recently progression (reviewed in Pitot and Dragan, 1991). As the process of carcinogenesis develops, the progression stage is an irreversible stage that is associated with increase in karyotypic instability, which is associated with the development of aneuploid malignant neoplasms (Pitot and Dragan, 1991). Thus, during the carcinogenic processes there are steps postinitiation that result in karyotypic instability. It is possible that dioxins are acting not only as promoters but also as progressors, and the mechanisms described above may provide some insight into how dioxins act.

8.2.2.1. A Potential Alternative Model for Promotion of Carcinogenesis By Dioxin:

PB-PK models permit estimation of tissue concentrations of dioxin and of various protein products regulated either directly or indirectly by dioxin and the Ah receptor. In developing a quantitative mechanistic model of the tumor responses to dioxin, it is necessary to propose some relationship between the concentration of these protein factors and the

critical cellular responses—mutation rates and cell proliferation rates—leading to tumor induction. One hypothesis, consistent with dioxin's primary mechanism as a promoter, is that dioxin, directly through effects on mitostimulatory hepatic growth factors, such as transforming growth factor- α , causes enhanced cell replication in normal and/or initiated cells. In some of the cancer modeling in this chapter, this approach has been followed at least indirectly by assuming that the reductions in cell surface ~~EGFR response~~ ^{EGFR concentrations} (EGFR) are an indication of enhanced levels of an activating ligand for this receptor and that the reduction in EGFR is a surrogate for increased cell proliferation rates.

Pitot et al. (1987) examined promotion of altered hepatic foci by dioxin in rats that were initiated with a DEN dose of 10 mg/kg following 70 percent partial hepatectomy. These altered hepatic foci are believed to be precursor lesions for overt hepatic malignancies. In this study, doses used were equal to the bioassay doses used by Kociba et al. (1978) with an additional low dose, 0.0001 μ g/kg/day. One important aspect of this study and the original dioxin bioassay is the dose range covered, 100-fold in the cancer bioassay and 1,000-fold in the promotion study. Pitot et al. (1987) found that the promotion dose-response behavior was U-shaped; the two lower doses gave a lower response for volume of liver as foci or total number of observed foci than did either the control or high-dose groups. This U-shaped dose-response behavior was also observed in the liver tumor incidence in the bioassay study, although the differences between control incidence and incidence at the lower doses were not statistically significant. These observations indicate a more complex relationship between dioxin exposure and response than expected for dose surrogates, whether dioxin concentrations, cellular EGFR concentrations, or protein product concentrations, that increase (or decrease) monotonically with dioxin concentration.

Although less marked than with dioxin, phenobarbital also had a U-shaped dose-response behavior for hepatic promotion when examined with the same experimental protocol over a dose range of 0.001 to 0.05 percent in the diet (Pitot et al., 1987). Jirtle and coworkers (1991) have examined the biology of phenobarbital promotion in detail. They propose that phenobarbital initially causes a mitogenic stimulus with proliferation of

pericentral hepatocytes. The liver responds to the persistent proliferative signal by increasing levels of mitoinhibitory growth factor, TGF- β 1, and periportal hepatocytes stain intensely for TGF- β 1. Down-regulation, presumably without activation, of EGFR also occurs in phenobarbital-treated livers. The hallmark of promotion in this hypothesis is the selection provided by the persistent increases in TGF- β 1 for initiated cells with particular alterations that decrease their responsiveness to these inhibitory growth factors. Consistent with this mechanism, the putative preneoplastic hepatocytes express significantly less TGF- β 1 than do the periportal hepatocytes (Jirtle et al., 1992). The suppression in growth of the normal cells in the tissue then is the critical alteration leading to promotion and really is more of a homeostatic response than a direct response of the cell to dioxin. In this model of hepatic promotion, the increases in mitoinhibitory factors serve to maintain the liver at constant size even in the continued presence of proliferative stimuli provided by dioxin, phenobarbital, or other xenobiotics that might share similar mechanisms of action. The clonal expansion of initiated cells results from a growth advantage because of their relative insensitivity to the antiproliferative environment produced by the promoter (Jirtle et al., 1991).

This general mechanism of promotion may also occur with dioxin (Mills and Andersen, 1993) and could give rise to the U-shaped dose-response curve seen by Pitot et al. (1987). Initiated cells might have differential sensitivity to apoptosis mediated by TGF- β 1 (Oberhammer et al., 1992) and to proliferation induced by other factors regulated by dioxin. If the apoptotic rates were increased at lower dose than doses that enhance proliferation, a U-shaped curve would be expected. Another possibility is that clones observed in control (PH/DEN; 0 μ g dioxin/kg/day) rats are derived from different precursor cells than are those clones arising in livers of rats treated with higher daily doses of dioxin (>0.01 μ g/kg/day). In the control rats no net increase in TGF- β 1 is expected. The initiated cells that become foci in these rats are potentially responsive to the antiproliferative activity of TGF- β 1 and, as the dose of dioxin is increased, the death rates of these cells increase; fewer clones grow out to become observable foci, and a lesser volume of the liver is occupied by these clones. At higher dioxin doses, tissue TGF- β 1 is increased and the selection environment favors growth

of a new population of initiated cells that are TGF- β 1 nonresponsive. At intermediate doses, the primary response is inhibition of the first type of initiated cells with a decrease in yield of observable clones. At these doses there is not yet any increase in the appearance of clones arising from the second population of initiated cells. This behavior would lead to a U-shaped dose-response curve. Early efforts to parameterize these promotion models (Andersen et al., 1993) indicate that the model assuming a different cellular origin of clones in control versus high-dose dioxin rats appears the more likely of these two hypotheses at this time.

8.2.3. Other Effects¹ Mammary/Uterine/Anticancer Endpoints

There are several lines of evidence that suggest that TCDD is anticarcinogenic in some organs of experimental animals. The most compelling data are from the Kociba bioassay (Kociba et al., 1978), which reported a dose-dependent decrease in several endocrine tumors in females. The most remarkable changes occurred in the incidence of carcinoma of the breast and uterus and all tumors of the pituitary. The decrease in these tumors coincided with the increase in hepatic tumors in the same animals. Tumor inhibition has also been reported in skin initiation-promotion experiments if TCDD administration precedes the initiator. This TCDD-induced inhibition occurs even if the complete initiator-promoter protocol is carried out following the TCDD administration (DeGiovanni et al., 1977; Marks et al., 1981).

Mechanistic explanations for these observations are incomplete. Several laboratories have attempted to elucidate the molecular events leading to the decrease in tumors particularly in the breast and uterus. Three lines of evidence are under great scrutiny at this time including (1) alteration of the estrogen receptor, (2) alteration of estrogen metabolism, and (3) alteration of binding of estrogen and estrogen receptor at the DNA level.

Down-regulation of the estrogen receptor has been reported by laboratories of Gallo (Gallo et al., 1986; DeVito et al., 1990), Safe (Romkes and Safe, 1987; Astroff and Safe, 1988, 1990), and Gierthy et al. (1987). This down-regulation occurs in a rank order

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relationship with the affinity of TCDD structural analogs to bind to the Ah receptor (Harris et al., 1990) and parenthetically with the ability of the compounds to induce cytochrome P-450IA1. There is differential regulation of the ER by TCDD in uterus and liver (liver being more sensitive) and the regulation of the uterine ER disappears in the adult animal (DeVito et al., 1992). Collectively, these observations suggest that there are at least two mechanisms involved in ER regulation by TCDD. Gierthy has supported the argument that, in MCF-7 cells, the alteration of estrogen metabolism following the induction of cytochrome P-450 is the explanation for the decreased activity of the ER. Safe has reported that the ED₅₀ for decreased ER in MCF-7 mice cells is several orders of magnitude below the ED₅₀ for induction of EROD in these same cells (Zacharewski et al., 1991). Gierthy's position is supported by Bradlow, who suggests that indolo(3,2b)carbazole, the active inducer of cytochrome P-450IA1 in cruciferae, decreases breast cancer in rodents by increasing the metabolism of estradiol (Bradlow et al. 19??). In vivo studies suggest that the uterine response is more responsive to changes in circulating estradiol (which may or may not hold up for breast tissue), but the hepatic ER response to TCDD is independent of circulating estradiol levels (DeVito et al., 1992). Another possibility is that TCDD induces enzymes that qualitatively change in situ estrogen metabolism and that these novel metabolites (or families of metabolites) alter estrogen action. Recent evidence suggests the presence of a 15-alpha metabolite of estradiol in liver microsomes, from TCDD-treated females, incubated with estradiol (Gallo and Conney ~~unpublished~~). This observation is similar to that of Gierthy and Lincoln (1990) in MCF-7 cells.

Another possible mechanism of the decrease in breast cancer is that the binding of the activated AhR complex ^{to GPRE} ~~to the Ah locus~~ on the DNA is in the upstream region of the ER gene or is in the vicinity of the binding region of the activated ER. Several lines of evidence also support this hypothesis since there is ample evidence that other steroid receptor complexes modify the DNA binding of different members of the steroid receptor superfamily (Gustafsson et al., 1987; Gutliff et al., 1987).

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The last area to be discussed here is the role of the pituitary in the regulation of the endocrine system and in particular the relationships between pituitary-hypothalamus and the estrogen receptors. The ER in liver and uterus is controlled by the pituitary (Lucier et al., 1972), and growth restores the albat of hypophysectomy. Hypophysectomy has little effect on TCDD induction of P-450, but TCDD does decrease ER in growth hormone-restored animals (DeVito et al., 1991). Interestingly, Peterson et al. (See Chapter on Reproduction) have made similar observations for regulation of testosterone and testicular function by TCDD.

The importance of this subsection is at least twofold. First, the regulation of the estrogen receptor is one of the most sensitive non-P-450 markers of TCDD exposure in immature females, and this regulation coincides in rank-order fashion with decreases in mammary, uterine, and pituitary tumors in TCDD-treated female Sprague-Dawley rats. Second, the removal of the ovaries (the primary source of estradiol) inhibits the formation of hepatic tumors in rats by TCDD (Lucier et al., 1991) without altering the ability of TCDD to induce cytochrome P-450IA1 (DeVito et al., 1992). However, ovariectomized animals developed lung tumors in a two-stage model in rats, whereas no lung tumors were detected in intact rats subjected to the same two-stage model (Clark et al., 1991). Taken together, these findings indicate that estrogens can either enhance or protect against TCDD-mediated cancer in a site-specific manner and that there is likely more than one mechanism for TCDD's carcinogenic action.

8.2.4. Noncancer Endpoints (~~DeVito et al. 1992~~)

Previous risk assessments have focused primarily on cancer as the most important and sensitive endpoint. This assumption has recently been questioned. For example, lead is carcinogenic in experimental paradigms, yet it is the neurotoxicity that drives the risk assessment. Past risk assessments of TCDD and its congeners have also focused on cancer as the primary toxic endpoint, although it produces adverse effects in a wide variety of tissues and cells. It is possible that the immunological, reproductive, or developmental

toxicities of TCDD are just as sensitive and important in the risk assessment process. For noncancer endpoints, risk assessments traditionally have used the safety factor method to estimate risk. Biologically based mathematical models for noncancer endpoints have not been extensively utilized and are not as developed as are cancer risk models. The development of biologically based models requires that the responses are well characterized, tissue doses have been established, and sufficient data are available to propose a mechanistic model. Many of the toxic effects of TCDD are well characterized with respect to dose-response relationships, time-course relationships, species differences, and the magnitude for the effects. Qualitative and quantitative evaluation of dose-response relationships for noncancer endpoints is presented in Chapter _____. This is a valuable exercise because it looks at consistencies and inconsistencies among species strain and exposure regimen. It helps to identify data gaps and provides a road map for future studies that will enable biologically based risk assessments for noncancer endpoints. The development of biologically based mechanistic models requires more extensive data sets on the putative mechanisms through which TCDD produces its toxic effects. However, it is important to note that many of the same molecular events involved in TCDD-mediated cancer may also be involved in the production of noncancer endpoints such as alterations in TGF, EGF receptor and estrogen receptor. Therefore, as we learn more about the mechanisms of TCDD-mediated, noncancer effects, we may be able to readily apply cancer mechanistic models to other toxic effects.

8.2.5. Neurological and Behavioral Toxicity

The neurotoxic effects of the dioxins and related compounds have not received much attention, in comparison to other target organs, despite a number of clinical and semianecdotal reports of neurotoxic signs and symptoms in exposed humans (see for instance, Ashe and Suskind's reports on the Monsanto workforce; Pazderova et al; Bleiberg and Poland, 1968). There are some reports on neurochemical changes in animals associated with exposures to PCBs and phenoxyacetic acids (Tilson and Agarwal; Mohammed et al.).

1977
PCB exposure also induced motor dysfunctions in some but not all mice (Tilson et al.), suggestive of effects on basal ganglia (circling and spinning).

1990
Recently, Seegal and coworkers have reported significant effects of certain PCBs on brain chemistry specifically on aminergic pathways (norepinephrine, dopamine, and serotonin). The structure-activity relationships of these effects suggest that they are not associated with the Ah receptor, since it is the noncoplanar, low-chlorinated, nondioxin-like PCBs that are neuroactive. These results are consistent with a report by Silbergeld (1991) that the Ah receptor was not detected in neurons although it was measurable in glia.

Dioxins may be neurotoxic through indirect actions that affect nervous system function and development. Some of the most exciting information in this area has been published by Peterson and coworkers. They have reported that very low level, single dose exposures of pregnant rats results in offspring with significant alterations in sexual behavior, characterized as a feminization of male rats (Mably et al., 1992a).^{b+c} It is well known that the fetal endocrine milieu is critical to the development of the mammalian brain, particularly the sexually dimorphic nuclei of the hypothalamus and cortex (Gorski, McEwen).¹⁹⁹² There are critical periods of vulnerability during which small alterations in levels of testosterone during gestation have profound and longlasting effects on brain and behaviors. Goy et al. and^{Mably et al 1992 a b c} Peterson et al.¹⁹⁹² Moore et al. have reported that these prenatal exposures to dioxin result in altered patterns of sexual behaviors in male rats. It is likely that the behavioral effects in this model are consequent to endocrine effects that impair the sexual "imprinting" of the brain, rather than direct neurotoxic actions of dioxin on the developing brain. Whether females are as sensitive to these intrauterine effects is not presently known; since the male must be differentiated over embryofetal development from the primordial female phenotype, it is possible that the male is more sensitive to these endocrine-mediated effects. The consequences of these exposures for behaviors other than sexual is also not known; however, other studies indicate that alteration in intrauterine imprinting can affect motor activity and learning in males. These findings open up important new areas of toxicological research on the dioxins.

8.2.6. Teratological and Developmental

8.2.6.1. *Cleft Palate*

Dioxins produce structural malformations and developmental toxicity in several species. Considerable information is becoming available on mechanisms of cleft palate formation, and it may be possible to construct mechanistic models for this effect. In mice, increases in the incidence of cleft palate is a well-characterized phenomena (Birnbaum et al., 1987a, b, 1991). The doses required to produce cleft palate in mice are well below doses that produce maternal toxicity or fetal mortality. In the normal developing palate, the peridermal medial epithelial cells cease to express EGF receptor, decrease cell proliferation, and eventually undergo programmed cell death while the basal cells differentiate into mesenchyme allowing the left and right palate to fuse. Temporal changes in the expression of EGF receptor, EGF, TGF-alpha, TGF-beta 1, and TGF-beta 2 are critical for the fusion of the palate. Experimental evidence indicates that changes in expression of these factors, induced by TCDD, results in cleft palate formation. The medial epithelial cells of cultured mouse embryonic palates exposed to TCDD express EGF receptor, incorporate [³H] thymidine, and differentiate into a stratified squamous oral-like epithelium in a dose-dependent manner (Abbott and Birnbaum, 1989). Changes in medial epithelial cell differentiation are associated with increased EGF receptor, TGF-beta 1, and TGF-beta 2 and decreased TGF-alpha levels (Abbott and Birnbaum, 1992).

The use of cultured embryo palates (Abbott and Birnbaum, 1991, 1990a; Abbott et al., 1989) has (1) led to a greater understanding of the mechanism of TCDD-induced cleft palate and (2) enabled researchers to compare TCDD-induced biochemical changes in palate tissue of several species. In vitro observations found that the human and rat palates are sensitive to the cleft palate formation through the same mechanisms seen in mice: changes in growth factors (i.e., EGF and TGFs) that are involved in the mechanism of altering programmed cell death in the medial epithelial cells of the palate. The response to TCDD in mouse palate cultures was about 100 to 1,000 times more sensitive than the response in human or rat palate cultures.

The available data provide substantial information to develop a qualitative model through which TCDD induces cleft palate. The induction of cleft palate in mice by TCDD is mediated through the Ah receptor. TCDD binds to the Ah receptor in the medial epithelial cells, and the activation of the Ah receptor initiates a cascade of events that increases TGF-beta 1 mRNA and protein, increases TGF-beta 2 and EGF receptor protein levels, and decreases TGF-alpha protein levels (Abbott and Birnbaum, 1992). These changes alter the normal signaling pathways in the medial epithelial cells. In control animals, the interaction between these signaling pathways results in the programmed cell death of the peridermal medial epithelial cells. The alterations in growth factor regulation by TCDD results in continued proliferation of the peridermal medial epithelial cells and the redifferentiation of the basal epithelial cells to stratified squamous oral-like epithelial cells, which subsequently prevents the fusion of the palate (Abbott and Birnbaum, 1989).

This preliminary model for the induction of cleft palate by TCDD requires better characterization of several steps. Structure-activity relationships indicate that the Ah receptor is involved, but there is limited evidence that the Ah receptor is present in the medial epithelial cells of the developing palate. Cytosolic fractions of embryonic palatal shelves do contain an Ah receptor but which cells are expressing the Ah-receptor is undetermined (Denker and Pratt, 1987). It is presently unknown if the increases in TGF-beta 1 mRNA is mediated by the interaction of the Ah receptor with a DRE directly activating transcription of the TGF-beta 1 gene or if the increases in TGF-beta 1 mRNA is due to the initiation of a cascade of cytosolic or plasma membrane events mediated through the Ah receptor. Further research is indicated into the interaction of the growth factors and their specific role in palate formation. Because many of the data used to formulate this model are from studies using cultured palates, the development of quantitative models would require dose-response data for the in vivo alterations of these growth factors by TCDD, which are unavailable at this time.

Cleft palate in rats (Schwetz et al., 1973; Couture et al., 1989) and hamsters (Olson et al., 1990) is induced at doses that result in significant maternal toxicity and fetal mortality, and maximal induction of cleft palate is between 10 and 20 percent; however, in the mouse,

cleft palate can reach 100 percent incidence before any fetal mortality or maternal toxicity is demonstrated. These data indicate that the mouse is extremely sensitive to this response. In vitro studies indicate that humans may be much less sensitive than mice to TCDD-mediated increases in cleft palate, so it is plausible that in humans cleft palate may occur only after high exposures.

8.2.6.2. *Hydronephrosis*

In mice, hydronephrosis is also produced by TCDD following prenatal exposure at doses that do not produce fetal mortality (Couture-Haws et al., 1991). Postnatal exposure prior to day 4 can also produce hydronephrosis in mice (Couture et al., 1989). The hydronephrosis induced by TCDD is due to occlusion of the ureter by epithelial cells (Abbott and Birnbaum, 1990). Increased proliferation of the epithelial cells by TCDD is associated with increased EGF receptor. Hydronephrosis has not been reported in any other species at doses that do not result in significant fetal mortality (Birnbaum et al., 1991).

Mice are the only species in which TCDD produces frank terata at doses that are not fetotoxic. At present, there is no evidence that indicates humans are as sensitive as mice to these effects. The only available data comparing the sensitivity of fetal tissue demonstrates that human and rat fetal tissue are equally sensitive to the effects of TCDD (~~Abbott and Birnbaum, 1991~~; Birnbaum, 1991). These data suggest that sublethal exposure to TCDD may not result in frank terata of the kidney.

8.2.6.3. *Thymic Atrophy*

Prenatal exposure to TCDD produces thymic atrophy in all species tested and occurs at doses well below those that cause maternal or fetal toxicity (Birnbaum, 1991). Thymic atrophy occurs at similar doses in rats, guinea pigs, and hamsters exposed prenatally despite a 5,000-fold difference in the LD₅₀ in the adult animals (Olson, et al., 1990). The sensitivity and interspecies consistency of this response indicates that prenatal exposure to TCDD may result in thymic atrophy in humans. The mechanism of thymic atrophy has not

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been elucidated sufficiently to incorporate into a biologically based mechanistic model. Current research has focused on the TCDD-induced alterations in thymocyte development and their role in immunotoxicity.

8.2.7. Immunotoxicity

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Although considerable research has focused on immunotoxicology of the dioxins (see chapter ⁴ ~~by Luster et al.~~), we are not at present able to develop or test a biologically based model for purposes of risk assessment. A major obstacle to this undertaking is uncertainty as to the outcome to be modeled. Susceptibility to infection or impairment of graft versus host response could be proposed as the outcome for risk assessment, but not all studies have used these responses as endpoints. Moreover, this may not be a sensitive indicator of immune function. Alterations in biological markers of disease in animals or humans are not known. Some of the measurable biochemical responses of animals to TCDD may reflect successful response to xenobiotic challenge rather than impairment in functional integrity. Thus, changes in immunoglobulins may reflect immune competence rather than dysfunction. Our inability to define outcome is not unique to immunotoxicology; the continuing controversies over the definition of acquired immunodeficiency syndrome reflects scientific uncertainty in this area. NIEHS has proposed a tier approach to the identification of potential immunotoxicants (Luster et al., 1992), and dioxin certainly tests positive in this system.

Our knowledge of basic immunobiology makes it difficult for us to integrate our findings on dioxin into an overall biologically based schematic of events. We do not know the quantitative relationships between a change in intercellular signaling and cell-mediated responses, although we know these events are fundamentally related. Many events in the immune system appear to have complex interactions, with biphasic relationships. Thus we have no quantitative context in which to develop predictive associations between events affected by dioxin and other events in immune system response.

We place high priority on improving our ability to develop risk assessment methods for immunotoxicants, not limited to dioxin. As noted above, progress has been made on

developing a consensus approach to the hazard identification of potential immunotoxicants, but as yet there are no methods for using dose-response data from such tests to develop quantitative risk assessments. Dioxin may be a prototype compound for developing such methods, and research should be directed toward designs that encompass many different events in immunology from early molecular and cellular events to whole animal response to immune challenge, in order to facilitate the overall evaluation of endpoints. Moreover, in such designs, sufficient dose ranges should be used to assist in the statistical evaluation of proposed models and to compare animal and human responses.

In clinical and epidemiological studies, much data collection should be done; given the accessibility of circulationⁿ³ lymphocytes and other markers in blood, it should be possible to increase our confidence in interspecies comparisons by examining the same parameters in exposed animals and well-characterized human populations. Because of the reported sensitivity of the developing organism to immunotoxic effects of dioxin, a priority should be placed on obtaining data on immunologic function in children with documented exposures to dioxins or related compounds. Clinical studies need to be well controlled and conditions of testing and sample collection carefully described in order to facilitate such comparisons.

8.2.8 Reproductive Toxicity

8.2.8.1. Female Reproductive Toxicity

Several studies have demonstrated that TCDD affects female reproductive function in mice, rats, and monkeys. TCDD reduces fertility, litter size, and uterine weights. TCDD also alters menstrual and estrus cycling in monkeys, mice, and rats. Uterine weight and menstrual/estrus cycling are regulated by estrogens. These data indicate that TCDD has antiestrogenic effects that result in decreased reproductive functioning.

The antiestrogenic actions of TCDD could be mediated either by changes in circulating estradiol, qualitative changes in estrogen metabolism, or through decreases in estrogen receptors. In mice, TCDD does not alter serum estradiol levels, and the antiestrogenic actions of TCDD are associated with decreases in uterine estrogen receptors

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(DeVito et al., 1992). Similarly, TCDD decreases rat hepatic and uterine estrogen receptor (Romkes et al., 1987) but does not affect serum estradiol levels (Shiverick and Muther, 1982). Structure-activity studies suggest that the Ah receptor mediates the down-regulation of the estrogen receptor. The estrogen is down-regulated by TCDD in several breast cancer cell lines (Safe et al., 1992). TCDD also decreases estrogen receptors in Hepa 1c1c7 cells but not in mutant cell types that do not express a high affinity form of the Ah receptor nor in cells that do not accumulate activated Ah receptors in their nucleus (Zacharewski et al., 1991). These studies provide further evidence that the Ah receptor is involved in the down-regulation of the estrogen receptor.

One possible mechanism for the antiestrogenic actions of TCDD is that TCDD binds to the Ah receptor in the target tissue and through a cascade of events decreases the amount of estrogen receptor in the cell, thus inhibiting the actions of estrogens. The down-regulation of the estrogen receptor by TCDD could be mediated either by decreased transcription of the estrogen receptor gene or possibly through nontranscriptional mechanisms. At present it is unclear how TCDD down-regulates the estrogen receptor other than it is mediated through the Ah receptor.

An alternative mechanism by which TCDD inhibits estrogenic actions is through increases in estradiol metabolism. Following TCDD exposure, estradiol metabolism is increased 100-fold in MCF-7 cells (Spink et al., 1990). Microsomal hydroxylation of estradiol is increased twofold to fourfold in rats treated with TCDD (Graham et al., 1988). The role of estrogen metabolism in the antiestrogenic actions of TCDD remains to be determined. While there is more evidence supporting the role for the down-regulation of the estrogen receptor mediating the antiestrogenic actions, further studies are required to determine the extent of estradiol metabolism in vivo following TCDD treatment.

Since TCDD alters immune function and a variety of growth factor pathways and in general is acting like a potent and persistent environmental hormone, research is needed to determine the relationships, if any, to endocrine-related disorders in women such as endometriosis, osteoporosis, and cancers of the reproductive tract.

8.2.8.2. *Male Reproductive Toxicity*

When administered to adult rats, TCDD decreases testis and accessory sex organ weights, decreases spermatogenesis, and reduces fertility (Moore et al., 1985; Moore and Peterson, 1988; Bookstaff et al., 1990a). These effects are associated with decreases in plasma testosterone (Moore et al., 1985). The decreases in circulating androgens are due to decreased testicular responsiveness to luteinizing hormone and increased pituitary responsiveness to feedback inhibition by androgens (Moore et al., 1989, 1991; Bookstaff et al., 1990a, b; Kleeman et al., 1990). Although the antiandrogenic effects occur within 24 hours, the doses required to produce these effects are overtly toxic and decrease food intake and body weight. The high doses needed demonstrate that the antiandrogenic effects are not very sensitive effects. However, epidemiological studies have demonstrated decreased testosterone in workers exposed to dioxin-like compounds (Dioxin92).

In contrast to the adults, the developing male reproductive system is very sensitive to the effects of TCDD. In rats, prenatal exposure to TCDD results in decreases in sex organ weight, impairs spermatogenesis and luteinizing hormone secretion, and demasculinizes and feminizes sexual behavior (Mably et al., 1991, 1992^c). Maternal doses as low as 0.064 ug/kg can produce these effects, indicating that the developing male reproductive system is one of the most sensitive endpoints for the toxic effects of TCDD. The alterations in male reproductive development are associated with decreases in testosterone levels. Sufficient testosterone levels are critical for sexual differentiation of the central nervous system; thus the decreases in testosterone levels may, in part, account for the TCDD-induced reproductive alterations in male rats. It is also possible that these effects are due in part to alterations in tissue sensitivity to testosterone (Mably et al., 1991, 1992^c).

In summary, there is ample evidence that noncancer endpoints are extremely sensitive to the toxic effects of TCDD. The available data do not provide enough information to develop biologically based mechanistic models for all noncancer endpoints. For some of the noncancer effects of TCDD there is sufficient evidence for which proposed mechanism may be modeled. Experimental evidence on cleft palate formation and male reproductive toxicity

provide sufficient evidence to propose qualitative models that can be developed into mechanistic models. However, for immunotoxicity, thymic atrophy, neurobehavioral toxicity, and female reproductive toxicity, the mechanism by which they occur are unknown and in some cases the target tissues remain undetermined. Furthermore, few if any of the molecular events beyond ligand binding to the Ah receptor are understood for these effects. The only information we have to develop mechanistic models is dose-response relationships. Future studies that better characterize target tissues and the molecular mechanisms underlying these events are indicated. Because of the importance of generating reliable estimates of the risk for noncancer effects, the development of biologically based dose-response models for these effects is an urgent research need.

8.3. COMPARATIVE ENDPOINTS/QUALITATIVE COMPARISONS

There has been considerable discussion concerning the relative sensitivity of the several toxicological endpoints modified by exposure to TCDD. Many scientists and regulators have stated that carcinogenicity may not be the most sensitive endpoint. However, before one can compare the magnitude of these various effects, one must understand the limitations imposed by both statistics and biology on these types of relative potency comparisons. This section briefly outlines some of the problems with discussing relative potency across different endpoints. It provides no simple solutions to these problems. These solutions will take a combination of research (in both statistics and biology) and management decisions where research cannot yet be used to fill the knowledge gaps in the comparison.

There are two basic problems with the comparison of toxic potency across endpoints. These are (1) comparison of measures of mortality (or serious threats to mortality) to measures of morbidity or to measures of biochemical modifications and (2) statistical issues concerning the power to detect effects and the inclusion of background responses. We will discuss each of these in detail using TCDD as an example of the problems involved in this undertaking.

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We will consider these issues in reverse order starting with the statistical problem first. Statements such as the one above sometimes reflect a statistical artifact due to the nature of the biological assay being analyzed. To illustrate this point, consider the data given in Table 8-4. This table considers four different responses to TCDD: liver cancer in female mice and rats, immunological changes in male mice, changes in protein concentrations in female rats, and rates of terata in mice. One practical way to address potency across endpoints is to rely on significant difference from control for each treatment group. On the basis of a statistical test, it is clear that the most sensitive endpoint is an effect on the immune system where a response was detected at a dose of 0.644 ng/kg. [↑]

^{more power}
Second, the endpoint being measured (plaque-forming cell response) has a greater numerical value than does other responses (such as tumor incidence) with a small variability allowing for much greater statistical power in obtaining a response. Also, the lowest dose for induction of CYPIA1 shows a significant effect on immune responses; it is impossible to know if lower doses might not have been significant also. Thus the location of a NOEL is dependent on the statistical properties of the endpoint being studied and the sensitivity of a particular response.

no observed effect level

^{One practical way}
~~A second approach would be~~ to consider the relative change in response over background in the TCDD-treated groups. For example, the drop in PFC response from control to low dose is 6 percent or -0.06 relative units of response per 0.322 units (pg/kg/day) increase in dose, or a slope of 0.0186. This is ~ 3 times greater than the relative increase in CYPIA1 (slope of 0.068) and about one-tenth the relative increase in liver tumors in female mice (National Technology Program, 1982). Even more dramatic is the infinite change in response from control (0.00) to low dose (0.019) for cleft palate in the ^{Abbott and Birnbaum} Birnbaum et al. (1989) study. Thus, using this measure of relative potency, cleft palate is the most sensitive endpoint, with liver cancer second and the immunological response third. Thus, a second practical measure of potency, changes relative to background, paints a very different picture than does the use of statistical p-values and is sensitive to zero response in the control population. In addition, you have to scale between relative drops and relative

plaque forming cells

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Table 8-4. Toxic endpoints database			
Endpoint	Dose ^a	Response	Reference
Liver cancer in female mice	0.0	0.041 ^c (3/73) ^d	NTP (1982 ^b)
	1.4	0.120 (6/50)	
	7.1	0.125 (6/48)	
	71.0	0.234 (11/47) ^e	
Liver cancer in female rats	0.000	0.051 ^g (3/58.4) ^h	Kociba et al., 1978 ^f
	1.0	0.029 (1/34.0)	
	10.0	0.272 (9/33.1) ^e	
	100.0	0.607 (19/31/3) ^e	
Plaque-forming cells per 10 ⁶ viable spleen cells	0.00	777 ± 88	Davis and Safe, 1988 ⁱ
	0.322	731 ± 190	
	0.644	438 ± 96 ^e	
	1.191	118 ± 46 ^e	
	3.091	65 ± 15 ^e	
Concentration of cytochrome P4501A1 in microsomal protein from hepatocytes (pmol/mg)	0.0	12.9 ± 11.3	Tritscher et al., 1992
	3.5	56.4 ± 26.7 ^e	
	10.7	111.5 ± 30.3 ^e	
	35.7	181.4 ± 18.4 ^e	
	125.0	293.3 ± 17.1 ^e	
Concentration of cytochrome P4501A2 in microsomal protein from hepatocytes (pmol/mg)	0.0	63.5 ± 38.4	Tritscher et al., 1992
	3.5	88.3 ± 23.0	
	10.7	161.0 ± 55.7 ^e	
	35.7	193.1 ± 60.2 ^e	
	125.0	297.4 ± 88.3 ^e	
Cleft palate in mice fetus	0.0	0.000 (0/159)	~~Birnbaum et al., 1989~~ Abbott and Birnbaum, 1989 ✓
	6000.0	0.019 (2/107)	
	9000.0	0.213 (26/122) ^e	
	12000.0	0.505 (50/103) ^e	
	15000.9	0.777 (84/108) ^e	

^aIn ng/kg/day

^bGavage dosing

^cProbability of getting a tumor prior to the end of the study

^dNumber with tumor/number examined for the tumor

^eSignificantly different from control response (p < 0.05)

^fTCDD in diet

^gSurvival-adjusted using poly-3 adjustment of Portier and Bailer (1989)

^hNumber with tumor/poly-3 adjusted number at risk of tumor

ⁱSingle intraperitoneal injection

Survival ✓

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increases, the one bounded by 0 and the other unbounded (although for practical purposes, this is easily dealt with). Finally, one could also use absolute (rather than relative) change over background², but then you would be faced with the problem of determining how a change of one unit in PFC response relates to a change of one unit in CYPIA1 response. ✓

The only practical manner in which a relative comparison of potency can be made is in terms of mortality. In this way, all responses are in the same units and have a common control response in terms of the background mortality in the population. However, this approach is currently infeasible. There are both technical and practical considerations that must be ironed out before an approach of this type can be applied. On the technical side, there are issues concerning life expectancy versus incidence of death. For example, suppose TCDD increased resorptions by 5 percent at some chosen dose. This would represent a 5 percent increase in mortality for potential fetuses and an overall loss of 5 percent of the total available number of animal lifetimes (resulting in a 5 percent drop in life expectancy). Suppose also that this same dose of TCDD increased mortality from cancer so that, by the end of the study, an additional 20 percent of the animals have died. Suppose also that this increase in mortality is late in life so that the overall drop in life expectancy is only 5 percent. Thus, on one scale (life expectancy), the two endpoints, resorptions and cancer, produce the same results, whereas on another scale, mortality by age, they are different. It is unclear which measure is most appropriate for ranking the observed effects of TCDD. On the more practical, biological side of the issue, one must relate increases in tumor incidence to changes in mortality, modifications in immune response and/or protein concentrations to changes in mortality, etc. The information needed to model these relationships is currently unavailable.

This is not to say that statements concerning the relative importance of certain endpoints for toxicity from exposure to TCDD cannot be addressed. However, caution must be used in this endeavor, and one must be careful to explain the methods by which the relative potencies were established. When this is done, the gaps in our knowledge become obvious and future research can be better directed to fill those gaps.

8.4. RELEVANCE OF ANIMAL DATA FOR ESTIMATING HUMAN RISKS

The reliability of using animal data to estimate human risks has been questioned, and this issue is especially important for TCDD. We know there are unusually wide species differences in acute toxic responses to TCDD, but we do not know if such wide differences exist for carcinogenic and other toxic effects. However, we do not know that the rank order of species differences in acute effects does not predict the rank order for all other toxic effects. For example, mice appear to be considerably more sensitive than rats to the teratogenic and immunotoxic effects of TCDD, but we do not know if dose-response relationships for immunotoxic effects in humans resemble those for rats, mice, or neither.

Although the human data are limited, it does appear that animal models are, in general, appropriate for estimating human risks, keeping in mind that for some responses, where wide species differences exist, the relative placement of human responses may not be possible at this time. However, humans contain a fully functional Ah receptor (Lorenzen and Orey, 1991; Manchester et al., 1987; Cook and Greenlee, 1989) and many of the biochemical effects produced by TCDD in animals also occur in humans. Data on effects of TCDD and its analogs in humans is based on in vitro (i.e., in culture) as well as epidemiological studies. A comparison of the effects of CDDs and CDFs on laboratory animals versus humans is given in Table 8-5. In vitro systems such as keratinocytes or thymocytes in culture have clearly shown that human cells possess Ah receptors, and they respond similarly to cells derived from rodents. Several reports in the literature suggest that exposure of humans to dioxin and related compounds may be associated with cancer at many different sites including malignant lymphomas, soft tissue sarcomas, hepatobiliary tumors, hematopoietic tumors, thyroid tumors, and respiratory tract tumors (Bertazzi et al., 1993², 1993³⁹, 1993¹⁴⁹³; Fingerhut et al., 1991; Manz et al., 1991; Zober et al., 1991⁵; Saracci et al., 1991). These studies are evaluated in Chapter 7 including discussion of confounding factors and strength of evidence. There is growing evidence from human cancer studies that TCDD is a multisite carcinogen, which is not unexpected if we assume that TCDD is acting like a potent and

Table 8-5. Similarities between laboratory animals and humans in biological effects of TCDD		
Effect	Laboratory animals	Human or human cells
<i>In vitro</i>		
Presence of Ah receptor	+	+
Enzyme induction	+	+
Altered pattern of growth and differentiation		+
Immunosuppression	+	+
Choracnogenic response	+	+
<i>In vivo</i>		
Presence of Ah receptor	+	+
Enzyme induction	+	+
Altered lipid metabolism	+	+
Immune effects	+	+/-
Cancer	+	+
Reproductive effects	+	+/-
Teratogenic effects	+	+/-
Altered epithelia cell differentiation	+	?
Tumor promotion	+	?

SOURCE: Silbergeld and Gasiewicz, 1989

The + indicates a clear association while +/- indicates conflicting or unclear associations; the ? indicates that not enough is known on the effects of TCDD on the system to evaluate.

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OK

persistent hormone agonist/antagonist. Likewise, TCDD is a multisite carcinogen in animals (Lucier, this document).

Several noncarcinogenic effects of chlorodibenzo dioxins (CDDs) and chlorodibenzo furans (CDFs) show good concordance between laboratory species and humans as well. For example, in laboratory animals, TCDD causes altered intermediary metabolism manifested by changes in lipid and glucose levels. ^{consistent} ~~In alliance~~ with these results, workers exposed to TCDD 7 to 8 years previously during the manufacture of trichlorophenol showed elevated total serum triacylglycerides and cholesterol with decreased high density lipoprotein (Walker, 1979). Recently, the results of a statistical analysis of serum dioxin analysis and health effects in Air Force personnel following exposure to Agent Orange was reported (Wolfe et al., 199⁰~~7~~). Significant associations between serum dioxin levels and several lipid-related variables were found (percent body fat, cholesterol, triacylglycerols, and HDL). Another interesting finding of these studies was a positive relationship between dioxin exposure and diabetes, possibly the first report of such an association.

The human to experimental animal comparison is confounded by at least two factors: (1) For most toxic effects produced by dioxin, there is marked species variation. An outlier or highly susceptible species for one effect (i.e., guinea pigs for lethality or mice for teratogenicity) may not be an outlier for other responses. (2) Human toxicity testing is based on epidemiological data comparing "exposed" to "unexposed" individuals. However, the "unexposed" cohorts contain measurable amounts of background exposure to CDDs and CDFs, ^{and dioxin-like PCBs.} Also, the results of many epidemiological studies are hampered by small sample size, and in many cases the actual amounts of dioxin and related compounds in the human tissues were not examined. However, based on the available information, it appears that humans respond to CDDs and CDFs like most experimental animals.

There is also relatively good concordance in the biochemical/molecular effects of TCDD between laboratory animals and humans. Placentas from Taiwanese women exposed to rice oil contaminated with ^{PCBs and} CDFs have markedly elevated levels of CYPIA1 (Lucier et al., 1987; ~~Wong et al., 1986~~). Comparison of these data with induction data in rat liver suggests

that humans are at least as sensitive as rats to enzyme-inductive actions of TCDD and its structural analogs (Lucier, 1991). Consistent with this contention, the in vitro EC_{50} for TCDD-mediated induction of CYP1A1-dependent enzyme activities is ~ 1.5 nM when using either rodent or human lymphocytes (Clark et al., 1992). However, binding of TCDD to the Ah receptor occurs with a higher affinity in rat cellular preparations compared to humans (Lorenzen and Okey, 1991; Okey, 1989). This difference may be related to the greater lability of the human receptor during tissue preparation and cell fractionation procedures (Manchester et al., 1987). In any event, it does appear that humans contain a fully functional Ah receptor (Cook and Greenlee, 1989) as evidenced by significant CYP1A1 induction in tissues from exposed humans, and this response occurs with similar sensitivity as observed in experimental animals.

One of the biochemical effects of TCDD that might have particular relevance to toxic effects is the loss of plasma membrane EGF receptor. There is evidence to indicate that TCDD and its structural analogs produce the same effects on the EGF receptor in human cells and tissues as observed in experimental animals. First, incubation of human keratinocytes with TCDD decreases plasma membrane EGF receptor, and this effect is associated with increased synthesis of TGF- α (Choi et al., 1991; Hudson et al., 1985). Second, placenta from humans exposed to rice oil contaminated with polychlorinated dibenzofurans, exhibit markedly reduced EGF-stimulated autophosphorylation of the EGF receptor, and this effect occurred with similar sensitivity as observed in rats (Lucier, 1991; Sunahara et al., 1987⁸⁹). The magnitude of the effect on autophosphorylation was positively correlated with decreased birth weight of the offspring.

In summary, animal models are reasonable surrogates for estimating human risks. However, it must be kept in mind that the animal to human comparison would be strengthened by additional mechanistic information, especially the relevance of specific molecular/biochemical changes to toxic responses. It is also important to note that the mechanism of carcinogenesis (sequence of molecular events) may be quite different at

different sites. For example, the mechanism responsible for TCDD-mediated lung cancer appears to be different than that responsible for liver cancer (see Chapter 6).

8.5. HUMAN MODELS

8.5.1. Introduction

Unlike animal data where recent studies have allowed modeling for dosimetry, induced proteins, cell proliferation, and toxic effects, human data are very sparse. With regard to toxic effects, Chapter 7 presents recent evidence suggesting TCDD's effects on human reproduction, neurotoxicity, diabetes, and cancer. From a modeling viewpoint, male reproduction, diabetes, and thyroid cancer appear to be good candidates for modeling from a "bottom-up" mechanistic approach, since TCDD's effects on male serum testosterone levels, the insulin receptor, and thyroid hormones have been documented. These, however, remain efforts for the future. The focus of this section will be on cancer, specifically liver cancer, respiratory cancer, and all cancers combined. There are two reasons for this. First, the emphasis on liver cancer in EPA's history of TCDD regulation demands it, and it is a logical sequence to Section 8.3. Second, the recent epidemiology evidence for respiratory cancer, soft tissue sarcoma, and all cancers combined suggests that dioxin is a human carcinogen.

Modeling for these cancers in humans, however, requires and receives different approaches than has been presented in earlier sections of this chapter. The approach used is one involving interspecies toxic equivalent liver doses, with extrapolation from a rat to human NOAEL. The modeling approach used for the human epidemiology data for lung cancer and all cancers combined involves estimating human intake dose associated with cancer response and curve fitting both additive and multiplicative risk models to the data.

FOR liver cancer

8.5.2. Modeling Toxic Effects in the Liver

One of the intrinsic features of PB-PK descriptions is that interspecies extrapolations can be attempted with reasonable confidence in the result, assuming that relevant changes are made in the configuration of the model to allow for changes in physiology, metabolism, and

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protein binding. The limiting factor with any modeling description is, however, the availability of relevant data sets. This is particularly true when attempting to model the pharmacokinetics of dioxins and dioxin-like chemicals in people. The use of a physiological pharmacokinetics description to analyze human TCDD exposure data was first attempted by Kissel and Rofbarger (1988). In this work the authors used a fugacity approach to examine the elimination of TCDD from humans using data derived from estimates of background exposure and tissue levels, half-lives from Ranch-Hand veterans, and a self-exposure experiment by Poiger (Poiger and Schlatter, 1986).

This fugacity-based model attributes the distribution of TCDD as a simple partitioning process with expected dose-independent linear kinetics. In both rats and mice, as noted earlier, a disproportionate amount of TCDD is found in the liver with increasing dose. This phenomenon cannot be described by simple solubility/partitioning alone. The dose-dependent liver-to-fat concentration ratio is a good indicator of this trend. In fact, analysis of data from people exposed to CDFs, congeners of TCDD, from consumption of contaminated oil show a higher liver:fat ratio with increasing body burden (Carrier and Brodeur, 1991). More complex models need to be constructed to account for the nonlinearities in dioxin disposition.

Carrier and Brodeur (1991) constructed a toxicokinetic model for HPAH in humans. This model is not a classical PB-PK model. The analysis begins with the observation that the tissue distribution of dioxin-like ~~HPAHs~~ in people and animals is body-burden dependent. As the body burden increases (C_{body}), the proportion of that body burden associated with the liver (F_h) increases towards a maximum value (F_{max}). This has been previously described for rats (Abraham et al., 1988). The data ^{on rats (Chiba et al. 1976)} from the Kociba et al. study (1976) in ~~rats~~ monkeys (McNulty et al., 1982) and ~~from~~ people, using toxic equivalency factor conversions (Kuroki and Masuda, 1978) were examined using an empirical, Michaelis-Menton type saturable binding isotherm:

$$\text{Liver fraction } (F_h) = F_{max} C_{body} / (K_d + C_{body})$$

halogenated
polycyclic aromatic
hydrocarbons

Considering the body burden as a surrogate for liver concentration, this equation can be loosely interpreted as the induction of binding species in the liver as dose increases. Indeed, analyses showed that the K_d was very similar for people and experimental animals, possibly indicative of similar protein-induction dynamics in various species. With different dioxin-like isomers F_{max} and K_d vary; this can be thought of as changes due to different binding affinities.

This empirical model fits the observed data in various species; however, it is a fitting exercise and not an examination of underlying biology. The model is not physiologically based. It in effect examines the steady state condition of a two-compartment model, consisting of the liver and "the rest of the body." The terms C_{body} and F_{max} are difficult to interpret in biological terms. C_{body} represents a body burden of chemical rather than a tissue concentration, and the term for maximum liver concentration F_{max} is derived empirically.

In its present form the model assumes that at very low doses the hepatic fraction is zero. This is unlikely due to the partitioning of dioxin into the liver and the low-dose binding characteristics of the Ah receptor and CYP1A2 in the liver. Also in this description the metabolism is modeled as saturable at maximal induction of liver TCDD sequestration. Given the experimental data in many species ~~(Olsen, this document)~~ ^{Chapter 1} this seems unlikely. The analysis of human dioxin kinetics does show that the dose-response curves for and induction of hepatic-binding species for dioxin in rodents and people appear to be very similar. If you assume that humans and rodents are also similarly sensitive in the toxic responses to TCDD, based on liver concentrations, some simple exposure calculations can be made. Interspecies comparisons of the daily intake of TCDD needed to reach equitoxic concentrations in the liver have been estimated by Carrier (1991). These are presented in Table 8-6. It is important to note that a number of assumptions are made to make these comparisons and considerable uncertainty exists.

The integration of the toxicokinetic description put forward by Carrier, and the accompanying data sets, with the more physiological approaches described for rats by

Terri add reference to Olsen revaluation

Table 8-6. Rat and Human Comparison of Daily TCDD Intakes and Body and Liver Concentration for Equitoxic Response				
Parameter	Low intake		High intake	
	Rat ^a	Human	Rat ^b	Human
Daily ~~Intake~~ ^{Intake} (TCDD eq)	1 ng/kg	40.7 pg/kg	100 ng/kg	1 ng/kg
Total body concentration (TCDD eq/body)	61 ng/kg	70 ng/kg	1.45 µg/kg	1.2 µg/kg
Liver concentration (TCDD eq/kg)	540 ng/kg	540 ng/kg	24 µg/kg ²	24 µg/kg ²

SOURCE: Carrier, 1991.

^aRat NOAEL (Kociba et al., 1978).

^bMarked liver toxicity; tumors in rats.

Andersen et al. (1992) and Kohn et al. (199³~~2~~), will provide an opportunity to further investigate the determinants of disposition of TCDD in humans.

The prediction for Carrier's model of 40 pg/kg-day for a no-effect dose ^{for cancer} is ~7,000 times as high as that currently used by EPA but only 6 to 7 times higher than that of other western countries (Kociba, 1991). However, this model assumes that humans are as sensitive as rats to a given tissue burden of dioxin, and it does not account for possible interindividual variation among humans. Also, it is specific for liver, so it does not predict cancer responses in other tissues such as lung.

8.5.3. Lung Cancer and All Cancers Combined

Data from four recent epidemiology retrospective cohort occupational studies provide evidence of the human carcinogenicity of dioxin. All showed increased mortality from respiratory cancer; the two largest (Saracci et al., 1991; Fingerhut et al., 1991) showed increases in mortality from soft tissue sarcoma, and three (Fingerhut et al., 1991; Zober et al., 1990; Manz et al., 1991) showed increased mortality from all cancers combined. In the Fingerhut et al. study all three cancer type results showed significance only for the high-exposure, long latent period subcohort. The largest study, with 18,000 total workers (Saracci et al., 1991), showed no increase in overall cancer mortality, but those authors have not presented the data allowing for a latent period. Furthermore, the Saracci et al. study, unlike the other three, provides no way to quantitatively estimate TCDD exposure to their cohort. Based on this lack of information, this modeling exercise will be restricted to the other three cohorts.

Three other recent cohorts were not included in this analysis for various reasons. Kuratsune et al. (1988) reported increased lung cancer in male victims (SMR=3.3, based on eight cases) of the Yusho PCB and CDF contamination rice poisonings. Although there are serum measurements and 37 TEF estimates available for this cohort, there was no actual TCDD in the contaminants. Thus, this cohort also will not be used in the modeling effort here. Collins et al. (1993) reported increased mortality for both lung cancer and all cancers

combined for a subcohort of 122 U.S. workers who developed chloracne following exposure to dioxin at a chemical plant during a 1949 accident. Their analysis, however, attributes this increase to co-exposure to 4-aminobiphenyl. Since that chemical plant is included in the Fingerhut et al. (1991) cohort, it will not be included in this analysis. The Seveso, Italy, community cohort is also not included in this analysis because of the limited observation period following the 1976 accident. All of these studies are discussed in much greater detail in Chapter 7.

The largest of the three studies used here is the Fingerhut et al. (1991) study of >5,000 U.S. workers from 12 U.S. plants producing chemicals contaminated with TCDD. Of 1,520 workers exposed to TCDD-contaminated processes for at least 1 year with a 20+ year latency, mortality was significantly increased for both respiratory cancer (SMR=142; 95% C.I. 103-192) and for all cancers combined (SMR=146; 95% C.I. 121-176). A similar-sized cohort with less than 1-year exposure with a 20+ year latency showed no increase in either all cancers or respiratory cancers. Manz et al. (1991), in a smaller cohort of 1,148 men in a herbicide manufacturing plant in Hamburg, Germany, also found increased mortality from lung cancer (SMR=141; 95% C.I. 95-201) and all cancers combined (SMR=124; 95% C.I. 100-152). Cancer mortality increased both among groups with increased duration of exposure and among groups with suspected highest levels of exposure. In the smallest of these recent studies, Zober et al. (1990) studied three subcohorts totaling 250 workers with potential exposure to dioxin during an industrial accident in 1953. Of the 127 who developed either chloracne or erythema, and who were considered among the most highly exposed, for those with a 20+ year latent period, mortality from all cancers (SMR=201; 95% C.I. 122-315) and from lung cancer (SMR=252; 95% C.I. 99-530) were both statistically increased. Furthermore, the increase in total cancer deaths in all these studies does not appear to be due totally to the increase in respiratory deaths. The SMRs for all cancer deaths not including lung cancer remain statistically significant in all three studies.

These findings are supported by recent animal evidence from Lucier et al. (1991) who found lung tumors in ovariectomized female Sprague-Dawley rats but not in intact female

rats following administration of TCDD. Increased lung tumors are also seen in the Kociba et al. (1978) study with female Sprague-Dawley rats, but not with male rats. Other animal data support the tumor-promoting ability of dioxin in the liver and skin (Pitot et al., 1980).

Based on the evidence of lung cancer and all cancers combined, a quantitative analysis of dioxin's cancer potency will be modeled from the three epidemiology studies. All three studies attempted to verify dioxin levels in samples of their working cohorts, although in all cases the subjects were tested decades after exposure ended. Thus, with the limited information available, assumptions must be made about the representativeness of both these sampled subjects and the dose-response models used to estimate risk. The details are presented below.

8.5.3.1. *Dose-Response Models*

The following analysis provides maximum likelihood and 95 percent lower confidence limits of incremental cancer risk based on the cancer death response in the lung and all cancers combined in the three recent cohort studies (Fingerhut et al., 1991; Zober et al., 1990; Manz et al., 1991). Both additive and relative risk models are used. This type of analysis has been used previously with epidemiologic studies in several EPA health assessments (e.g., methylene chloride, nickel, and cadmium). For this report the analyses will be done both separately for each study and for all studies combined. A description of the models follows.

8.5.3.1.1. *Excess or additive risk model.* This model follows the assumption that the excess cause-age-specific death rate at age t due to dioxin exposure, $h_1(t)$, is increased in an additive way by an amount proportional to exposure at some age $t-k$. In mathematical terms, this is:

$$h_1(t) = \beta X_{t-k}$$

where X_{t-k} is the exposure at age $t-k$, and β , the parameter to be estimated, is the proportional increase. The total cause-age-specific rate $h(t)$ is then additive to the background cause-age-specific rate $h_0(t)$ as follows:

$$h(t) = h_0(t) + h_1(t)$$

For an individual i observed from $t_{0,i}$ until age t_i , the cumulative death rate expected is:

$$H_i(t) = \sum_{t=t_{0,i}}^{t_i} S(t)h_0(t) + \beta \sum_{t=t_{0,i}}^{t_i} X_{t-k}$$

where $S(t)$ = survival function to age t . For N_j individuals in exposure group J the expected number of deaths is:

$$E_j = \sum_{i=1}^{N_j} H_i(t) = E_{0j} + \beta \sum_{i=1}^{N_j} \bar{X}_i \cdot W_i = E_{0j} + \beta X_j W_j$$

where E_j is the total number of expected cancer deaths in the observation period from the group exposed to average exposure X_j . E_{0j} is the expected number of cancer deaths due to background causes (lifetable "expected" rates); W_j is the number of person-years of observation for the j th exposure group; and the parameter β represents the slope of the dose-response model. To estimate β , the observed number of cause-specific deaths in group j , O_j , is assumed to be distributed as a Poisson random variable with expected value E_j . The parameter estimate, b , can be tested for being significantly > 0 . A statistically significant result is evidence of an additional cancer effect due to dioxin exposure.

Under the above assumptions, the solution by maximum likelihood proceeds as follows: The likelihood equation is

$$L = \prod_{j=1}^N [\exp(-(E_{0j} + \beta X_j W_j)) (E_{0j} + \beta X_j W_j)^{O_j} / O_j!]$$

where N = the number of separate exposure groups. The maximum likelihood estimate (MLE) of the parameter β is obtained by taking the first derivative of the log likelihood equation, setting it equal to 0 and solving for b :

$$\frac{d \ln L}{d\beta} = \sum_{j=1}^N [-X_j W_j + (O_j X_j W_j / (E_{0j} + b X_j W_j))] = 0$$

The asymptotic variance for the parameter estimate b is:

$$[\sum_{j=1}^N X_j^2 W_j^2 / (E_{0j} + b X_j W_j)]^{-1}$$

where b is the MLE. This variance can then be used to obtain approximate 95 percent upper and lower bounds for β . Lifetime incremental cancer risk estimates for continuous exposure are estimated by multiplying b by 70 if X is in units of lifetime continuous exposure (i.e., lifetime average daily dose [LADD]).

8.5.3.1.2. Multiplicative or relative risk model. This model follows the assumption that the background cause-age-specific rate at any age t is increased in a multiplicative way by an amount proportional to the cumulative dose up to that age. In mathematical terms this is:

$$h(t) = h_0(t)(1 + \beta X_t)$$

As above, summing over the observed and expected experience yields, for each exposure group,

$$E_j/E_{0j} = 1 + \beta X_j$$

Again, to estimate β , the observed number of cause-specific deaths, O_j , assumed to be a Poisson random variable, is substituted for E_j . Following the same procedure as above, the MLE, b , is the solution to

$$\frac{d \ln L}{d\beta} = \sum_{j=1}^N -E_{0j}X_j + (O_j / (1 + bX_j)) = 0$$

with asymptotic variance

$$[\sum_{j=1}^N (E_{0j}X_j^2)/(1 + bX_j)]^{-1}$$

If X_j is in units of LADD, then lifetime incremental risk estimates per unit under this model are obtained by multiplying b by the background lifetime cause-specific risk of death, P_0 . The values P_0 are derived using life table methods for competing risks and 1973-1977 U.S. death rates. For lung and all cancers combined these are 0.038 and 0.185, respectively.

8.5.3.2. *Exposure and Dose Estimates*

Exposure estimates are derived from serum dioxin levels in workers sampled long after exposure ended and extrapolated backward using a first-order model for elimination (U.S. EPA, 1993) with a biological half-life of 7.1 years (Pirkle et al., 1989). In humans, dioxin deposits primarily in adipose tissues at normal exposure levels, although body deposition dose-dependency has been shown in animal studies (see Chapter 2 and Section 8.5.2). In those rat studies, liver/fat concentration ratios increase with increasing dose, due to TCDD induction of the binding protein P4501A2 in the rat liver. However, although there is evidence that dioxin causes some human liver toxicity (Di Domenico and Zopponi, 1986) and that the dioxin-like PCB and dibenzofuran compounds can also cause liver cancer in humans (Kuratsune et al., 1988) (see Chapter 7), there is no direct evidence that TCDD induces cancer in human liver. Thus, although Table 8-6 presents rat-to-human liver concentration toxic equivalents for various rat exposures, this section uses only human data. In humans, all estimates suggest that adipose tissue is the major storage compartment. Schlatter (1991) estimated a liver/fat concentration ratio of about 1/6. With a body adipose

tissue fat weight of 15 to 20 percent and a liver weight of 2.5 percent, over 90 percent of stored dioxin will be in adipose tissue.

Direct initial exposure to the lung in these studies is also difficult to estimate. Both inhalation and skin absorption are the equally likely routes of initial exposure, but the exposure scenario cannot be distinguished. Di Domenico and Zapponi (1986) estimated that ~ 50 to 90 percent of TCDD exposure to the Seveso residents following the 1976 accident occurred via the dermal route, but they assumed 100 percent dermal and inhalation absorption. A more likely 1 to 10 percent dermal and 75 percent inhalation absorption estimate (U.S. Environmental Protection Agency, 1985) would project that the inhalation route provided the major TCDD exposure. To further complicate the situation, the cohorts discussed below are all occupational so that both dermal and inhalation exposure are highly likely.

The data on body concentration levels in the three studies are presented in Table 8-7. Fingerhut et al. (1991) measured serum levels adjusted for lipids in a sample of 253 of the workers from 2 of the 12 plants approximately 20 years after last known exposure. They found a highly statistically significant correlation ($r=0.72$; $p<0.0001$) between the logarithm of number of years of exposure to processes involving TCDD contamination and the logarithm of individual TCDD serum levels. Based on this correlation, they divided the sample into a high-exposure group (defined as those exposed more than 1 year) and a low-exposure group (those exposed <1 year). The mean TCDD level of the low-exposure group was 69 ppt, while that of the high exposure group was 418 ppt. Among the 176 sampled workers last exposed >20 years before, those with under 1 year of exposure ($n=81$) had a mean level of 78 ppt, and those with over 1 year of exposure ($n=95$) had a mean level of 462 ppt.

For the Zober et al. (1991) study, the serum-level data are based on a nonrandom sample of 28 survivors tested 32 years after the 1953 accident. These subjects were then classified into three groups by scenario of high (C1), medium (C2), or low (C3) chance of TCDD exposure, with the mean (median) levels of 60 (24.5), 25 (9.5), and 25 (8) ppt,

Table 8-7. Measured serum TCDD levels and estimated levels at time of last occupational exposure to TCDD, based on first-order elimination kinetics and a half-life for elimination of 7.1 years					
Study	Sample	Sample size	Concentration at test time (ppt)		Estimated ^b median concentration at time of last exposure adjusted for background (ppt)
			Including background	Median adjusted for background ^c	
Fingerhut et al., 1991	Surviving cohort of workers from Plants 1 and 2 tested approximately 20 years after last occupational exposure	253			Range 2,000-32,000 ^e
	Exposed > 1 year, all	119	Mean 418 Median 231	226	
	Exposed > 1 year, ≥20-year latency	95	Mean 462	226 ^d	1,770
	Exposed < 1 year, all	134	Mean 69 Median 24	19	
	Exposed < 1 year, ≥20-year latency	81	Mean 78	19 ^d	150
Zober et al., 1991	Sample of survivors tested 32 years after the accident	28			
Breakdown A	High-exposure scenario	10	Mean 60 Median 24.5	19.5	450
	Medium-exposure scenario	7	Mean 25 Median 9.5	4.5	105
	Low-exposure scenario	11	Mean 25 Median 8.4	4.4	100
Breakdown B	Either chloracne or erythema	16	Mean 50 Median 15	10	230
	Neither chloracne nor erythema	12	Mean 25 Median 5.8	0.8	20

^aBackground adjustment by subtracting 5 ppt from median value.

^bEstimation based on adjusted medians, since serum levels are not normally distributed.

^cCalculated mean levels by Fingerhut et al. (1991) not adjusted for background

^dEstimated based on median for all latency periods, since estimate not given for ≥20-year latency.

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Table 8-7 (cont.)					
Study	Sample	Sample size	Concentration		Estimated ^b median concentration at time of last exposure adjusted for background (ppt)
			Including background	Median adjusted for background ^a	
Manz et al., 1991	Sample of nonselected members of cohort tested approximately 31 years after 1954 TCP production stopped	48			2,750 ^a
	High-exposure scenario	37	Mean 296 Median 137	132	
	Medium- and Low-exposure scenario	11	Mean 83 Median 60	55	
Schechter et al., 1989	Sample of U.S. veterans of Operation Ranch Hand	10	Mean 22 Median 17 Range 7-55	12	250 ^f
Schechter, 1991	Sample of U.S. general population	100	Mean 5.2	0	5.2 ^g

^aAssumes no TCDD exposure after 1954.^fAssumes no TCDD exposure after 1969.^gContinuous background exposure only, no adjustment.

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respectively. An alternative breakdown (B) (see Table 8-7) by the 16 sample subjects who exhibited either chloracne or erythema versus those 12 who did not yields estimates of 50 (15) and 25 (5.8) ppt.

For the Manz et al. (1991) study, serum levels were measured in 48 unselected members of the cohort who had what the authors believed to be similar exposures to TCDD as the cohort. Workers were classified into three groups, having either high-, medium-, or low-exposure opportunities, and interviews with these 48 members led to a division of 37 into the high group (mean 296 ppt, median 137 ppt) and 11 into the medium- and low-exposure groups combined (mean 83 ppt, median 60 ppt).

Also included in Table 8-7 are measured serum levels of U.S. veterans of Operation Ranch Hand and a sample of 100 U.S. men from the general population. The mean U.S. estimate of 5 ppt is identical to that reported from four controls in the German population (Schechter et al., 1988). The Fingerhut et al. (1991) referent controls had a mean level of 7 ppt.

Table 8-7 also presents estimates of median TCDD concentrations at time of last exposure for the median levels of various cohorts, based on first-order elimination kinetics, assuming a 7.1 year half-life. To be consistent with the requirements of the model, background levels of 5 ppt are subtracted from each median before back extrapolation. The formula used is:

$$C_t = C_0 e^{-k_e t}$$

where

C_t = concentration at time of measurement

C_0 = estimated concentration at time of last exposure

k_e = elimination constant (per year) = .098

t = years since last exposure

For the Zober (1991) and Manz (1991) studies and the Fingerhut short-exposure subcohort, these concentrations C_0 can be considered to be from short-term exposure, and average fat concentrations can be calculated from the formula:

$$\bar{C} = \frac{1}{T} \int_0^T C_0 e^{-kt} dt$$

where T = length of study (see Table 8-8). For the Fingerhut long-exposure subcohort with an average exposure period of 6.8 years, average concentration during the exposure period is estimated as 50 percent of the calculated $C_0 = 1770$ ppt. Since the average length of followup for this subcohort is 30 years, the total time from start of exposure to end of exposure is estimated as 9 (30-21) years. This leads to a total concentration $\times$ time for this subcohort of (Table 8-8):

$$9 \times 850 + \int_0^{21} 1770 e^{-kt} dt = 24,750 \text{ ppt} - \text{years}$$

Table 8-8 presents calculations of equivalent exposure estimates to convert from the dose metric of total fat concentration $\times$ time to intake dose. The process is to (1) calculate the continuous lifetime average daily *uptake* dose that will produce an equivalent total concentration $\times$ time and (2) to estimate the *intake* (oral) lifetime average daily dose (LADD) that would result in the continuous *uptake* dose.

To calculate (1) the assumptions of steady state discussed in Chapter 6 of U.S. EPA (1993) appear appropriate. These lead to their eq. 6-11 which is:

$$D = \left[\ln \frac{2}{t_1} \right] V_f C_{f,ss}$$

where

D = uptake dose

Table 8-8: Estimates of Lifetime Average Daily Dose for Oral Intake Equivalence for TCDD based on Total Concentration x Time Equivalence. Estimates of TCDD concentration adjusted for background at time of sampling and back-calculated using first order elimination kinetics, by cohort.

Study	Estimated Median Fat Concentration at Time of Last Exp. Adjusted for Background ^f ppt Year	Average Time from Start of Exposure to End of Study Years	Average Age at End of Study Years	Total Conc. x Time ppt - Years	Average Concentration From Start of Exposure to End of Study ppt	Equivalent Average Conc. for Exposure from Age 21 to Age at End of Study ppt	Constant Daily Uptake for Equivalent Average Conc. pg/kg-day	Lifetime Average Daily Dose for Oral Intake Equivalence pg/kg-day ^a
Fingerhut et al, (1991) ≥ 20 year latency								
Exposed > 1 year	1770 (1966)	30 ^a	63 ^c	24,720	790	589	31.5	63.0
Exposed < 1 year	150 (1966)	28 ^a	56 ^c	1,430	51	41	2.2	4.4
Zober et al, 1991								
<u>Breakdown A</u>								
High	450 (1953)	34 ^b	70 ^d	4,430	130	90	4.9	9.7
Medium	105 (1953)	34 ^b	70 ^d	1,030	30	21	1.1	2.3
Low	100 (1953)	34 ^b	70 ^d	980	29	20	1.1	2.2
<u>Breakdown B</u>								
Either Chlorance or erythema	230 (1953)	34 ^b	70 ^d	2,260	67	46	2.5	5.0
Neither Chloracne or erythema	20 (1953)	34 ^b	70 ^d	200	6	4	0.2	0.4
Manz et al, 1991								
High exp. scenario	2750 (1954)	37 ^b	70 ^d	27,300	738	557	30.0	60.0
Medium and low exp. scenario	1150 (1954)	37 ^b	70 ^d	11,422	309	233	12.5	25.0

^a From Table 8-9. Person Years/Cohort Size + 20 years

^b Estimated from Study Descriptions

^c From Fingerhut et al, 1991

^d Estimated from study descriptions and comparison with Fingerhut et al

^e Assumes 50% absorption by oral route

^f From Table 8-7, dates in parentheses are estimated dates of last exposure

V_f = volume of distribution of fat

$C_{f,ss}$ = steady state concentration of dioxin in fat

$t_{1/2} = 7.1 \times 365 = 2,591.5$ days

To calculate D, set $V_f = 14L$ and first calculate $C_{f,ss}$ from age 21 to the age at the end of study, which will yield the same average concentration for each of the subcohorts described. For the Fingerhut et al. long-exposure subcohort, the equivalent $C_{f,ss}$ is (24,750/42 years) = 589 ppt. Constant daily uptake, D, for this subcohort is then 30.2 pg/kg-b.w. Daily intake, or calculation (2), assuming 50 percent absorption from dietary, is then estimated as 63 pg/kg-b.w.

8.5.3.3. *Calculation of Risk Estimates*

Table 8-9 presents the LADDs from Table 8-8, the estimated relative risks, and sample size information for the various subcohorts. Whenever the data could be found, the subcohorts with at least 20-year latency are presented, in order to coincide as closely as possible with the Fingerhut et al. (1991) cohort. For the Manz et al. (1991) study, no data on person-years at risk are available, so only the relative risk model could be used to estimate risk. For the other studies, both models could be used. The data are shown in Figure 8-11 and indicate trends with increasing LADDs for all three studies and for both respiratory cancers and all cancers combined.

Calculations of the incremental unit risk estimates for lung cancer and all cancers combined are presented in Tables 8-10 and 8-11, respectively, for each of the three cohorts separately and all cohorts combined, for both the additive and multiplicative risk models. The results show statistically significant estimates of the slope parameter for the Fingerhut (1991) study, the Manz (1991) study, and all studies combined. Although the slope estimates for the Zober (1990) study are greater than those for the Fingerhut (1991) and Manz (1991) studies, the cohort is smaller and statistical significance is seen only for all cancer deaths combined in the subcohort with chloracne or erythema. Since the Fingerhut (1991) data

Table 8-9. Estimated lifetime average daily doses and relative risks by individual study cohort										
Study	Cohort	LADD (pg/kg-day)	Cohort size N _i	Person years	Respiratory cancer deaths			All cancer deaths		
					Obs.	Exp.	Rel. Risk	Obs.	Exp.	Rel. Risk
Fingerhut et al. (1991)	Exposed > 1 year all		5,172	116,748	96	84.5	1.13	265	229.9	1.15
	Exposed < 1 year all									
	Exposed > 1 year, ≥ 20-year latency	63.0	1,520	15,136	43	30.2	1.42	114	78.0	1.46
	Exposed < 1 year, ≥ 20-year latency	4.4	1,516	12,299	19	18.4	1.03	48	46.8	1.02
Zober et al. (1990)	(≥ 20-year latency)									
	High-exposure scenario	9.7	57	673	3	1.25	2.52	7	4.20	1.67
	Medium-exposure scenario	2.3	74	563	2	1.03	1.94	8	3.37	2.38
	Low-exposure scenario	2.2	81	676	0	0.97	0	1	3.39	0.29
	Either chloracne or erythema	5.0	109	1,209	5	2.09	2.39	14	6.96	2.01
	Neither chloracne nor erythema	0.4	103	703	0	1.16	0	2	4.00	0.50
Manz et al. (1991) ^a	(≥ 20-year latency) ^b				N = 1,148			(Entry before 1955)		
	High-exposure exposure scenario	60.0	96 ^c	No data	30	21.3	1.41	18	8.5	2.11
	Medium- and low- exposure scenario	25.0	200 ^c	No data				33	23.1	1.43

^aWest German reference controls used for consistency with Fingerhut and Zober studies.

^bAssumed because of entry before 1955; actual data unavailable.

^cEntry before 1955.

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**Relative risks of lung cancer and all cancer mortality
in three recent cohort studies of workers exposed to TCDD,
by estimated Lifetime Average Daily Dose intake (LADD)**

Figure 8-11. Relative risks of lung cancer and all cancer mortality in three recent cohort studies of workers exposed to TCDD by estimated LADD

SOURCE:

Table 8-10. Calculation of incremental unit cancer risk estimates and 95 percent lower limits for both the additive and relative risk models based on the lung cancer deaths response in the Fingerhut, Zober, and Manz studies						
Study	Model used	Parameter estimates b^a	Asymptotic variance estimates	p-Value for slope ^b	Lifetime incremental cancer risk per 1 pg/kg-day LADD intake	
					Lower 95% limit	Maximum likelihood estimate ^c
Fingerhut et al., 1991	Additive	1.3×10^{-2}	4.69×10^{-5}	0.02	7.6×10^{-5}	4.7×10^{-4}
	Multiplicative	6.730	11.83	0.02	4.1×10^{-5}	2.6×10^{-4}
Zober et al., 1990	Additive	2.18×10^{-1}	5.28×10^{-2}	0.17	0	7.6×10^{-3}
	H-M-L scenario	4.45×10^{-1}	1.30×10^{-1}	0.11	0	1.6×10^{-3}
	Multiplicative	126.55	16476.6	0.16	0	4.8×10^{-3}
	H-M-L scenario	258.13	43519.2	0.11	0	9.8×10^{-3}
Manz et al., 1991 ^d	Multiplicative	11.67	53.98	0.06	0	4.4×10^{-4}
All studies combined	Additive ^e	1.38×10^{-2}	4.73×10^{-5}	0.02	8.8×10^{-5}	4.8×10^{-4}
	Multiplicative ^f	7.862	9.90	0.01	1.0×10^{-4}	3.0×10^{-4}

^aEstimates for additive risk model given in (ng/kg-day)⁻¹. Estimates for multiplicative risk model given in (ng/kg-day)⁻¹/ $h_0(t)$ where $h_0(t)$ = background age-specific hazard rate.

^bOne-sided test based on asymptotic variance.

^cMLE for additive risk model approximated by multiplying b by 35 since risk before age 35 is close to zero. MLE for multiplicative risk model approximated by multiplying b by $P_0 = 0.038$.

^dDataset in Table 8-9 with estimated LADD = 40 pg/kg-day.

^eFingerhut and Zober (chloracne) cohorts only.

^fFingerhut, Zober (chloracne), and Manz. See also footnote d for Manz.

Table 8-11. Calculation of incremental unit risk estimates and 95 percent lower limits for both the additive and relative risk models based on the total cancer deaths response in the Fingerhut, Zober, and Manz studies

Study	Model used	Parameter estimates b^a	Asymptotic variance estimates	p-Value for slope estimate ^b	Lifetime incremental cancer risk per 1 $\mu\text{g}/\text{kg}\cdot\text{day}$ LADD intake	
					Lower 95% limit	Maximum likelihood estimate ^c
Fingerhut et al., 1991	Additive	3.76×10^{-2}	1.24×10^{-4}	0.0004	1.4×10^{-3}	2.6×10^{-3}
	Multiplicative	7.32	4.70	0.0004	7.0×10^{-4}	1.4×10^{-3}
Zober et al., 1990	Additive	4.62×10^{-1}	1.46×10^{-1}	0.11	0	3.2×10^{-3}
	H-M-L scenario	1.11	0.37121	0.03	8.1×10^{-3}	7.8×10^{-3}
	Multiplicative	78.72	3950.1	0.11	0	1.5×10^{-3}
	H-M-L scenario	192.62	11207.05	0.03	3.9×10^{-3}	3.6×10^{-3}
Manz et al., 1991 ^d	Multiplicative	18.03	40.57	0.002	1.4×10^{-3}	3.3×10^{-3}
All studies combined	Additive ^e	3.83×10^{-2}	1.25×10^{-4}	<0.001	1.4×10^{-3}	2.7×10^{-3}
	Multiplicative ^f	8.93	4.33	<0.0001	1.0×10^{-3}	1.7×10^{-3}

^aEstimates for additive risk model given in $(\text{ng}/\text{kg}\cdot\text{day})^{-1}$; estimates for multiplicative risk model given in $(\text{ng}/\text{kg}\cdot\text{day})^{-1}/h_0(t)$ where $h_0(t)$ = background age-specific hazard rate.

^bOne sided, based on asymptotic variance; likelihood ratio tests generally gave smaller p-values.

^cMLE for additive risk model approximated by multiplying b by 70; MLE for multiplicative risk model approximated by multiplying b by $P_0=0.185$.

^dSee footnote d, Table 8-10.

^eFingerhut and Zober (chloracne) cohorts only.

^fFingerhut, Zober (chloracne), and Manz.

provide the bulk of the weight, the estimates from the combined studies are closer to those based on the Fingerhut (1991) study alone than to the others.

Also shown in Tables 8-10 and 8-11 are estimates of the lifetime incremental cancer risk for 1 pk/kg-day LADD intake. These are derived by substituting the MLE estimates of B back into the age-specific hazard rates and deriving lifetime incremental risk estimates based on lifetable probabilities with competing risks (for practical purposes the procedure described in the table footnotes produces nearly the same results). For lung cancer these unit risk estimates range from 3×10^{-4} to 2×10^{-2} (pg/kg-day)⁻¹, with the estimates for all studies combined between 3×10^{-4} and 5×10^{-4} (pg/kg-day)⁻¹. For all cancers combined the range of MLE estimates is between 1×10^{-3} and 8×10^{-2} (pg/kg-day)⁻¹ with the estimates based on all studies combined between 2×10^{-3} and 3×10^{-3} (pg/kg-day)⁻¹.

These estimates from all studies combined (both lung cancer and total cancers) range from 3×10^{-4} to 3×10^{-3} (pg/kg-day)⁻¹. They exceed the upper-limit estimate of 1.6×10^{-4} (pg/kg-day)⁻¹ previously derived by EPA (U.S. EPA, 1985) based on the total cancer response in the female Sprague-Dawley rat in the Kociba et al. (1978) lifetime feeding study, and the LMS model. Using the same Kociba (1978) study and LMS model but with the liver histopathology rereadings from a recent reanalysis (Sauer and Goodman, 1992) and original Kociba readings for the other tumor sites, the upper-limit estimate is 0.8×10^{-4} (pg/kg-day)⁻¹. Based on the LMS model and only the liver tumors (Sauer and Goodman reanalysis), the upper-limit estimate is 0.5×10^{-4} (pg/kg-day)⁻¹. This compares closely with the MLE estimate of 0.24 (pg/kg-day)⁻¹ provided by the two-stage model in Section 8.2.2, which uses the same liver tumor pathology, together with additional liver foci data. These estimates are shown in Table 8-12.

8.5.3.4. *Low-Dose Deviation From Linearity*

Based on the LADDs and the relative risk estimates presented in Table 8-9, some idea of the degree of nonlinearity in the dose response for these cancers can be derived. For the Fingerhut et al. (1991) cohort, the ratio of 14.3 for high to low LADDs (63.0/4.4)

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Table 8-12: Estimates of U.S. EPA Unit Cancer Risk for TCDD Oral Intake, Based on Animal and Human Studies and U.S. EPA Current and Proposed Estimates							
Source	Cancers	Oral Dose Range	Model	Estimates of Unit Risk (pg/kg-day) ⁻¹		Comments	Ref. for Calculation
				MLE	95% Upper Limit		
<u>Animal</u> (Female Sprague-Dawley rat) All Based on Kociba et al. (1978)	Liver	1-100 ng/kg-day	2-Stage ^a	0.24x10 ⁻⁴	—	Liver Pathology Readings by Sauer and Goodman (1992)	U.S. EPA (1994)
			LMS	—	0.5x10 ⁻⁴		U.S. EPA (1992) ^b
	All (liver, lung, hard palate/nasal turbinate)		LMS	—	0.8x10 ⁻⁴	Liver Pathology by Kociba (1978) and Squire (1980)	U.S. EPA (1992) ^b
			LMS	1.2x10 ⁻⁴	1.6x10 ⁻⁴		U.S. EPA (1985)
				Multistage Weibull (Incidental Tumor Analysis)	2.1x10 ⁻⁴	3.1x10 ⁻⁴	U.S. EPA (1988)
<u>Human</u> (Males) Fingerhut et al. (1991) Zober et al. (1990) Manz et al. (1991)	Lung All	1-60 pg/kg-day ^c	Additive Risk	4.8x10 ⁻⁴	—	Calculations based on combined cohorts	U.S. EPA (1994) Chapter 8
			Multiplicative Risk	3.0x10 ⁻⁴	—		
			Additive Risk	2.7x10 ⁻⁴	—		
			Multiplicative Risk	17x10 ⁻⁴	—		
U.S. EPA Proposed (1988) U.S. EPA Currently Proposed (1994)			Based on reciprocal of risk specific dose for 10 ⁻⁴ incremental risk	1x10 ⁻⁴		External Review Drafts	U.S. EPA (1988)
					1x10 ⁻⁴		

^aAnimal to human correction on the basis of target tissue dose.^bUnpublished.^cEstimates based on total conc. x time equivalence.^dEstimate currently used by U.S. EPA.

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corresponds to a ratio of increased risk of 14 (0.42/0.03) for respiratory cancer and 23 (0.46/0.02) for all cancer mortality combined. For the Manz et al. (1991) cohort, the comparisons are also consistent with linearity; the LADD ratio of 2.4 (60/25) corresponds to an increased risk ratio of 2.6 (1.11/0.43). The Zober et al. (1990) chloracne versus no-chloracne cohort with a high-to-low dose ratio of 12 suggests some low-dose sublinearity, but the low dose estimate is very close to background and no quantitative comparisons can be derived, since the relative risk estimates for this low-dose group are < 1 . For the Saracci et al. (1991) cohort, no direct comparison can be made either, except to note that the relative risk for lung cancer for the low-exposure group was actually higher than that for the high-exposure group. Thus, for two of the cohorts there is some suggestion of sublinearity in dose response, but for the other two linearity is appropriate.

Estimates of LADDs of TCDD intake in the general U.S. population range from 0.3-1.0 pg/kg-day (U.S. EPA, 1993b). The LADDs estimated in three epidemiology studies analyzed range from 0.4 to 63 pg/kg-day above background, with increased risks suggested in the 2-5 pg/kg-day. The LADD estimates, themselves are just too imprecise for more definitive statements.

8.5.3.5. *Uncertainties in Estimates From Human Epidemiology*

There are many uncertainties associated with the unit risk estimates just derived from the epidemiology studies, the two largest being hazard identification and dose estimation. The evidence for a dioxin lung cancer hazard in humans is suggestive but not conclusive (see chapter 7), while that for all cancers combined has less certainty. The estimates of dose, while based on actual body measurements, may lack both representativeness and precision. Although 253 subjects were sampled in the Fingerhut study, they were all taken decades after last exposure and were from two plants. Subjects from the larger plant, plant 1, had the higher dioxin levels, but a lung cancer SMR=72 based on seven deaths, while the smaller plant had only one death from lung cancer (SMR=155). Analysis by plant in the Fingerhut study would have been possible if body measurements at these other 10 plants had been available.

Also, the body levels measured were quite variable and not symmetrically distributed within each study. This led to the selection of a median rather than a mean fat concentration for back extrapolation of the dioxin levels. If the mean had been used, the unit risk estimates based on these studies would have been approximately 50% to 70% less and much closer to those from the animal studies. Also, the estimated exposure in the Zober chloracne subcohort is lower than levels thought to cause chloracne. This could explain why the unit risk estimates based on the Zober study are higher than those of the other two.

Another uncertainty is that of a possible interaction between dioxin and tobacco smoking. In mice, dioxin and 3-methylcholanthrene (3-MC, one of the many polycyclic aromatic hydrocarbons in tobacco smoke) have been shown to be cocarcinogenic (Kouri et al., 1978; U.S. EPA, 1985). Other studies of mouse skin tumors have shown that dioxin can have anticarcinogenic properties when administered before initiation with either 3-MC or benzo(a)pyrene (U.S. EPA, 1985). Furthermore, dioxin's tumor-promoting ability suggests that two-stage models would be more appropriate if individual smoking histories were known. Individual smoking histories are presented only for the 37 cancer cases and deaths in the Zober cohort; only 2 were stated as being nonsmokers. All seven men with lung or larynx cancer were smokers. While characteristics of the two subcohorts in Fingerhut et al., 1991, suggest similar smoking prevalence, the effects with higher levels of dioxin could be synergistic.

A synergism of dioxin with 4-aminobiphenyl, a known human bladder carcinogen, has been suggested (Collins et al., 1993) for one of the plants in the Fingerhut study, but tobacco smoking would be present in all the plants and would seem to be a more likely universal effect modifier.

8.5.3.6. Conclusions

Epidemiology studies suggest that the lung in the human male is a much more sensitive target organ for TCDD than is the liver and that the human is a sensitive species for cancer response, probably more sensitive than is the rat. The studies also show increases for

all cancers combined. Estimates derived from the human data suggest a unit risk for lung cancer of $3 \text{ to } 5 \times 10^{-4} \text{ (pg/kg-day)}^{-1}$; for all cancers combined the unit risk estimate is $2 \text{ to } 3 \times 10^{-3} \text{ (pg/kg-day)}^{-1}$. While unit risk estimates based on rat tumors are somewhat less, they are within the range of uncertainty of those based on human data. Both animal and human responses are consistent with low-dose linearity.

8.6. KNOWLEDGE GAPS

Considerable information is now available on the mechanisms of action responsible for TCDD's effects in experimental animals and humans, and important new information is now being generated. These data are, of course, essential to the development of reliable biologically based models for the estimation of human risks as a consequence of exposure to TCDD and its structural analogs such as the CDFs and co-planar PCBs. Uncertainty in such models reflects incomplete knowledge of mechanisms and inadequacies in exposure/tissue dose relationships. In the process of developing and evaluating biologically based models, we can identify those knowledge gaps that create uncertainty. The idea that interactions of TCDD with the Ah receptor is an essential first step in most, if not all, of dioxin's effects has been considered as a reasonable assumption for over a decade. The recent Banbury Conference on dioxin formalized this as a general consensus among dioxin researchers. The development of models that accurately predict risks also requires tissue and cell dosimetry data in experimental animals and humans. This kind of dosimetry information is available for blood, liver, and adipose tissue, but dosimetry data in other target tissues such as the lung, skin, pituitary, and reproductive tract are not available or incomplete. It would be especially relevant to the development of biologically based dose-response models to have dosimetry data (relationship between exposure, dose, and cell-specific dose) in target cells when the target cell is known. For example, the lung is composed of numerous cell types, but the identity of the target cell(s) for TCDD-mediated lung cancer is not known nor are there many data on dose-response relationships for concentrations of TCDD in whole lung or discrete cell types. Since the vast majority of dioxin is found in the liver and adipose tissue

under chronic exposure-steady state conditions and the lung is clearly a target organ for biochemical and toxic effects, it would seem that the lung and perhaps other organs require far less tissue/cell levels of dioxin to exhibit toxic effects than in liver.

One of the most confounding yet important knowledge gaps in the development of mechanistic models is the evaluation of the adverse health consequences, if any, of current background exposure to the CDDs and CDFa, which is estimated at 1 to 3 pg TCDD equivalents/kg/day. More accurate information on the potency of dioxin-like PCBs is also an essential component in evaluating the health impact of background exposure to chemicals that bind the Ah receptor.

Many of the molecular events that follow binding of TCDD to the Ah receptor are now known for transcriptional activation of the CYP1A1 gene. However, there is little information on the characterization of analogous events for dioxin's many other effects on gene expression such as Ah receptor-mediated alterations in the EGF or estrogen receptor. Most of the mechanistic or dose-response information on dioxin's effects has been generated on changes in gene expression of single genes such as CYP1A1 induction. There is only limited information on the complex interaction of biochemical, molecular, and biological events that are necessary to produce a frank toxic effect such as cancer, developmental defects, reproductive effect, or neurological effects. Figure 8-12 from Greenlee et al. (1992) summarizes the series of interconnected steps within the three major components of receptor-mediated events (recognition, transduction, and response). Although this scheme is simplified (i.e., each step may comprise several events), it does provide a framework for identifying knowledge gaps that create uncertainty. Clearly, interactions with other endocrine systems are involved in some effects, and our ability to construct accurate dose-response models for noncancer endpoints would be enhanced if we had a better understanding of TCDD/endocrine interactions.

One of the more active areas of research on hormone action is directed at identifying the cell specific factors that produce diversity of responses for receptor-mediated responses, that is, how do a single receptor and a single ligand produce the wide spectrum of cell-

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Figure 8-12

specific responses characteristic of exposure to a given hormone. Since TCDD is acting like a potent and persistent hormone agonist/antagonist, the mechanisms responsible for qualitative and quantitative differences in dose-response relationships for Ah receptor-mediated events might be similar to those mechanisms identified for steroid hormones. Fuller (1991) has summarized some of the mechanisms responsible for generating diversity and these are listed below:

ligand < ----- agonist, antagonist

target tissue < ----- receptor gene expression
activating or inactivating enzymes
binding proteins (extra of intracellular)

receptor < ----- cytoplasmic versus nuclear
isoforms-differential
splicing
gene duplication

dimers < ----- hetero or homodimers
DNA binding factors

nuclear factors < ----- antagonist isoforms
squenching

response elements < ----- consensus versus nonconsensus
number of copies
position
proximity of other response elements

transactivation < ----- gene specific factors
cell-specific factors

In addition to the above considerations, there is considerable speculation regarding the normal cellular functions of the Ah receptor and the identity of any endogenous ligands for

the Ah receptor. If sound scientific information were available on the normal functions of the receptor, especially if those functions involve regulation of cell proliferation and differentiation, it would greatly enhance our ability to predict the health consequences of low-level dioxin exposure. It would also help considerably in the selection of appropriate animal models for estimating dioxin risks.

Interindividual variation in human responses to TCDD and its structural analogs is one of the most difficult issues to accommodate in the development of biologically based dose-response models. We know from epidemiology studies that some individuals develop chloracne from a given exposure to dioxin, whereas other individuals exposed to the same amount of dioxin do not develop chloracne. The mechanisms responsible for sensitivity or resistance to the chloracnegenic actions of dioxin are not known, nor is there any information on the relationship of chloracne to other toxic effects. For example, are individuals who are susceptible to chloracne also susceptible to the carcinogenic actions of dioxin? Likewise, there are considerable differences in the magnitude of enzyme induction when human cells are cultured with dioxin. We need to understand the molecular mechanisms responsible for these differences and whether high inducers are more or less susceptible to the toxic effects of dioxin and its structural analogs. These kinds of data would allow the development of epidemiologic and laboratory approaches for evaluating health consequences in both sensitive or resistant populations.

In summary, we have gained considerable and valuable insights regarding mechanisms of dioxin and dose-response relationships for dioxin effects. These data are not yet complete but are appropriate for the development of preliminary biologically based models that may eventually be useful for estimating dioxin's risks to humans. When sufficiently developed, these models should provide increased confidence and decreased uncertainty than are present with the current default approaches (LMS or safety factor). They should also accommodate new scientific information from research directed at filling knowledge gaps to further reduce uncertainty. Based on the model structures presented in this chapter, it should be possible to design specific experiments to fill key knowledge gaps.

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A Mechanistic Model of Effects of Dioxin on Gene Expression in the Rat Liver

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A Mechanistic Model of Effects of Dioxin on Gene Expression in the Rat Liver. KOHN, M. C., LUCIER, G. W., CLARK, G. C., SEWALL, C., TRITSCHER, A. M., AND PORTIER, C. J. (1993). *Toxicol. Appl. Pharmacol.* 120, 138-154.

Improved methods for estimating the shape of the response curve for effects of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are needed in order to evaluate possible adverse health effects of TCDD. A mathematical model has been constructed to describe TCDD-mediated alterations in hepatic proteins in the rat. In this model it was assumed that TCDD mediates increases in the liver concentration of transforming growth factor- α (TGF- α) by a mechanism which requires the aryl hydrocarbon (Ah) receptor. TGF- α subsequently binds to the epidermal growth factor (EGF) receptor, a process which is known to cause internalization of this receptor in hepatocytes. This action is thought to be an early event in the generation of a mitogenic signal. Because TCDD decreases binding of EGF in the livers of intact female rats but not in ovariectomized rats, this effect was further assumed to be dependent on estrogen action. The model postulates Ah receptor-dependent effects on the concentration of cytochrome P450 1A2 (CYP1A2), which is involved in the metabolism of estradiol, and on the concentration of the estrogen receptor. The model also incorporates information on induction of cytochrome P450 1A1 (CYP1A1) by TCDD. The biochemical response curves for all these proteins were hyperbolic (Hill exponents in the equations for their expression were found to be 1), indicating a proportional relationship between target tissue dose and protein concentration at low administered doses of TCDD. The model successfully reproduced the observed tissue distribution of TCDD, the concentrations of CYP1A1 and CYP1A2, and the effects of TCDD on the Ah, estrogen, and EGF receptors over a wide dose range. © 1993 Academic Press, Inc.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent carcinogen which is associated with increased incidence of liver tumors in female rats but not in male rats (Kociba *et al.*, 1978; National Toxicology Program, 1982; Clark *et al.*, 1991). Similarly, TCDD enhances hepatocyte proliferation and stimulates development of enzyme-altered hyperplas-

tic foci in intact female rats but not in ovariectomized rats (Lucier *et al.*, 1991), suggesting that estrogens play a major role in TCDD-mediated hepatocarcinogenesis.

Biochemical signals regulating cellular proliferation are mediated in many tissues, including the liver, by the epidermal growth factor (EGF) receptor (Schlessinger *et al.*, 1983). The EGF receptor is a member of a family of plasma membrane receptors that, on binding of ligand, transduce signals by tyrosine kinase activity and by internalization of the liganded receptor (Hunter and Cooper, 1985). Phosphorylation of various proteins by this tyrosine kinase leads to alterations in cellular regulation and mitotic activity.

Livers of TCDD-treated rats show a dose-dependent decline in the maximal binding of EGF (Lucier, 1991) although TCDD has no effect on the amount of mRNA for the EGF receptor in mouse liver (Lin *et al.*, 1991a). The effect of TCDD on mRNA for the EGF receptor is a tissue-specific response. TCDD affects the amount of mRNA for the EGF receptor in the uterus (Astroff *et al.*, 1990) but not in keratinocytes (Osborne *et al.*, 1988). Antibodies raised against the EGF receptor stain the plasma membranes of hepatocytes in control rats, but in TCDD-treated rats there is an apparent redistribution of the receptor into the cytosol (C. Sewall and A. M. Tritscher, unpublished results). This is consistent with the notion that the loss of EGF binding capacity in liver plasma membranes is due to internalization of the liganded receptor.

The liver does not produce EGF, but there is evidence that it does produce several other EGF-like peptides such as transforming growth factor- α (TGF- α), another ligand of the EGF receptor (Mead and Fausto, 1989). TCDD induces expression of TGF- α in keratinocytes (Choi *et al.*, 1991), suggesting that TCDD may also induce TGF- α in the liver. Increased production of this peptide would subsequently stimulate EGF receptor-mediated events in that organ.

In order to obtain a quantitative relationship between exposure to TCDD and consequent alterations in the properties of the EGF receptor, we have constructed a mathematical model of the tissue distribution of TCDD in the rat

and its effect on the concentrations of several important liver proteins. The model includes equations for the aryl hydrocarbon (Ah) receptor-dependent induction of cytochrome P450 isozymes 1A1 (CYP1A1) and 1A2 (CYP1A2) and of the Ah receptor itself. The complex between this receptor and TCDD (Ah-TCDD) is treated as inducing expression of TGF- α although the induced ligand of the EGF receptor actually may be another EGF-like peptide. It is also assumed that estrogen action is required for TCDD-mediated induction of TGF- α . TGF- α is modeled as released into the liver interstitium. From there it binds to the EGF receptor, causing its internalization. The model calculates the distribution of the receptor between the plasma membrane and cytosol.

Because estrogens appear to be required for TCDD-mediated effects on the EGF receptor, production of the estrogen receptor, CYP1A2-catalyzed formation of catechol estrogens, and deactivation of estrogens by glucuronidation were included in the model. In this effort, data on the stimulation of estrogen receptor synthesis by estradiol and the inhibition of estrogen receptor synthesis by TCDD were incorporated.

The model's predictions were compared to the data of Tritscher *et al.* (1992) and Sewall *et al.* (1993). Their experiments were a two-stage protocol in which female Sprague-Dawley rats were injected with an initiating dose of diethylnitrosamine (DEN). After 14 days the rats were subjected to biweekly gavage with TCDD in corn oil at doses equivalent to 3.5–125 ng/kg/day for 30 weeks. The rats were killed 1 week following the last dose, and their livers were assayed for TCDD, CYP1A1, CYP1A2, and plasma membrane binding of EGF. All such measurements were performed using samples from the same livers.

This model was used to predict tissue concentrations of TCDD and concentrations of induced proteins following administration of TCDD. A model which reproduces the dose-response relationships of experimental data and is consistent with the biochemical and physiological processes known to occur in rats exposed to TCDD might permit extrapolation of responses beyond the range obtained from experimental data and lead to scientifically sound approaches for estimating risks of adverse health effects of exposure to TCDD.

METHODS

The present model, hereafter referred to as the NIEHS model (see Appendices 1 and 2 for a complete description), was adapted from the physiologically based pharmacokinetic model of Leung *et al.* (1990), hereafter referred to as the LPMA model. To fit the data of Tritscher *et al.* (1992) and Sewall *et al.* (1993), the LPMA model has been modified as described below. A flowchart of the resulting model is given in Fig. 1.

TCDD in the gut compartment is periodically increased by orally administered TCDD according to the experimental schedule (Tritscher *et al.*, 1992). TCDD is extracted from the gut into the blood compartment, where

some of it binds to unspecified serum protein. From the blood it is distributed to the tissue compartments.

TCDD in the liver is distributed between "metabolically available" and protein-bound pools. The latter category includes binding to CYP1A2 and to the Ah receptor, which is presumed to mediate all of the tissue's responses to TCDD. Unbound TCDD is converted to metabolites which are either transferred to the gut via the bile or released into the blood. The metabolites are treated as partitioning between the liver and blood in the same manner as TCDD. Gut metabolites ultimately appear in the feces, and blood metabolites appear in the urine. TCDD is also cleared from the liver by lysis of dead cells consequent to prolonged exposure.

Equations for the pharmacodynamic effects of TCDD described above were also included in the NIEHS model. Proteins whose synthesis is represented in the model are removed by proteolysis or, in the case of the EGF receptor, by endocytosis.

In many cases quantities that were reported in a variety of units had to be converted to those units (nanomoles of material, volume in liters, time in days) used in the model. These calculations were performed using conversion factors of 100 mg cytosolic protein/g liver (Poland and Knutson, 1982), 18 mg microsomal protein/g liver (based on average yield of membrane protein; Tritscher *et al.*, 1992), and 4.2 mg plasma membrane protein/g liver (based on average yield of membrane protein; Sewall *et al.*, 1993). Rates obtained from *in vitro* measurements conducted at temperatures other than body temperature were adjusted to 37°C using a doubling of the rate for every 10°C increase in temperature, a factor which is typical of enzymatic reactions.

The animals used in these experiments (Tritscher *et al.*, 1992) grew, on average, from 220 to 390 g during the 31 weeks of the investigation. To account for the dilution of material with growth the parameters in the model were expressed per liter in the appropriate compartment. Because of the increase in compartment volume, a steady-state concentration of a protein whose synthesis is represented in the model actually corresponds to an increase in the tissue content of the protein with time.

TCDD distribution and clearance. The LPMA model is said to be flow-limited: the rate of uptake by tissues is limited by the rate of delivery via the blood. In such a model, material is transferred from the arterial blood to various tissues at a rate given by

$$\frac{\text{Blood Content} \times \text{Flow}}{\text{Blood Content}}$$

where Flow represents the rate of blood flow through the particular tissue. Transfer of material from the tissue to the venous blood is given by

$$\frac{\text{Tissue Content} \times \text{Flow}}{\text{Tissue Volume} \times \text{Partition Coeff.}}$$

where the partition coefficient is the ratio of tissue to blood concentrations at equilibrium. The LPMA model includes compartments for gut, blood, fat, muscle (plus skin), liver, and other viscera. The cell membranes of these tissues constitute a barrier to transport of TCDD. In the NIEHS model the rates of transport given by the above expressions were multiplied by a "diffusion coefficient" to account for the membrane permeability. Different compartments were assigned different coefficient values as required to match the time course data of Abraham *et al.* (1988). For these calculations the dose was absorbed directly into the blood from the site of injection with a scaled rate constant of 0.37 kg^{0.75}/day, bypassing the gut compartment.

The observed blood levels of TCDD (A. M. Tritscher, unpublished results) were an order of magnitude smaller than those predicted by the LPMA model. The LPMA model includes binding of TCDD to unspecified blood lipoproteins and uses a factor of 2.5 for the ratio of protein-bound to free TCDD in the blood. This constant was replaced in the

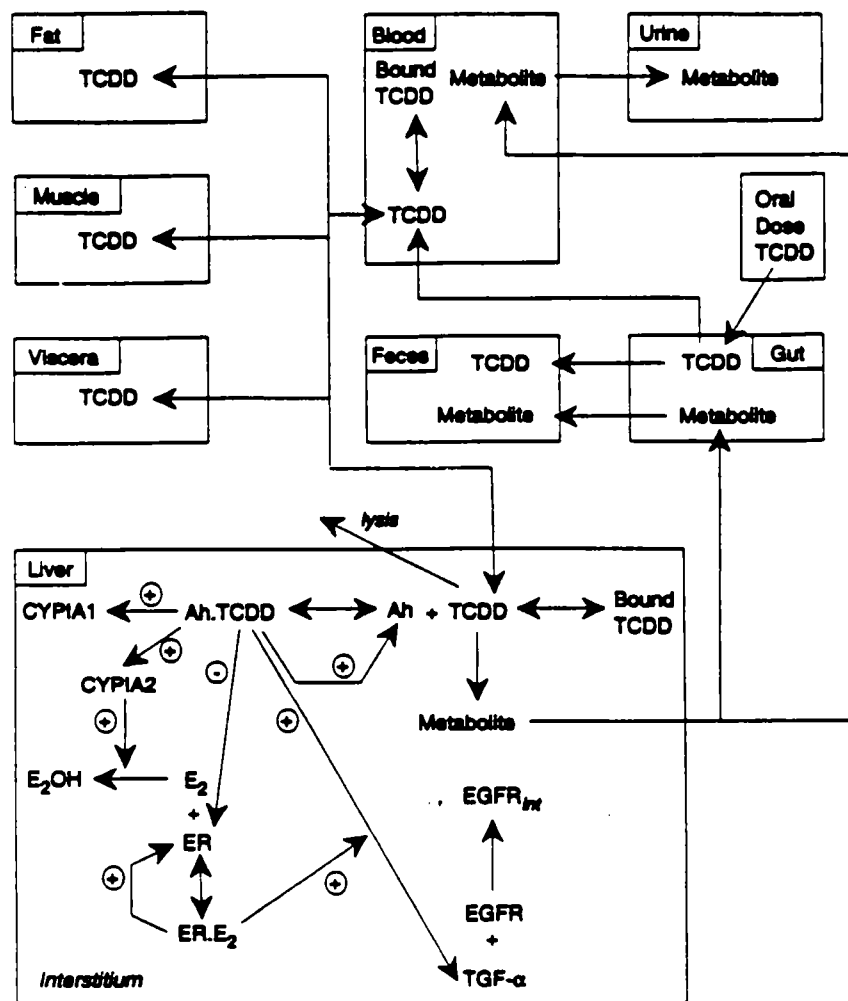


FIG. 1. Flowchart of the NIEHS model of TCDD distribution and consequent effects on gene expression in the rat liver.

NIEHS model by a reverse hyperbolic function (Appendix 1) for the maximal amount of TCDD-binding blood protein. Although the form of this equation suggests that TCDD decreases the hepatic production of blood lipoprotein, the equation is intended as just an empirical relationship.

TCDD has been found to bind to CYP1A2 in rat liver (Voorman and Aust, 1989) and P450d in the mouse liver (Poland *et al.*, 1989) with an apparent K_d of 30 nM. The estradiol 2-hydroxylase activity of this cytochrome is inhibited as a consequence of this binding (Voorman and Aust, 1987). As Poland *et al.* (1989) concluded that TCDD congeners bind at or near the active site on this enzyme, such binding is treated as competitive against estradiol in the NIEHS model. It was further assumed that TCDD binds only to the CYP1A2 that is not complexed with NADPH:cytochrome P450 reductase.

The appearance of unabsorbed TCDD in feces and TCDD metabolite(s) in both feces and urine is included in the NIEHS model. Birnbaum *et al.* (1980) administered a single oral dose of ^{14}C -labeled 2,3,7,8-tetrachlorodibenzofuran (TCDF) to rats. They calculated rate constants of 0.381 day^{-1} for the appearance of metabolites in the feces and 0.536 day^{-1} for the appearance of metabolites in the urine. However, the calculated rate constant for clearance of radiolabel from the blood during the first 3 hr in their experiments (32 day^{-1}) was an order of magnitude higher than after 3 hr (0.6 day^{-1}). Because excretion of metabolites of TCDF is limited by their rates of formation, the rate constants for clearance at late time most likely reflect this limitation. This notion is supported by nearly identical calcu-

lated rate constants for clearance of radiolabel from liver and from blood after 3 hr (Birnbaum *et al.*, 1980). The higher rate constant for clearance at early time more likely reflects the rate of uptake of labeled material from the blood by the various tissues, which therefore cannot be rate limiting for clearance. The rates of transport of TCDFs and TCDD's metabolites across cell membranes should be similar. This leads to the conclusion that the specific rates of transport of TCDD's metabolites from liver to bile and from blood to urine must be larger than the rate constants for net clearance for times greater than 3 hr. Therefore, the above rate constants were increased by a factor of 10 for this model to ensure that clearance of metabolites is not limited by transport.

Rose *et al.* (1976) found as much TCDD in the livers of rats after 7 weeks of chronic dosing as Tritscher *et al.* (1992) found after 31 weeks at comparable doses. A preliminary version of the NIEHS model indicated that parameter values which enabled the model to reproduce the results of Tritscher *et al.* (1992) would uniformly underestimate the results of Rose *et al.* (1976) by about 50%. The preliminary model included a first-order rate constant for metabolism of TCDD whose value could not be altered to fit the data of Rose *et al.* (1976) without destroying the fit to the data of Tritscher *et al.* (1992). Therefore, an additional mechanism for clearance from the liver was necessary to permit fitting both data sets simultaneously.

The cellular proliferation rate in the livers of rats exposed to biweekly doses of $1.4 \mu\text{g/kg}$ of TCDD for 30 weeks is 7.3% of the liver cells/week (Lucier *et al.*, 1991). At this rate, one would expect a 226% increase in the

weight of the liver over the course of the experiment. Only a 90% increase was observed (Lucier *et al.*, 1991; A. M. Tritscher, unpublished results), suggesting that toxic effects of cumulative exposure to TCDD result in cell death. The NIEHS model includes loss of TCDD from the liver by lysis of dead cells. The specific rate of clearance by cell lysis was assumed to increase as a hyperbolic function of the cumulative exposure to unbound liver TCDD (Appendix 1). No information regarding the fate of TCDD from lysed cells is available. Therefore, this feature of the model merely represents the net contribution to clearance by cell turnover.

TCDD-induced changes in gene expression. The complex formed by binding of TCDD to the Ah receptor has been clearly implicated in induction of CYP1A1 (Fisher *et al.*, 1990). The liganded Ah receptor modulates expression of CYP1A1 by forming a ternary complex with at least one other transcription factor (Hoffman *et al.*, 1991), and this complex then binds to enhancer sequences for the CYP1A1 gene. This mechanism was used as a prototype for all Ah receptor-dependent protein synthesis in the model. There are no data for the concentrations of the additional transcription factors required for expression nor for the competition among various receptors for binding to them. Therefore, the NIEHS model combines binding of additional transcription factors to the Ah-TCDD complex into the equations for the rate of Ah-dependent induction by treating induction of this protein as proceeding with saturation kinetics subsequent to binding of the Ah-TCDD complex itself to the appropriate enhancer sequences. That is, the Ah-TCDD complex was treated as though it were the "substrate" of the rate-limiting reaction in expression.

TCDD increases the liver concentration of the Ah receptor itself (Poland and Knutson, 1982; Sloop and Lucier, 1987). In the NIEHS model, increased synthesis of the Ah receptor is postulated to occur by an Ah-dependent mechanism similar to that outlined above. Bradfield *et al.* (1988) observed the Ah receptor's K_d for 2-iodo-7,8-dibromodibenzo-*p*-dioxin to decline from 0.16 to 0.012 nM on 32-fold dilution of the Ah receptor protein. A dissociation equilibrium constant depends on ligand solubility, pH, ionic strength, etc. The hydrophobicity of TCDD apparently makes the apparent K_d very sensitive to the above conditions. Therefore, the model uses the value of 0.27 nM reported by Poland and Knutson (1982) for the K_d of TCDD under conditions more representative of the intracellular milieu.

A rate law as described above treats the synthesis of gene product as occurring by a single event. The appearance of gene product is actually the result of many steps and requires some time before measurable amounts of protein are synthesized. The data of Sloop and Lucier (1987) show that 6 hr elapses between administration of TCDD and increased production of Ah receptor protein. To account for such a delay, the concentration of the Ah-TCDD complex at time $t - 6$ hr is substituted into the rate law to compute the rate of synthesis of protein at time t . The same approach was used for binding of all receptor complexes in all gene expression processes in the model. The model's predictions were observed to be fairly insensitive to reasonable numerical values of this delay.

Fisher *et al.* (1990) found four Ah-responsive enhancer sites associated with the CYP1A1 structural gene. This finding raises the possibility of cooperative binding of Ah-TCDD complexes to these binding sites and, by analogy, to the other Ah-TCDD binding sites postulated in the model. To test for such cooperativity, Hill exponents were included in the rate equations for protein synthesis and those values which would enable the model to fit experimental observations were identified.

Proteins in the model were treated as being removed from the system with first-order kinetics. The rate constant for the proteolytic degradation of all proteins whose synthesis is represented in this model was determined from the data of Lucier *et al.* (1972).

Effects of TCDD on epidermal growth factor receptor. Choi *et al.* (1991) found TCDD to induce expression of TGF- α by an Ah-dependent mechanism in keratinocyte cultures. The TGF- α produced was exported to the extracellular fluid. A similar mechanism has been included in the

NIEHS model, representing the product peptide as appearing in the interstitial space of the liver.

Intact female rats subjected to 30 weeks of oral dosing with 125 ng TCDD/kg body weight/day show a 65% reduction in the maximal binding capacity (B_{max}) of the EGF receptor in liver plasma membranes (Sewall *et al.*, 1993) at a liver TCDD content of 0.063 nmol/g (Tritscher *et al.*, 1992). Ovariectomized rats exhibit only a 19% reduction of EGF B_{max} at a liver TCDD content of 0.106 nmol/g after 30 weeks of oral dosing at 100 ng TCDD/kg/day (Clark *et al.*, 1991). Sunahara *et al.* (1989) observed a 56% reduction in the B_{max} in female rats 10 days following gavage with 10 μ g TCDD/kg. Madhukar *et al.* (1984) observed a 45% decline in the B_{max} of the EGF receptor of male rats 10 days after a single intraperitoneal injection of 10 μ g TCDD/kg. The NIEHS model predicts a liver TCDD content of 0.217 nmol/g for females and 0.223 nmol/g for males at the end of the 10-day period. The responses of males, ovariectomized females, and intact females may indicate different sensitivities for transcriptional activation of the TGF- α gene by the Ah-TCDD complex as a consequence of differences in the amount of hepatic estrogen (or some other ovarian hormone). Intact females, having much more circulating estrogen than either males or ovariectomized females, should be the most sensitive.

Both the Ah-TCDD and estrogen receptor-estrogen (ER-E) complexes may be acting as transcriptional activators of the TGF- α gene, but a synergistic interaction between them appears to be necessary to fully activate transcription. In the NIEHS model, binding of the ER-E complex is treated as activating the TGF- α gene for subsequent binding of the Ah-TCDD complex. However, binding of this complex to the TGF- α gene has not been proven, and other mechanisms are possible.

Estrogen metabolism and the production of the estrogen receptor are included in the NIEHS model to account for the effects of estrogen on alterations in the EGF receptor. The kinetic constants for estradiol 2-hydroxylase activity (Appendix 2) would predict an increase in enzymatic activity comparable to the ninefold rise in CYP1A2 resulting from exposure to TCDD. Only a threefold increase in activity was actually observed (Graham *et al.*, 1988) in female rats. The smaller increase in activity was attributed to rate limitation by the availability of NADPH:cytochrome P450 reductase.

This reductase forms a complex with cytochrome P450 (Coon *et al.*, 1977). The complex binds molecular oxygen and is reduced by NADPH to eliminate water, leaving a single oxygen atom to combine with substrate (Estabrook and Wernigloer, 1977). As all P450 isozymes compete for binding to reductase, there might not be sufficient reductase to bind to all the increased CYP1A2. Indeed, there is evidence (Ullrich and Duppel, 1975) that P450 reductase is limiting for other mono-oxygenase reactions that are not induced. Therefore, binding of the reductase to both CYP1A1 and CYP1A2 was included in the NIEHS model. Estradiol and reductase were allowed to bind to CYP1A2 in random order, and oxygen and NADPH were treated as saturating.

The catechol estrogens produced by the activity of CYP1A2 bind to the estrogen receptor an order of magnitude more weakly than does estradiol (Li *et al.*, 1985). Such binding is included in the NIEHS model, and the resulting complex was allowed to function identically to the ER-estradiol complex. This means that the ER-estradiol and ER-catechol estrogen complexes produce the same effect on gene expression, but that 10 times as much catechol estrogen as estradiol is required to achieve the same quantitative response. Therefore, the symbol ER-E is used in this report to represent complexes between the estrogen receptor and either estradiol or catechol estrogen.

The estrogen-binding capacity of the estrogen receptor in the livers of ovariectomized or immature female rats is increased by administration of estradiol (Romkes *et al.*, 1987). One interpretation of this observation is that there is synthesis of additional receptor protein consequent to binding of the ER-E complex to the estrogen receptor gene. Female rats treated with 100 ng TCDD/kg/day exhibit a 55% reduction in the estrogen receptor (Clark *et al.*, 1991). A 40% reduction in estrogen receptor levels was

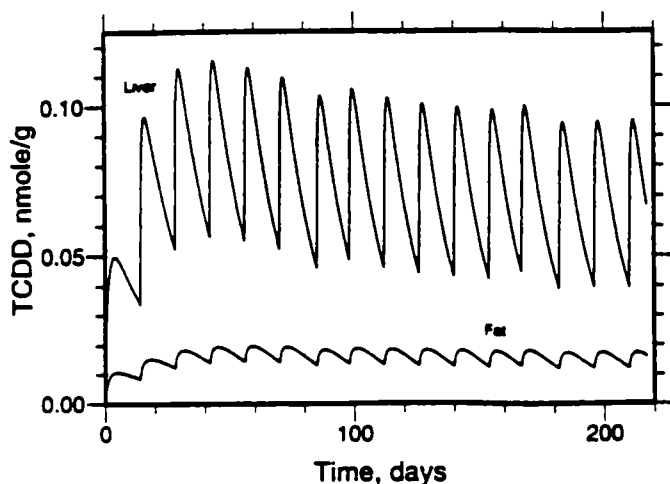


FIG. 2. Computed time courses of the accumulation of TCDD in liver and fat of rats treated with 125 ng TCDD/kg body weight/day.

observed in the livers of mice exposed to a single 100 ng/kg dose of TCDD (Lin *et al.*, 1991b). "Ah-unresponsive" mice having defective or insufficient Ah receptors require higher TCDD exposures than do normal mice to achieve a given reduction in estradiol binding capacity (Lin *et al.*, 1991b). These observations may be interpreted as indicating that induction of estrogen receptor is inhibited by binding of the Ah-TCDD complex at another DNA binding site. This mechanism is represented in the NIEHS model as noncompetitive inhibition of receptor synthesis by the Ah-TCDD complex (Appendix 1).

The NIEHS model was implemented in the SCoP simulation program (Kootsey *et al.*, 1986; Kohn *et al.*, 1993). Values for parameters that could not be obtained from the literature were adjusted to make the model reproduce the observations of Tritscher *et al.* (1992) and Sewall *et al.* (1993). Where experimental data were available, parameters were estimated using the "praxis" algorithm (Brent, 1973) in the SCoPfit program (part of the SCoP package).

The fully assembled model was composed of 33 first-order ordinary differential equations. There were 77 constants in these equations, of which 15 were freely adjustable parameters. The remaining 62 constants were obtained from the literature, constrained by experimental data, or mathematically determined by the law of conservation of mass. The model's equations are listed in Appendix 1, and all parameter values (estimated or from the literature) are given in Appendix 2. The experiments of Tritscher *et al.* (1992) and Sewall *et al.* (1993) were simulated by integration of the differential equations to 217 days of biweekly exposure to TCDD using a C language translation of the LSODA Gear integration package from Lawrence Livermore Laboratory.

RESULTS

The NIEHS model predicts that 92.5% of the ingested TCDD is absorbed into the bloodstream and 7.5% appears unchanged in the feces. As some of the TCDD cleared from the liver by cell lysis may be returned to the gut (the model does not treat the fate of this material), this result may be an underestimation of the amount of TCDD in the feces. Rose *et al.* (1976) report an average value of 90.3% of ^{14}C -TCDD extracted from the guts of female rats. The model predicts that 92.2% of the metabolite appears in the feces and 7.8%

appears in the urine at a dose of 125 ng/kg/day. The dose of TCDD did not have a significant effect on these predictions. Urinary radioactivity at doses comparable to those of Tritscher *et al.* (1992) was often undetectable in the chronic dosing experiments of Rose *et al.* (1976).

The computed time courses for TCDD in the liver and fat for biweekly oral doses of 125 ng/kg body weight/day (Tritscher *et al.*, 1992) and for a single subcutaneous injection of 300 ng/kg (Abraham *et al.*, 1988) are given in Figs. 2 and 3, respectively. From the fit to the data of Abraham *et al.* (1988) the NIEHS model predicts an initial half time of 11.8 days for clearance of TCDD from the liver and an overall liver half time of 13.5 days. Abraham *et al.* (1988) report an initial half time of 11.5 days and an overall half time of 13.6 days in the liver. The model predicts a half time of 22.3 days in the fat; Abraham *et al.* (1988) report a half time of 24.5 days in fat.

The predicted dose dependency of TCDD concentration in the blood, liver, and fat after 217 days of exposure (biweekly oral doses of TCDD for 31 weeks) is given in Table 1. The NIEHS model predicts a linear relationship between administered dose and the concentration in the liver at doses between 3.5 and 125 ng/kg/day (Fig. 4) in agreement with the experimental observations (Tritscher *et al.*, 1992). Table 2 gives the model's predictions for repeated doses, and Table 3 gives the model's predictions for single doses.

The Hill exponents in the equations for the rates of induction of CYP1A1 and CYP1A2 were estimated from the

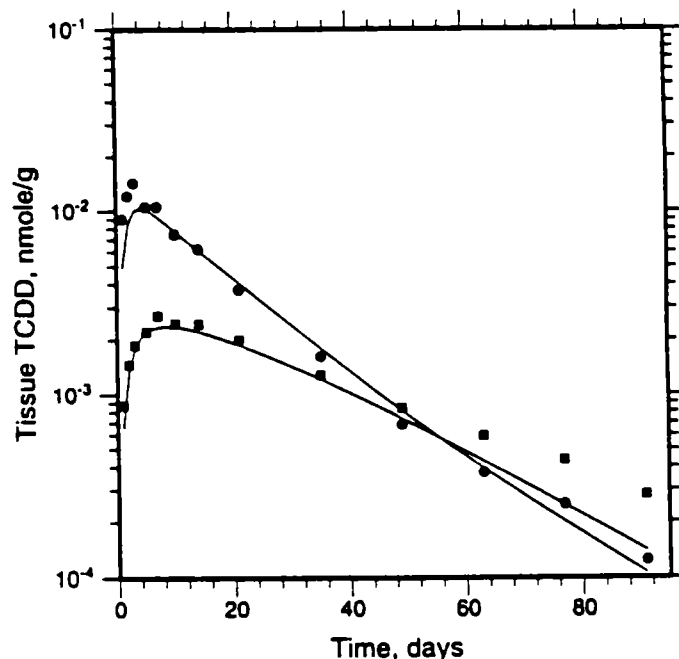


FIG. 3. Fit to the time courses in rats given a subcutaneous injection of 300 ng/kg body weight of TCDD (Abraham *et al.*, 1988). Circles are for observations in liver, and squares are for observations in fat.

TABLE 1
Computed TCDD Distribution

Dose, ng/kg/day	Blood, nm ^a	Liver, nmol/g ^b	Fat, nmol/g
0.01	0.0000967	0.00000394	0.00000432
0.1	0.000925	0.0000395	0.0000421
0.3	0.00254	0.000118	0.000120
1.0	0.00672	0.000389	0.000350
3.5	0.0150 (0.0127–0.0174)	0.00142 (0.000652–0.00267)	0.000954
7.0	0.0218	0.00313	0.00162
10.7	0.0269 (0.0143–0.0251)	0.00515 (0.00435–0.00683)	0.00223
15.0	0.0315	0.00754	0.00289
20.0	0.0361	0.0105	0.00361
25.0	0.0400	0.0134	0.00431
30.0	0.0435	0.0165	0.00496
35.7	0.0471 (0.0323–0.103)	0.0199 (0.0155–0.0311)	0.00568
45.0	0.0523	0.0252	0.00683
55.0	0.0573	0.0307	0.00804
65.0	0.0618	0.0360	0.00925
75.0	0.0661	0.0417	0.0104
85.0	0.0700	0.0468	0.0115
100.0	0.0754	0.0543	0.0133
115.0	0.0806	0.0618	0.0149
125.0	0.0839 (0.0401–0.134)	0.0673 (0.0385–0.134)	0.0160

^a Range of observed values (A. M. Tritscher, unpublished results) is given in parentheses.

^b Range of observed values (Tritscher *et al.*, 1992) is given in parentheses.

data of Tritscher *et al.* (1992). Hill exponents of 1.0 for induction of CYP1A1 (Fig. 5) and CYP1A2 (Fig. 6) reproduced the observed responses. Because binding of TCDD to

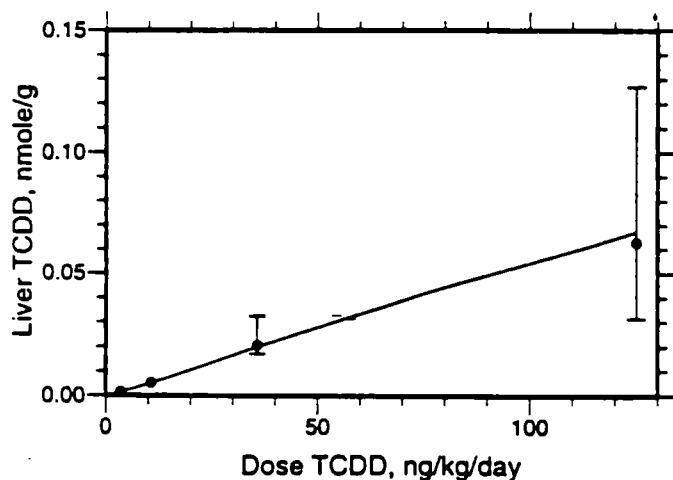


FIG. 4. Computed relationship between liver TCDD concentration and administered dose. Experimental data points (filled circles) are the average values of Tritscher *et al.* (1992). Error bars denote the range of observed values.

TABLE 2
Fit of Model to Observed TCDD Concentrations Following Repeated Doses^a

	Dose, ng/kg		
	10	100	1000
Dosing 5 days/week for 1 week			
Liver	0.000898 (0.0)	0.0141 (0.00870–0.0155)	0.147 (0.143–0.165)
Fat	0.0004063 (0.0)	0.00302 (0.000932–0.00466)	0.0249 (0.0217–0.0404)
Dosing 5 days/week for 3 weeks			
Liver	0.00235 (0.00217–0.00280)	0.0373 (0.0298–0.0435)	0.302 (0.227–0.457)
Fat	0.00108 (0.000932)	0.00726 (0.0059–0.0109)	0.0586 (0.0497–0.0963)
Dosing 5 days/week for 7 weeks			
Liver	0.00408 (0.00342–0.00652)	0.0505 (0.0519–0.0711)	0.329 (0.471–0.796)
Fat	0.00170 (0.000620–0.00124)	0.0107 (0.0118–0.0161)	0.0766 (0.0767–0.305)

^a Concentrations in nmol/g tissue; observed values (Rose *et al.*, 1976) are in parentheses.

the Ah receptor is the initial step in the induction of cytochrome P450 isozymes, the fractional occupancy of the receptor will have a strong effect on the predictions of the model. As fractional occupancy is dramatically affected by cooperativity in ligand binding, estimation of the Hill exponent for this binding was included in the course of estimating the gene induction parameters for CYP1A1 and CYP1A2. The optimal value of the exponent was 0.96, indicating a lack of cooperativity (i.e., hyperbolic binding). This value agrees with the estimate of 1.0 obtained by Gaisiewicz (1984) and by Bradfield *et al.* (1988).

Table 4 compares the NIEHS model's predictions to the observed terminal concentrations of the Ah receptor and of the induced P450 isozymes. The model predicts that the fractional occupancy of the Ah receptor by TCDD (not included in Table 4) rises from 13.4% at a dose of 3.5 ng TCDD/kg/day to 69.3% at 125 ng/kg/day. The liver concentration of CYP1A1 is 0.02 nmole/g in the absence of administered TCDD (Tritscher *et al.*, 1992), consistent with (but not proof of) the notion of an endogenous ligand or a ligand of dietary origin for the Ah receptor. A liver concentration of 1.5 pM (in TCDD equivalent units) of this ligand reproduces the observed basal level of CYP1A1. Needham *et al.* (1991) detected a background level of TCDD of 2 pM in human livers.

Table 5 gives the predicted concentration of interstitial TGF- α after 217 days of exposure to TCDD and the conse-

TABLE 3
Fit of Model to Observed TCDD Concentrations Following
Single Doses^a

Dose, ng/kg	Liver, nmol/g	Fat, nmol/g
22 days following gavage ^b		
1000	0.0114 (0.00869–0.0145)	0.00389 (0.0010–0.0222)
4 days following gavage ^c		
0.25	0.00000366 (0.0000035)	
0.5	0.00000734 (0.0000035)	
1	0.0000147 (0.0000078)	
10	0.000155 (0.000066)	
100	0.00198 (0.0011)	
7 days following subcutaneous injection ^d		
1	0.0000119 (0.0000096)	0.0000101 (not detected)
3	0.0000365 (0.000032)	0.0000302 (0.000043)
10	0.000129 (0.000126)	0.0000995 (0.000153)
30	0.000456 (0.000503)	0.000290 (0.000432)
100	0.00218 (0.00217)	0.000885 (0.00104)
300	0.00928 (0.0105)	0.00234 (0.0025)
1000	0.0377 (0.0332)	0.00699 (0.0063)
3000	0.116 (0.087)	0.0201 (0.0114)

^a Observed values are in parentheses.

^b Rose *et al.* (1976).

^c J. Vanden Heuvel, NIEHS, personal communication.

^d Abraham *et al.* (1988).

quent distribution of the EGF receptor between the plasma membrane and cytosol. Figure 7 gives the decline in EGF binding capacity of the plasma membranes with the dose of TCDD and corresponds to that fraction of the receptor which had not been internalized consequent to binding of TGF- α . Figure 8 relates the predicted degree of internalization of the EGF receptor to the TGF- α concentration. The points on this curve corresponding to the predicted responses for the four dose groups of Sewall *et al.* (1993) are also shown. Note that the TGF- α concentration is given in pmol/g liver; multiplying by 10 gives the concentration in the interstitial fluid in nanomolar. These interstitial concentrations are comparable to the concentrations produced by TCDD in the extracellular fluid of keratinocyte cultures exposed to TCDD (Choi *et al.*, 1991). These results were

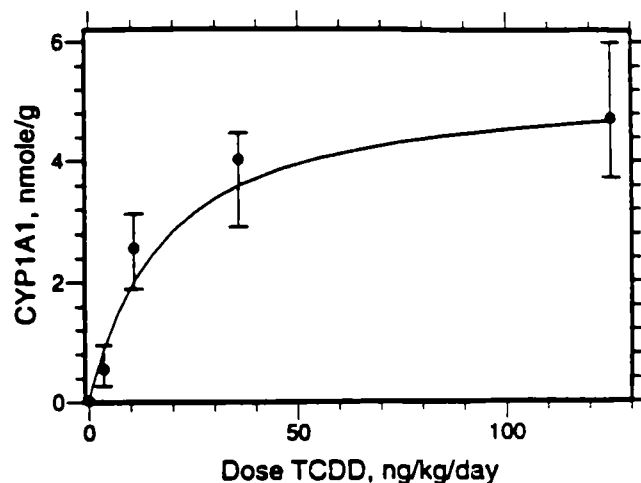


FIG. 5. Induced CYP1A1 concentration vs administered dose of TCDD. Experimental data points (filled circles) are the average values of Tritscher *et al.* (1992). Error bars denote the range of observed values.

obtained with a model which required prior binding of the ER-E complex to activate binding of the Ah-TCDD complex to the TGF- α gene. An alternative model which postulates random-order binding of the ER-E and Ah-TCDD complexes produced quantitatively similar results.

The NIEHS model predicts that 10 days after administration of a single dose of 1 μ g TCDD/kg there should be a 20.1% decrease in the B_{max} of the plasma membrane EGF receptor in female rat livers (14% observed; Sunahara *et al.*, 1989), but only a 9.6% decrease in male rat livers (10% observed on average; Madhukar *et al.*, 1984). This computed twofold greater responsiveness of the female over the male persisted over all doses in the range of 0.1 to 10 μ g/kg.

As estrogen plays a major role in regulating TCDD's effects on the EGF receptor, estrogen action was examined in

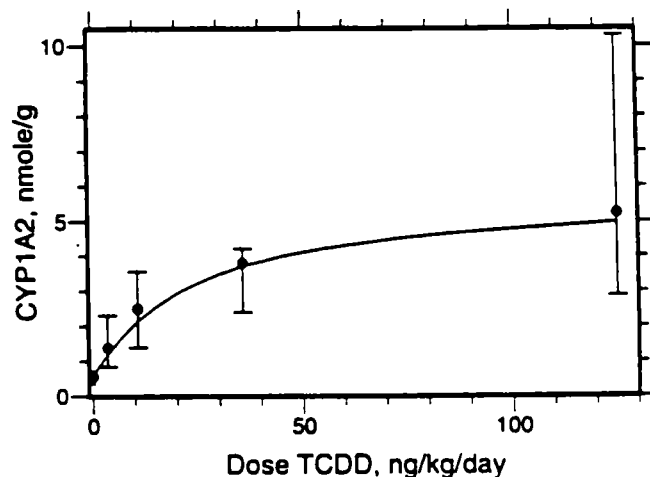


FIG. 6. Induced CYP1A2 concentration vs administered dose of TCDD. Experimental data points (filled circles) are the average values of Tritscher *et al.* (1992). Error bars denote the range of observed values.

TABLE 4
Ah Receptor and Induced Cytochrome P450 Isozymes

Dose, ng/kg/day	Ah receptor, pmol/g	CYP1A1, nmol/g ^a	CYP1A2, nmol/g ^a
0.0	2.09 (2.1) ^b (2.1–3.2) ^c	0.0234 (0.0081–0.0351)	0.557 (0.352–0.714)
0.01	2.10	0.0271	0.561
0.1	2.14	0.0591	0.583
0.3	2.23	0.126	0.628
1.0	2.45	0.328	0.771
3.5	3.21	0.881 (0.269–0.953)	1.190 (0.840–2.31)
7.0	4.01	1.501	1.699
10.7	4.68 (3.0–5.7) ^c	2.011 (1.89–3.13)	2.145 (1.39–3.56)
15.0	5.25	2.443	2.544
20.0	5.77	2.841	2.927
25.0	6.16	3.135	3.224
30.0	6.50 (5.5–7.8) ^c	3.377	3.476
35.7	6.80	3.589 (2.91–4.47)	3.704 (2.39–4.21)
45.0	7.16	3.845	3.990
55.0	7.43	4.038	4.211
65.0	7.63	4.183	4.380
75.0	7.82	4.314	4.537
85.0	7.95	4.409	4.652
100.0	8.11 (4.9–10.5) ^c	4.519	4.785
115.0	8.24	4.612	4.899
125.0	8.32 (8.5) ^d	4.672 (3.72–5.99)	4.975 (2.86–10.3)

^a Experimental data from Tritscher *et al.* (1992) are given in parentheses.

^b Poland and Knutson (1982).

^c Sloop and Lucier (1987).

^d Maximal induction from Poland and Knutson (1982).

the model. Chronic exposure to TCDD was found to reduce the concentration of the hepatic estrogen receptor in DEN-initiated female rats from 5.1 to 2.3 pmol/g (Clark *et al.*, 1991). The model reproduces this decrease in the estrogen receptor level (Table 6). It also predicts that the reduction is dependent on the dose of TCDD with a curve shape similar to that obtained for mouse liver by Lin *et al.* (1991b).

The calculated liver concentrations of estradiol and catechol estrogen and the calculated rate of the estradiol 2-hydroxylase activity of CYP1A2 at various doses are given in Table 6. At a dose of 125 ng TCDD/kg/day the calculated rate of the estradiol 2-hydroxylase is 3.1-fold greater than in the absence of administered TCDD whereas CYP1A2 is computed to increase 8.9-fold over its basal level. The NIEHS model predicts that only 44.9% of the CYP1A2 is complexed with reductase in the absence of administered TCDD. This percentage decreases slightly as more enzyme

TABLE 5
Effects of TCDD on the EGF Receptor

Dose, ng/kg/day	EGF receptor, pmol/g ^a	TGF- α , pmol/g	Internalized EGF receptor, pmol/g
0.0	2.553 (2.06–2.61)	0.0	0.0
0.01	2.553	0.00000995	0.000849
0.1	2.545	0.0000999	0.00854
0.3	2.53	0.000291	0.0246
1.0	2.48	0.000901	0.0747
3.5	2.34 (1.45–2.95)	0.00280	0.219
7.0	2.16	0.00540	0.391
10.7	2.01 (1.18–2.45)	0.00806	0.541
15.0	1.88	0.0107	0.674
20.0	1.75	0.0138	0.807
25.0	1.65	0.0165	1.00
30.0	1.55	0.0194	
35.7	1.46 (0.899–1.64)	0.0222	1.09
45.0	1.36	0.0263	1.20
55.0	1.28	0.0299	1.28
65.0	1.22	0.0326	1.34
75.0	1.15	0.0361	1.40
85.0	1.11	0.0386	1.44
100.0	1.07	0.0429	1.49
115.0	1.02	0.0446	1.53
125.0	0.990 (0.702–1.13)	0.0470	1.56

^a Experimental data from Sewall *et al.* (1993) are given in parentheses.

is induced; at a dose of 125 ng/kg/day, 39.8% of CYP1A2 is calculated to be complexed with reductase. The lack of stoichiometric amounts of bound reductase is consistent with

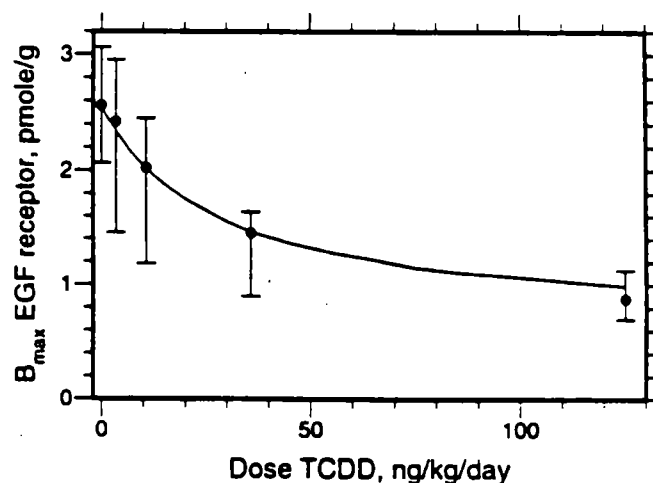


FIG. 7. Reduction of B_{max} of EGF receptor vs administered dose of TCDD. Experimental data points (filled circles) are the average values of Sewall *et al.* (1993). Error bars denote the range of observed values.

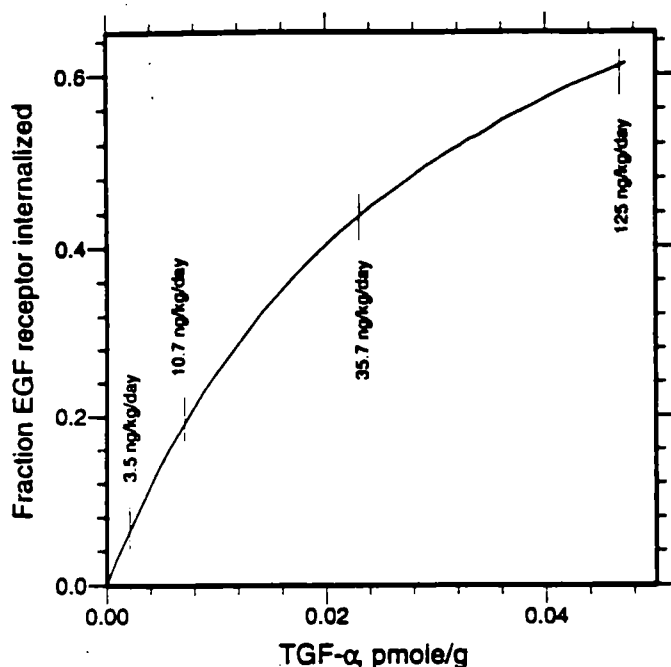


FIG. 8. Calculated fraction of EGF receptor redistributed from the plasma membrane into the cytosol as a function of the concentration of TGF- α . Vertical bars indicate model's predictions for the four dose groups of Sewall *et al.* (1993).

the conclusion of Graham *et al.* (1988) that P450 reductase is limiting for the hydroxylation of estradiol. However, at a dose of 125 ng/kg/day, the estradiol concentration in the liver is computed to fall to 39% of its level in the absence of administered TCDD. As the computed estradiol concentration is below its K_m for this enzyme, the enzymatic rate is probably limited more by depletion of substrate than by availability of reductase.

DISCUSSION

The purpose of this modeling effort was to suggest plausible biochemical mechanisms which may explain the observed tissue effects of TCDD in female rats. Internalization of the EGF receptor in response to induction of TGF- α is a possible origin for the mitogenic signal important to carcinogenesis. The NIEHS model's prediction of TGF- α concentrations that are comparable to those observed in cell cultures is consistent with this mechanism, and the model's success in reproducing observed responses to TCDD supports the proposed mechanism. However, other EGF-like peptides may be involved although we have treated TGF- α as the nominal ligand for our modeling. In addition, the production of a considerable quantity of catechol estrogens may play a role in carcinogenesis (Li *et al.*, 1985) either by covalent binding to protein or DNA (Tsibris and McGuire, 1977) or by generation of active oxygen spe-

cies (Cerutti, 1978). The proliferative effect of EGF receptor-mediated events could also provide genetically altered cells with a selective growth advantage. This mechanism is consistent with the general agreement that TCDD is a tumor promoter, but not an initiator, in two-stage models for hepatocarcinogenesis (Kociba *et al.*, 1978; Pitot and Sirica, 1980; Lucier *et al.*, 1991) and a complete carcinogen in the 2-year chronic animal bioassay (Huff *et al.*, 1991).

EGF Receptor and Cellular Proliferation

Figure 7 shows the predicted and observed responses of the maximal binding of EGF to liver plasma membranes vs dose of TCDD. Although the liver TCDD concentration is linear in the administered dose over the range 3.5–125 ng/kg/day, the response of the EGF receptor resembles a hyperbolic curve. As this response may be involved in the mechanism of tumorigenesis in TCDD-treated rats, it would be expected that it would correlate with tumor incidence better than does tissue dose. If this is true, linear extrapolation of effects at high doses to low doses by a line that passes through the background incidence would underestimate low-dose effects. However, such hyperbolic curves are approximately linear in the low-dose range, indi-

TABLE 6
Effects of TCDD on Estrogen Metabolism in Liver

Dose, ng/kg/day	Estrogen receptor, pmol/g ^a	Estradiol, pmol/g	Catechol estrogen, pmol/g	Estradiol 2-hydroxylase, nmol/g/day
0.0	5.19 (5.1)	0.154	0.610	0.0347
0.01	5.19	0.154	0.613	0.0348
0.1	5.17	0.153	0.633	0.0359
0.3	5.12	0.151	0.672	0.0381
1.0	4.98	0.145	0.785	0.0446
3.5	4.59	0.130	1.067	0.0605
7.0	4.18	0.114	1.336	0.0757
10.7	3.85	0.103	1.517	0.0860
15.0	3.58	0.0962	1.641	0.0931
20.0	3.33	0.0878	1.734	0.0984
25.0	3.16	0.0828	1.792	0.102
30.0	3.01	0.0792	1.828	0.104
35.7	2.88	0.0760	1.854	0.105
45.0	2.73	0.0722	1.877	0.106
55.0	2.62	0.0692	1.888	0.107
65.0	2.54	0.0670	1.893	0.107
75.0	2.46	0.0653	1.896	0.107
85.0	2.41	0.0638	1.895	0.107
100.0	2.35	0.0642	1.891	0.107
115.0	2.30	0.0610	1.889	0.107
125.0	2.26 (2.3)	0.0601	1.888	0.107

^a Experimental data of Clark *et al.* (1991) are given in parentheses.

cating that linear extrapolation from low doses to extremely low doses should still be valid.

Because the interstitial space occupies about 10% of the liver volume, the accumulated TGF- α is 10 times more concentrated in the interstitium than it would be in the cytosol. This concentration effect has significant consequences for the predicted reduction in the $B_{\max}$ of the EGF receptor. If the TGF- α were actually produced in some other tissue, it would have to be transported to the liver interstitium via the blood. This would greatly dilute the concentration of the peptide and require synthesis of 15 times as much material to result in the observed decrease in EGF-binding capacity of the plasma membrane.

Although production of TGF- α by the liver was modeled as being induced by the Ah-TCDD complex, there is evidence (Gaido *et al.*, 1992) for post-transcriptional regulation as well. TCDD may act to "stabilize" the mRNA for that peptide. If information regarding the mechanism of such an effect were available, it could readily be incorporated into this model. As it is, the model merely states that TCDD stimulates the rate-limiting step in the net synthesis of hepatic TGF- α .

Under normal conditions, one of the functions of TGF- α is as a signal for regenerative hyperplasia of injured tissue (Burgess, 1989; Mead and Fausto, 1989). The substrates for the internalized EGF receptor's tyrosine kinase may be involved in modulating this process, and their enzymatic activities may be regulated by phosphorylation. According to the NIEHS model, TCDD causes inappropriate stimulation of this process. Cells which had previously sustained nonlethal DNA damage could be stimulated to reproduce before the damage could be repaired. With the damage fixed in the genome, clonal expansion of the transformed cells could provide a necessary step in tumor development.

Different populations of cells may differ in the sensitivity of their proliferative responses to TCDD. The small population of polyploid cells in the livers of rats treated with DEN and ethinylestradiol are especially rich in EGF receptors (Vickers and Lucier, 1991). It is possible that these cells would show a correspondingly larger concentration of internalized EGF receptors in response to induction of TGF- α and, hence, a stronger mitogenic signal. If the population of polyploid cells includes preneoplastic cells, proliferation of these previously transformed cells may represent the most important effect of TCDD in hepatocarcinogenesis.

The NIEHS model predicts that after 31 weeks of treatment, as much EGF receptor is internalized in the male at a dose of 125 ng TCDD/kg/day as is internalized in the female at a dose of 15 ng/kg/day. This behavior is consistent with the observed lower sensitivity of the male (Madhukar *et al.*, 1984). It arises because serum estradiol in the male is about 10% of that in the female (D. Schomberg, Duke University, personal communication), resulting in concentrations of the ER-E complex which only slightly stimulates

production of TGF- α . Because the male rat is predicted to produce far less TGF- α than the female, the rate of cellular proliferation in the male at low doses of TCDD should be indistinguishable from that at zero dose. This prediction of the model could explain the absence of excess liver tumors in male rats exposed to TCDD (Kociba *et al.*, 1978; National Toxicology Program, 1982).

Cytochrome P450 and Estrogen Metabolism

As estrogen is necessary for TCDD-mediated internalization of the EGF receptor, induction of CYP1A2 may also be an important index of cancer risk because this cytochrome catalyzes oxidation of estradiol to catechol estrogen. The predicted response curve for this indicator (Fig. 6) is hyperbolic. This behavior is attributable to the Hill coefficient of 1 determined for induction of this protein.

The finding of at least four enhancer sequences for induction of CYP1A1 (Fisher *et al.*, 1990) suggests the possibility that several enhancer sites must be occupied to enable transcriptional activation of the CYP1A1 gene. Such a mechanism could lead to sigmoidal response curves for the induction of this cytochrome. The Hill exponent of 1, which gave the best fit to the data of Tritscher *et al.* (1992), yields a hyperbolic response curve (Fig. 5). Because many factors other than cooperative interactions among binding sites (e.g., heterogeneous uptake of TCDD in the liver and recycling of TCDD among Ah receptor molecules) could influence the shape of the response curve, the value of the Hill exponent is not necessarily related to the number of binding sites for the Ah-TCDD complex.

Using steady-state kinetics and a simpler model, Portier *et al.* (1993) estimated Hill exponents of 1.86 for CYP1A1 induction and 0.534 for CYP1A2 induction. A possible origin of the difference in Hill exponents is the steady state model's neglect of the increase in the concentration of the Ah receptor with increasing dose of TCDD. The maximal slope of the curve for induction rate vs inducer concentration is steeper for cooperative kinetics (Hill exponents not equal to 1) than for hyperbolic kinetics. The increase in the concentration of the Ah receptor with dose can mimic the steeper rise of rate of synthesis with dose exhibited by cooperative kinetics.

Portier *et al.* (1993) showed the importance of the mechanism by which putative alternative ligands bind to the Ah receptor and/or modulate protein synthesis. In the NIEHS model, constitutive expression of all proteins except CYP1A1 was assumed to be independent of the Ah receptor. Background CYP1A1 levels were treated as being produced by an additional Ah receptor-dependent mechanism. An "independent" mechanism accentuates any sigmoidicity in the response curve. An "additive" mechanism minimizes such sigmoidicity and leads to linearity in the low-dose response. Such an effect may be involved in producing

the hyperbolic response of CYP1A1 (Fig. 5). It is also possible that CYP1A1 is constitutively expressed at a low rate by a mechanism that does not involve the Ah receptor. Additional experiments on the mechanism of expression of CYP1A1 in the absence of administered TCDD could resolve this issue.

As catechol estrogens bind more weakly to the estrogen receptor than does estradiol, it is conceivable that their production from estradiol by the estradiol 2-hydroxylase activity of CYP1A2 could result in reduced ligation of the estrogen receptor. Because it was assumed that the ER-E complex induces synthesis of the estrogen receptor protein, a reduced calculated rate of synthesis of the receptor could result from the enzymatic activity of CYP1A2. An alternative model, in which the ER-E complex was a transcriptional activator of the estrogen receptor gene, but the Ah-TCDD complex was not a transcriptional inhibitor of the gene, predicted only an 18.9% decrease in the estrogen receptor concentration. As a 55% reduction in the estrogen receptor concentration was observed (Clark *et al.*, 1991), an Ah receptor-mediated effect of TCDD on expression of the estrogen receptor such as that incorporated into the NIEHS model is likely. This result is consistent with the observation (Lin *et al.*, 1991b) of only a 10% decrease in receptor level in Ah-deficient mice.

Serum estradiol in the female rat oscillates from 15 to 120 pg/ml over a 5-day estrus cycle. Including such oscillations in the NIEHS model could make as much as a 12% difference in the model's predictions, depending on where in the cycle the simulation was begun. The serum levels, 50–60 pg/ml, reported by Shiverick and Muther (1983) are close to the average level over the entire cycle, and the experimental data are the average results for nine individuals. Therefore, use in the model of Shiverick and Muther's value for the circulating estradiol concentration is justified.

TCDD Tissue Dose

The LPMA model predicts a liver TCDD content 2.4-fold higher than observed at a dose of 125 ng/kg/day (Tritscher *et al.*, 1992). The LPMA model assumes that TCDD binds to total CYP1A2 with a K_d of 7 nM, and it predicts a liver CYP1A2 concentration 60% higher than

observed at a dose of 125 ng/kg/day (Tritscher *et al.*, 1992). The resulting large calculated amount of bound TCDD is most likely responsible for the overprediction of liver TCDD.

Although the NIEHS model accurately reproduces the observed CYP1A2 concentration, much less binding had to be assumed in order to reproduce the experimental data. An alternative model in which TCDD is bound nonspecifically to an unspecified protein with K_d between 5 and 8 nM required only about 0.2 nmol/g liver of that protein to reproduce the observed liver TCDD content. Setting the binding protein concentration to values comparable to that of CYP1A2 necessitated a higher rate constant for clearance of TCDD from the liver in order to match the experimental data and resulted in a half time in the liver of only 7 days, half the experimentally observed value.

Livers of TCDD-treated rats often show an increase in lipid content. From the data of Tritscher *et al.* (1992) the relationship (fraction lipid) = $0.01865 \times (\text{nmol total liver TCDD}) + 0.1443 \times (\text{kg body weight})$ was obtained. Allowing the TCDD to partition between the aqueous phase of the liver cytosol and lipid droplets with partition coefficients between 140 and 350 did not sequester enough TCDD to match the data without also requiring induction of TCDD-binding protein.

The dose-response relationships illustrated by the NIEHS model are important for understanding the risks of adverse health effects following exposure to TCDD. Sigmoidicity in the response requires a higher concentration to produce a given response at low dose than does hyperbolic response exhibiting the same concentration for half-maximal effect. Such behavior can have dramatic consequences for the estimated minimum exposure to TCDD that should produce an unacceptable risk of adverse health effects (Portier *et al.*, 1993). All of the amounts of protein calculated in this model show hyperbolic dependence on dose. The fact that the model presented here does not predict sigmoidicity in the observed responses indicates that the response is approximately linear at very low doses. The model's success in reproducing these responses in a biochemically realistic way suggests that it should also be useful in the analysis of more complex dose-response relationships, such as for cellular proliferation rates and tumor incidence.

APPENDIX 1: MODEL EQUATIONS

Compartment and Flow Characteristics

CardiacOutput	= $404 \times \text{BodyWeight}^{0.75}$ (Delp <i>et al.</i> , 1991)
StartWeight	= 0.237 kg (Tritscher <i>et al.</i> , 1992)
BodyWeight	= $\text{StartWeight} + 0.219674 \times e^{-0.002839 \times \text{dose} \times \text{time}} / (116.345 + \text{time})$ (BodyWeight was estimated from the data of Tritscher <i>et al.</i> (1992) as a function of dose and time by nonlinear regression)
BloodVolume	= $0.054 \times \text{BodyWeight}$ (Delp <i>et al.</i> , 1991)

APPENDIX 1: Continued

FatVolume	= $0.08 \times \text{BodyWeight}$ (average of literature values: Leung <i>et al.</i> , 1990; Delp <i>et al.</i> , 1991)
MuscleVolume	= $0.59 \times \text{BodyWeight}$ (Delp <i>et al.</i> , 1991) (this compartment also includes skin and other slowly perfused tissues)
VisceraVolume	= $0.083 \times \text{BodyWeight}$ (Delp <i>et al.</i> , 1991) (this compartment includes all richly perfused tissues)
LiverVolume	= $\text{BodyWeight} \times (0.03324 + 0.01927 \times \text{dose}/(88.91 + \text{dose}))$ (LiverVolume was estimated from the data of Tritscher <i>et al.</i> (1992) as a function of dose by nonlinear regression)
LiverInterstitialVolume	= $\text{frac\_interstitium} \times \text{LiverVolume}$
LiverFlow	= $0.15 \times \text{CardiacOutput}$ (Delp <i>et al.</i> , 1991)
FatFlow	= $0.07 \times \text{CardiacOutput}$ (Delp <i>et al.</i> , 1991)
MuscleFlow	= $0.36 \times \text{CardiacOutput}$ (Delp <i>et al.</i> , 1991)
VisceraFlow	= $0.40 \times \text{CardiacOutput}$ (Delp <i>et al.</i> , 1991)
<i>Distribution of TCDD</i>	
GutTCDD	= $\text{GutTCDD} + \text{dose}(t) \times \text{BodyWeight}/322$ (doses at time t —in ng/kg—were introduced into the gut compartment according to the experimental schedule; $\text{dose}(t) = 0$ if no dose is scheduled at time t)
GutTCDD'	= $-k\_absorption \times \text{GutTCDD}/\text{BodyWeight}^{0.75} - k\_feces \times \text{GutTCDD}$
FecesTCDD'	= $k\_feces \times \text{GutTCDD}$
cum_exposure'	= LiverTCDD
BloodProtein	= $\text{MaxBloodProtein} \times \text{LiverVolume} \times K\_BloodProtein/(K\_BloodProtein + \text{LiverTCDD}/\text{LiverVolume})$
BloodTCDD'	= $k\_absorption \times \text{GutTCDD}/\text{BodyWeight}^{0.75} - \text{BloodTCDD} \times \text{MuscleDiffusion} \times \text{MuscleFlow}/\text{BloodVolume} - \text{BloodTCDD} \times \text{FatDiffusion} \times \text{FatFlow}/\text{BloodVolume} - \text{BloodTCDD} \times \text{VisceraDiffusion} \times \text{VisceraFlow}/\text{BloodVolume} - \text{BloodTCDD} \times \text{LiverDiffusion} \times \text{LiverFlow}/\text{BloodVolume} + \text{MuscleTCDD} \times \text{MuscleDiffusion} \times \text{MuscleFlow}/(\text{MuscleVolume} \times \text{MusclePartition}) + \text{FatTCDD} \times \text{FatDiffusion} \times \text{FatFlow}/(\text{FatVolume} \times \text{FatPartition}) + \text{VisceraTCDD} \times \text{VisceraDiffusion} \times \text{VisceraFlow}/(\text{VisceraVolume} \times \text{VisceraPartition}) + \text{LiverTCDD} \times \text{LiverDiffusion} \times \text{LiverFlow}/(\text{LiverVolume} \times \text{LiverPartition}) - k\_binding \times \text{BloodTCDD} \times \text{BloodProtein} + k\_binding \times K\_BloodProtein \times \text{BloodVolume} \times \text{BloodBoundTCDD}$
BloodBoundTCDD'	= $k\_binding \times \text{BloodTCDD} \times \text{BloodProtein} - k\_binding \times K\_BloodProtein \times \text{BloodVolume} \times \text{BloodBoundTCDD}$
MuscleTCDD'	= $\text{BloodTCDD} \times \text{MuscleDiffusion} \times \text{MuscleFlow}/\text{BloodVolume} - \text{MuscleTCDD} \times \text{MuscleDiffusion} \times \text{MuscleFlow}/(\text{MuscleVolume} \times \text{MusclePartition})$
FatTCDD'	= $\text{BloodTCDD} \times \text{FatDiffusion} \times \text{FatFlow}/\text{BloodVolume} - \text{FatTCDD} \times \text{FatDiffusion} \times \text{FatFlow}/(\text{FatVolume} \times \text{FatPartition})$
VisceraTCDD'	= $\text{BloodTCDD} \times \text{VisceraDiffusion} \times \text{VisceraFlow}/\text{BloodVolume} - \text{VisceraTCDD} \times \text{VisceraDiffusion} \times \text{VisceraFlow}/(\text{VisceraVolume} \times \text{VisceraPartition})$
LiverTCDD'	= $\text{BloodTCDD} \times \text{LiverDiffusion} \times \text{LiverFlow}/\text{BloodVolume} - \text{LiverTCDD} \times \text{LiverDiffusion} \times \text{LiverFlow}/(\text{LiverVolume} \times \text{LiverPartition}) - k\_metabolism \times \text{LiverTCDD}/\text{BodyWeight}^{0.3} - k\_binding \times \text{LiverTCDD} \times \text{LiverCYP1A2} + k\_binding \times K\_TCDD \times \text{LiverVolume} \times \text{LiverCYP1A2} - \text{TCDD} - k\_binding \times \text{LiverTCDD} \times \text{LiverAhReceptor} + k\_binding \times K\_AhR \times \text{LiverVolume} \times \text{LiverAhR} - \text{TCDD} + k\_proteolysis \times (\text{LiverAhR} - \text{TCDD} + \text{LiverCYP1A2} - \text{TCDD}) - k\_lysis \times \text{cum\_exposure}/(\text{crit\_exposure} + \text{cum\_exposure}) \times \text{LiverTCDD}$
LiverCYP1A2_TCDD'	= $k\_binding \times \text{LiverTCDD} \times \text{LiverCYP1A2} - k\_binding \times K\_TCDD \times \text{LiverVolume} \times \text{LiverCYP1A2} - \text{TCDD}$
<i>TCDD Metabolite Clearance</i>	
LiverMetabolite'	= $k\_metabolism \times \text{LiverTCDD}/\text{BodyWeight}^{0.3} - k\_bile \times \text{LiverMetabolite} + \text{BloodMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow}/\text{BloodVolume} - \text{LiverMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow}/(\text{LiverVolume} \times \text{LiverPartition})$

APPENDIX 1: Continued

BloodMetabolite'	= -BloodMetabolite × LiverDiffusion × LiverFlow/BloodVolume + LiverMetabolite × LiverDiffusion × LiverFlow/(LiverVolume × LiverPartition) - k _{_urine} × BloodMetabolite
UrineMetabolite'	= k _{_urine} × BloodMetabolite
GutMetabolite'	= k _{_bile} × LiverMetabolite - k _{_feces} × GutMetabolite
FecesMetabolite'	= k _{_feces} × GutMetabolite

Ah Receptor and P450 Induction

LiverAhR _ TCDD'	= k _{_binding} × LiverTCDD × LiverAhReceptor - k _{_binding} × K _{_AhR} × LiverVolume × LiverAhR _ TCDD - k _{_proteolysis} × LiverAhR _ TCDD
LiverAhR _ Inducer'	= k _{_binding} × LiverVolume × Natural _ Inducer × LiverAhReceptor - k _{_binding} × K _{_AhR} × LiverVolume × LiverAhR _ Inducer - k _{_proteolysis} × LiverAhR _ Inducer
LiverAhReceptor'	= V _{_AhRinduction} × LiverVolume/(1 + (K _{_AhRinduction} × LiverVolume)/LiverAhR _ TCDD(t - lt)) + AhRexpression × LiverVolume - k _{_binding} × LiverTCDD × LiverAhReceptor + k _{_binding} × K _{_AhR} × LiverVolume × LiverAhR _ TCDD - k _{_binding} × LiverVolume × Natural _ Inducer × LiverAhReceptor + k _{_binding} × K _{_AhR} × LiverVolume × LiverAhR _ Inducer - k _{_proteolysis} × LiverAhReceptor
LiverCYP1A1'	= V _{_CYP1A1induction} × LiverVolume/(1 + (K _{_CYP1A1induction} × LiverVolume)/(LiverAhR _ TCDD(t - lt) + LiverAhR _ Inducer(t - lt))) - k _{_proteolysis} × LiverCYP1A1 - k _{_binding} × LiverCYP1A1 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A1 _ R
LiverP450Reductase'	= P450Red _ expression × LiverVolume - k _{_proteolysis} × LiverP450Reductase - k _{_binding} × LiverCYP1A1 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A1 _ R - k _{_binding} × LiverCYP1A2 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R - k _{_binding} × LiverCYP1A2 _ E2 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R _ E2
LiverCYP1A1 _ R'	= k _{_binding} × LiverCYP1A1 × LiverP450Reductase - k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A1 _ R

Estradiol Metabolism

LiverE2'	= ConcBloodE2 × LiverFlow - LiverE2 × LiverFlow/LiverVolume - k _{_binding} × LiverCYP1A2 × LiverE2 + k _{_binding} × K _{_E2} × LiverVolume × LiverCYP1A2 _ E2 - k _{_binding} × LiverCYP1A2 _ R × LiverE2 + k _{_binding} × K _{_E2} × LiverVolume × LiverCYP1A2 _ R _ E2 + k _{_proteolysis} × LiverER _ E2 - k _{_binding} × LiverEReceptor × LiverE2 + k _{_binding} × K _{_ER} _ E2 × LiverVolume × LiverER _ E2
LiverCYP1A2'	= V _{_CYP1A2induction} × LiverVolume/(1 + (K _{_CYP1A2induction} × LiverVolume)/(LiverAhR _ TCDD(t - lt) + CYP1A2expression × LiverVolume - k _{_proteolysis} × (LiverCYP1A2 + LiverCYP1A2 _ E2) - k _{_binding} × LiverCYP1A2 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R - k _{_binding} × LiverCYP1A2 × LiverE2 + k _{_binding} × K _{_E2} × LiverVolume × LiverCYP1A2 _ E2
LiverCYP1A2 _ R'	= k _{_binding} × LiverCYP1A2 × LiverP450Reductase - k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R + V _{_E2H} × LiverCYP1A2 _ R _ E2
LiverCYP1A2 _ E2'	= k _{_binding} × LiverCYP1A2 × LiverE2 - k _{_binding} × K _{_E2} × LiverVolume × LiverCYP1A2 _ E2 - k _{_binding} × LiverCYP1A2 _ E2 × LiverP450Reductase + k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R _ E2
LiverCYP1A2 _ R _ E2'	= k _{_binding} × LiverCYP1A2 _ E2 × LiverP450Reductase - k _{_binding} × K _{_Reductase} × LiverVolume × LiverCYP1A2 _ R _ E2 + k _{_binding} × LiverCYP1A2 _ R × LiverE2 - k _{_binding} × K _{_E2} × LiverVolume × LiverCYP1A2 _ R _ E2 - V _{_E2H} × LiverCYP1A2 _ R _ E2
LiverE2OH'	= V _{_E2H} × LiverCYP1A2 _ R _ E2 - k _{_binding} × LiverEReceptor × LiverE2OH + k _{_binding} × K _{_ER} _ E2OH × LiverVolume × LiverER _ E2OH - k _{_conjugation} × LiverE2OH

APPENDIX 1: Continued

$$\begin{aligned}
 \text{LiverERReceptor}' &= V\_ERinduction \times \text{LiverVolume} / ((1 + K\_ERinduction \times \text{LiverVolume} / (\text{LiverER\_E2}(t - lt) + \text{LiverER\_E2OH}(t - lt))) \times (1 + \text{LiverAhR\_TCDD}(t - lt) / (K\_ERinduction \times \text{LiverVolume}))) + ERexpression \times \text{LiverVolume} - k\_proteolysis \times \text{LiverERReceptor} - k\_binding \times \text{LiverERReceptor} \times \text{LiverE2} + k\_binding \times K\_ER\_E2 \times \text{LiverVolume} \times \text{LiverER\_E2} - k\_binding \times \text{LiverERReceptor} \times \text{LiverE2OH} + k\_binding \times K\_ER\_E2OH \times \text{LiverVolume} \times \text{LiverER\_E2OH} \\
 \text{LiverER\_E2}' &= -k\_binding \times \text{LiverERReceptor} \times \text{LiverE2} + k\_binding \times K\_ER\_E2 \times \text{LiverVolume} \times \text{LiverER\_E2} - k\_proteolysis \times \text{LiverER\_E2} \\
 \text{LiverER\_E2OH}' &= -k\_binding \times \text{LiverERReceptor} \times \text{LiverE2OH} + k\_binding \times K\_ER\_E2OH \times \text{LiverVolume} \times \text{LiverER\_E2OH} - k\_proteolysis \times \text{LiverER\_E2OH}
 \end{aligned}$$

EGF Receptor and TGF- α

$$\begin{aligned}
 \text{LiverEGFReceptor}' &= EGFRexpression \times \text{LiverVolume} - k\_endocytosis \times \text{LiverEGFReceptor} \\
 \text{LiverTGF}' &= V\_TGFinduction \times \text{LiverVolume} / (1 + K\_TGFinduction \times \text{LiverVolume} / (\text{LiverAhR\_TCDD}(t - lt) \times (1 + K\_TGFinduction \times \text{LiverVolume} / (\text{LiverER\_E2}(t - lt) + \text{LiverER\_E2OH}(t - lt)))) - k\_proteolysis \times \text{LiverTGF} - k\_binding \times \text{LiverEGFReceptor} \times \text{LiverTGF} + k\_binding \times K\_TGF \times \text{LiverVolume} \times \text{LiverInternalEGFR} \\
 \text{LiverInternalEGFR}' &= k\_binding \times \text{LiverEGFReceptor} \times \text{LiverTGF} - k\_binding \times K\_TGF \times \text{LiverVolume} \times \text{LiverInternalEGFR} - k\_endocytosis \times \text{LiverInternalEGFR}
 \end{aligned}$$

Initial Conditions

$$\begin{aligned}
 \text{LiverAhReceptor} &= 0.017806 \text{ nmol (Sloop and Lucier, 1987)} \\
 \text{LiverAhR\_Inducer} &= 0.000674 \text{ nmol (determined from conservation of mass)} \\
 \text{LiverCYP1A1} &= 0.063174 \text{ nmol (Tritscher et al., 1992)} \\
 \text{LiverCYP1A1\_R} &= 0.094902 \text{ nmol (determined from conservation of mass)} \\
 \text{LiverCYP1A2} &= 2.4199 \text{ nmol (Tritscher et al., 1992)} \\
 \text{LiverCYP1A2\_R} &= 1.9799 \text{ nmol (determined from conservation of mass)} \\
 \text{LiverP450Reductase} &= 0.602536 \text{ nmol (Miwa et al., 1978)} \\
 \text{LiverE2} &= 0.00123 \text{ nmol (assumed at equilibrium with blood)} \\
 \text{LiverERReceptor} &= 0.01495 \text{ nmol (Clark et al., 1991)} \\
 \text{LiverER\_E2} &= 0.02325 \text{ nmol (determined from conservation of mass)} \\
 \text{LiverER\_E2OH} &= 0.0066792 \text{ nmol (determined from conservation of mass)} \\
 \text{LiverEGFReceptor} &= 0.022563 \text{ nmol (Sewall et al., 1993)} \\
 \text{(All other state variables start at zero)}
 \end{aligned}$$

APPENDIX 2

Physiological Parameters

$$\begin{aligned}
 \text{frac\_interstitium} &= 0.1 \\
 lt &= 0.25 \text{ day (Sloop and Lucier, 1987) (delay between binding of liganded receptor to DNA and appearance of induced proteins in the liver cell)} \\
 \text{ConcBloodE2} &= 0.185 \text{ nmol/liter (Shiverick and Muther, 1983)} \\
 \text{crit\_exposure} &= 0.3 \text{ nmol}
 \end{aligned}$$

Production of Blood Proteins

$$\begin{aligned}
 \text{MaxBloodProtein} &= 5 \text{ nmol/liter (determined from the data of Tritscher et al., 1992)} \\
 K\_BloodProtein &= 0.1 \text{ nM (determined from the data of Tritscher et al., 1992)} \\
 K\_BloodProtein &= 0.4 \text{ nM (determined from the data of Tritscher et al., 1992)}
 \end{aligned}$$

APPENDIX 2: Continued

Receptor Binding Constants

K_{AhR}	= 0.27 nM (apparent K_d in biological materials; Poland and Knutson, 1982)
K_{ER-E2}	= 0.13 nM (Vickers <i>et al.</i> , 1987)
$K_{ER-E2OH}$	= 1.3 nM (Li <i>et al.</i> , 1985)
K_{TGF}	= 0.28 nM (Hudson <i>et al.</i> , 1985)
K_{TCDD}	= 30 nM (Poland <i>et al.</i> , 1989; Voorman and Aust, 1989)

Constitutive Expression Rates

AhRexpression	= 1.4553 nmol/liter/day (determined from conservation of mass)
ERexpression	= 0.67273 nmol/liter/day (determined from conservation of mass)
CYP1A2expression	= 213.4 nmol/liter/day (determined from conservation of mass)
P450Reductase - expression	= 47.45 nmol/liter/day (determined from conservation of mass)
EGFRexpression	= 0.69376 nmol/liter/day (determined from conservation of mass)

Endogenous Ligand of Ah Receptor

Natural - Inducer	= 0.0015 nmol/liter (determined from the data of Tritscher <i>et al.</i> , 1992; assumed same K_d as for TCDD)

Gene Induction Parameters

$V_{AhRinduction}$	= 7.2 nmol/liter/day (Lower bound of 4 nmol/liter/day; Sloop and Lucier, 1987)
$K_{AhRinduction}$	= 4 nM (Sloop and Lucier, 1987)
$V_{CYP1A1induction}$	= 3319 nmol/liter/day (determined from the data of Tritscher <i>et al.</i> , 1992)
$K_{CYP1A1induction}$	= 4.279 nM (determined from the data of Tritscher <i>et al.</i> , 1992)
$V_{CYP1A2induction}$	= 4197 nmol/liter/day (determined from the data of Tritscher <i>et al.</i> , 1992)
$K_{CYP1A2induction}$	= 7.458 nM (determined from the data of Tritscher <i>et al.</i> , 1992)
$V_{ERinduction}$	= 3.2488 nmol/liter/day (Romkes <i>et al.</i> , 1987)
$K_{ERinduction}$	= 0.35 nM (determined from the data of Clark <i>et al.</i> , 1991)
$K_i_{ERinduction}$	= 3.1 nM (determined from the data of Clark <i>et al.</i> , 1991)
$V_{TGFinduction}$	= 1.5 nmol/liter/day (determined from the data of Sewall <i>et al.</i> , 1993)
$K_{TGFinduction}$	= 9 nM (determined from the data of Sewall <i>et al.</i> , 1993)
$K_a_{TGFinduction}$	= 0.75 nM (determined from the data of Sewall <i>et al.</i> , 1993)

Metabolic Parameters

V_{E2H}	= 8496 day ⁻¹ (Graham <i>et al.</i> , 1988)
K_{E2}	= 9400 nM (Voorman and Aust, 1987)
$K_{Reductase}$	= 83.5 nM (Miwa <i>et al.</i> , 1987)

Rate Constants

$k_{absorption}$	= 4.8 kg ^{0.73} /day (Leung <i>et al.</i> , 1990)
$k_{binding}$	= 10 ⁵ nmol ⁻¹ day ⁻¹ (value selected to ensure that binding reactions are always close to equilibrium)
$k_{proteolysis}$	= 0.693 day ⁻¹ (Lucier <i>et al.</i> , 1972)
$k_{endocytosis}$	= 0.271 day ⁻¹ (determined from the data of Sewall <i>et al.</i> , 1993)
$k_{metabolism}$	= 2.75 day ⁻¹
k_{urine}	= 5.36 day ⁻¹ (Birnbbaum <i>et al.</i> , 1980)
k_{bile}	= 3.81 day ⁻¹ (Birnbbaum <i>et al.</i> , 1980)
k_{feces}	= 1.152 day ⁻¹ (Lutz <i>et al.</i> , 1977)
$k_{conjugation}$	= 56.693 day ⁻¹ (Lucier and McDaniel, 1977)
k_{lysis}	= 9.5 day ⁻¹

APPENDIX 2: Continued

Partition and Diffusion Coefficients

FatPartition	= 350 (Leung <i>et al.</i> , 1990)
MusclePartition	= 40 (Leung <i>et al.</i> , 1990)
VisceraPartition	= 20 (Leung <i>et al.</i> , 1990)
LiverPartition	= 20 (Leung <i>et al.</i> , 1990)
FatDiffusion	= 0.1 (selected to fit the data of Abraham <i>et al.</i> , 1988)
MuscleDiffusion	= 0.5 (selected to fit the data of Abraham <i>et al.</i> , 1988)
VisceraDiffusion	= 0.5 (selected to fit the data of Abraham <i>et al.</i> , 1988)
LiverDiffusion	= 0.4 (selected to fit the data of Abraham <i>et al.</i> , 1988)

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Modeling Receptor-Mediated Processes with Dioxin: Implications for Pharmacokinetics and Risk Assessment

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Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD), a widespread polychlorinated aromatic hydrocarbon, caused tumors in the liver and other sites when administered chronically to rats at doses as low as 0.01 $\mu\text{g/kg/day}$. It functions in combination with a cellular protein, the *Ah* receptor, to alter gene regulation, and this resulting modulation of gene expression is believed to be obligatory for both dioxin toxicity and carcinogenicity. The U.S. EPA is reevaluating its dioxin risk assessment and, as part of this process, will be developing risk assessment approaches for chemicals, such as dioxin, whose toxicity is receptor-mediated. This paper describes a receptor-mediated physiologically based pharmacokinetic (PB-PK) model for the tissue distribution and enzyme-inducing properties of dioxin and discusses the potential role of these models in a biologically motivated risk assessment. In this model, ternary interactions among the *Ah* receptor, dioxin, and DNA binding sites lead to enhanced production of specific hepatic proteins. The model was used to examine the tissue disposition of dioxin and the induction of both a dioxin-binding protein (presumably, cytochrome P4501A2), and cytochrome P4501A1. Tumor promotion correlated more closely with predicted induction of P4501A1 than with induction of hepatic binding proteins. Although increased induction of these proteins is not expected to be causally related to tumor formation, these physiological dosimetry and gene-induction response models will be important for biologically motivated dioxin risk assessments in determining both target tissue dose of dioxin and gene products and in examining the relationship between these gene products and the cellular events more directly involved in tumor promotion.

KEY WORDS: PB-PK modeling; dioxin; gene regulation; cytochrome P450; risk assessment; pharmacokinetics; pharmacodynamics.

1. INTRODUCTION

Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD), a carcinogen^(1,2) and tumor promoter⁽³⁾ in ro-

dents, also causes immunotoxic,⁽⁴⁾ reproductive,⁽⁵⁾ and teratogenic⁽⁶⁾ effects. The present U.S. EPA cancer risk assessment, based on the incidence of liver tumors in female rats dosed with dioxin for 2 years,⁽¹⁾ utilizes the standard linearized multistage (LMS) approach, with a one in a million risk level, to derive a virtually safe dose of 6 femtograms dioxin/kg body weight per day.⁽⁷⁾ Other Western countries treat dioxin as a tumor promoter, with a threshold for its effects, and arrive at acceptable or tolerable daily intake values up to 1000-fold higher than that used by the United States.⁽⁸⁾

All of the toxic effects of dioxin noted above are believed to be dependent on the interaction of dioxin with a specific cellular protein, the *Ah* (aryl hydrocarbon) receptor. The *Ah* receptor-dioxin complex binds to

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specific sites on DNA modifying regulation of various genes, some of which can alter cell growth and differentiation, while others affect metabolic processes (Fig. 1). Due to the obligate role of the *Ah* receptor in its toxic effects, dioxin has been referred to as a "receptor-mediated" carcinogen. Dioxin is only one of many polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls that alter cell growth characteristics *via* interactions with the *Ah* receptor. The EPA is reviewing the current dioxin risk assessment with the stated intention of developing a generic, biologically realistic approach to risk assessment for these "receptor-mediated" agents.⁽⁹⁾ With improved knowledge of the biological basis of the action of dioxin at the molecular level, it may well prove possible to reconcile the present disparity among different countries.

Dioxin demonstrates dose-dependent kinetics: as the administered dose increases, a larger proportion of the total dose is found in the liver.⁽¹⁰⁾ Among the hepatic proteins induced by dioxin is a cytochrome, P4501A2, which has a high affinity for dioxin.^(11,12) It appears likely that dose-dependent hepatic sequestration is related to induction of P4501A2, but other proteins may also be involved. Any comprehensive model of dioxin pharmacokinetics must include the induction of these dioxin-binding proteins, mediated by the interaction of a dioxin-*Ah*-receptor complex with specific binding sites on DNA.

Leung and co-workers developed a physiologically based pharmacokinetic (PB-PK) model for dioxin and congeners in mice⁽¹³⁾ and rats.⁽¹⁴⁾ Their model included induction of a single hepatic binding species occurring in direct proportion to the fractional occupancy of the available *Ah* receptor by dioxin, but did not include direct interactions of the *Ah*-TCDD complex with DNA. In mice, dioxin pretreatment increased metabolic clearance of dioxin-like analogs.⁽¹⁵⁾

This paper extends the earlier PB-PK models by including induction of binding proteins/enzymes and of dioxin metabolism in response to ternary interactions of dioxin, the *Ah* receptor, and DNA binding sites and analysis of repeated dose exposure situations. This physiological model for dioxin disposition and enzyme induction is discussed in relation to its potential role for estimating dosimetry and gene regulation in receptor-based risk assessment strategies for dioxin.

2. PB-PK MODEL

2.1. Model Structure

The dioxin PB-PK model (Fig. 2) consists of five compartments—liver, fat, slowly perfused tissues (e.g.,

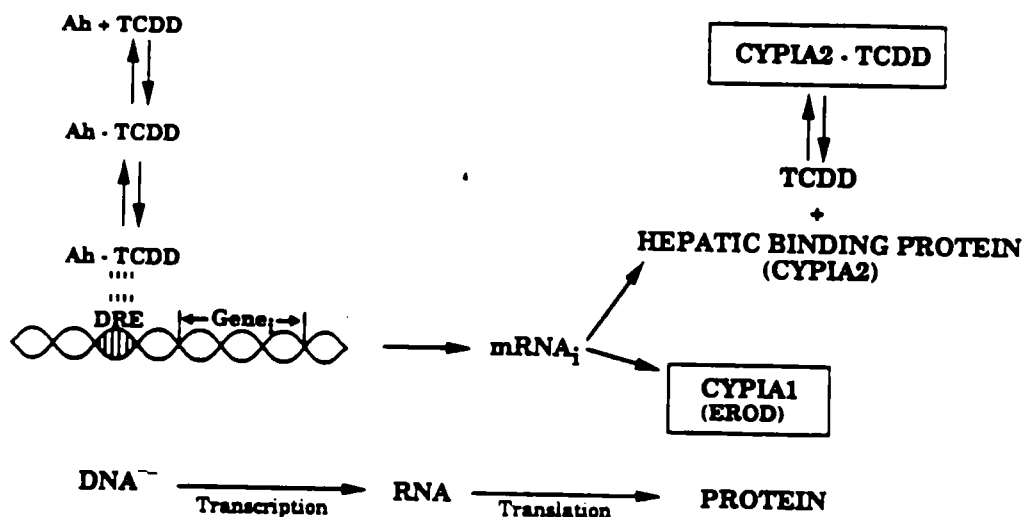


Fig. 1. Schematic of the molecular mechanisms of action of dioxin. Dioxin binds to a cellular protein, the *Ah* receptor, and the dioxin-receptor complex interacts with DNA sites in regulatory regions of certain genes. Interaction with these sites, called dioxin-responsive elements (DREs), leads to changes in rates of gene transcription (i.e., the rate at which mRNA is produced from these genes). The mRNAs serve as templates for protein synthesis. Changes in cellular mRNA levels lead to altered protein concentrations for cytochrome P4501A1, hepatic, dioxin-binding proteins, toxic effects of dioxin.

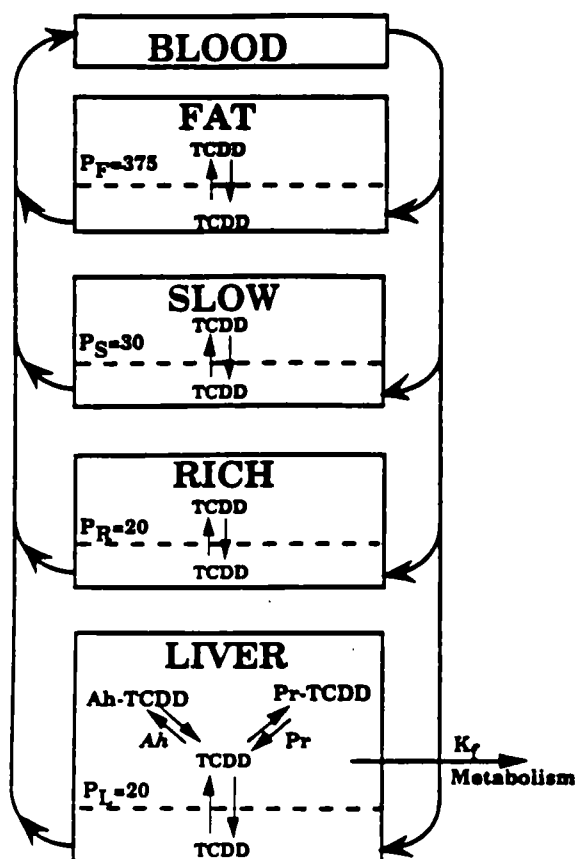


Fig. 2. The PB-PK model for dioxin. A five-compartment diffusion-limited model was developed with metabolism and protein binding included in the liver. Uptake after subcutaneous administration was described as a first-order process with the chemical appearing in mixed venous blood. Symbol definitions are in Table I.

muscle, skin, etc.), richly perfused tissues (e.g., kidney, brain, etc.), and blood. The relevant mass-balance differential equations appear in the Appendix and the terms are defined in Table I. Each of the four tissue compartments (denoted by subscript, i) has both a specified blood flow (Q_i), tissue compartment volume (V_i), and a tissue blood volume (V_{ib}). The tissue blood volumes were estimated from Bischoff and Brown.⁽¹⁶⁾ Movement of chemical from the tissue blood into the tissue is modeled to be proportional to a permeation coefficient-surface area cross-product (PA) for the tissue (i). Tissue uptake is diffusion-limited when $PA_i < Q_i$. Each tissue has a specified volume, blood flow, PA product, and partition coefficient (P_i). In the Leung *et al.* models,^(13,14) tissues had no specified blood volume and the tissue/tissue blood aggregates were described as flow-limited compartments. In addition, there was binding in the blood com-

Table I. Parameters in the Physiological Dosimetry Model for Dioxin^a

Model parameters body weight (kg)	Abbreviations bw	Wistar rat ^b 0.215
Volumes^c (liters)		
Liver	V_l	$0.0375 \times bw$
Fat	V_f	$0.07 \times bw$
Richly perfused (viscera)	V_r	$0.0525 \times bw$
Slowly perfused (muscle/skin)	V_s	$0.75 \times bw$
Blood	V_b	$0.05 \times bw$
Tissue blood volumes		
Liver	V_{lb}	$0.01 \times V_l$
Fat	V_{fb}	$0.05 \times V_f$
Richly perfused	V_{rb}	$0.01 \times V_r$
Slowly perfused	V_{sb}	$0.05 \times V_s$
Cardiac output (liters/hr)		
	Q_c	4.4
Tissue blood flow (% Q_c)		
Liver (portal and arterial)	Q_l	0.25
Richly perfused	Q_r	0.51
Fat	Q_f	0.09
Slowly perfused	Q_s	0.15
Diffusional tissue clearance (liters/hr)		
Liver	PA_l	$0.5 \times Q_l$
Fat	PA_f	$0.2 \times Q_f$
Richly perfused	PA_r	$0.5 \times Q_r$
Slowly perfused	PA_s	$0.5 \times Q_s$
Metabolic constants		
Metabolism (hr^{-1})	k_{fc}	1.65
Induction (fold over basal rate)	Fold	1.00
Partition coefficients		
Liver/blood	P_l	20
Fat/blood	P_f	375
Richly perfused/blood	P_r	20
Slowly perfused/blood	P_s	30
Protein binding		
Ah maximum (pmol/liver)	EM_1	3.75
Ah affinity (pM)	KB_1	35
1A2 basal level (nmol/liver)	$BM2_0$	10
1A2 maximum (nmol/liver)	$BM2_\infty$	85
1A2 affinity (nM)	KB_2	6.5
1A2 Hill term	n	1.0
1A2 Hill binding constant (pM)	K_d	50
1A1 Hill term	n_1	2.3
1A1 Hill binding constant (pM)	K_{d1}	180
1A1 degradation rate constant (hr^{-1})	k_1	0.035
1A1 synthesis-basal rate (units/hr)	K_0	0.7
1A1 maximum induction (fold)	K_{onmax}	50

^a Deviations from this base parameter set are noted in the legends to each figure.

^b Parameters are for the 215 g rat at the start of the dose-response study in Fig. 3.

^c Tissue volumes are given as liters and referenced to tissue as a proportion of the total body weight, assuming unit density (1 kg = 1 L).

partment which effectively decreased the rate of tissue uptake. Our revised model, because of the diffusion-limited tissue compartments, does not require blood binding to match tissue uptake time-course behavior.

Initial estimates of partition coefficients were obtained from Leung *et al.*⁽¹⁴⁾ and were adjusted in fitting the data sets here. As expected, the fat has the highest partition coefficient due to the highly lipophilic nature of dioxin. In addition, metabolism and protein binding are included in the liver, where both the *Ah* receptor and the inducible binding protein act to sequester dioxin *via* capacity-limited binding processes. The binding protein was assumed to be cytochrome P4501A2. The concentrations of P4501A2 in naive and induced rats differ by about 10-fold.⁽¹⁷⁾ The differential equation for the liver (Eq. 5) is solved for the total amount of dioxin in liver, which is related to partitioned dioxin, dioxin bound to the *Ah* receptor, and dioxin bound to P4501A2 (Eq. 6). This conservation equation is used to solve for the free dioxin in the liver tissue, C_{lf} , which is then utilized in calculating the concentration of the *Ah* receptor-dioxin complex available for inducing cytochrome P4501A2 and cytochrome P4501A1 activity. This latter protein, an oxidative microsomal enzyme induced by dioxin, is frequently used as a marker of *Ah* receptor-mediated protein induction. All these binding interactions are described by simple, reversible equilibrium relationships. This approach is valid as long as the rate constants for association/dissociation are large (i.e., the processes are rapid). This description, like all models, is really a simplification of the more complex series of biological events. The model can readily be extended to include new information on these biological associations as it becomes available.

2.2. Enzyme/Protein Induction

The induction process is described by assuming that the *Ah*-TCDD complex is formed based on *Ah* receptor-binding parameters (BM_1 and KB_1) and free dioxin in the liver (C_{lf}). This complex then binds to unspecified sites on DNA with an affinity, K_d . Since the binding to DNA does not directly alter the *Ah*-TCDD concentration in this present model configuration, it is tacitly assumed that the DNA sites are present at much lower concentrations than the *Ah*-TCDD complex. The induction is modeled with a Hill plot binding relationship (Eq. 6), where n provides a measure of interaction for multiple *Ah*-TCDD complex binding sites. The value of n is estimated by comparison of model simulations to data on the dose-dependence of liver sequestration of dioxin or on the

induction of P4501A1 activity (Figs. 3 and 4). Basal levels of P4501A1 are described by a zero-order production rate (K_0) and a first-order elimination rate constant (k_1). The induction process increases the production rate as specified by the Hill relationship and the maximum observable enhancement (K_{0max}) (Eq. 7). Data on the time course of cytochrome P4501A1 from Abraham *et al.*⁽¹⁰⁾ were used to estimate the first-order degradation rate constant (k_1) for P4501A1 degradation.

Induction of hepatic binding capacity was also mod-

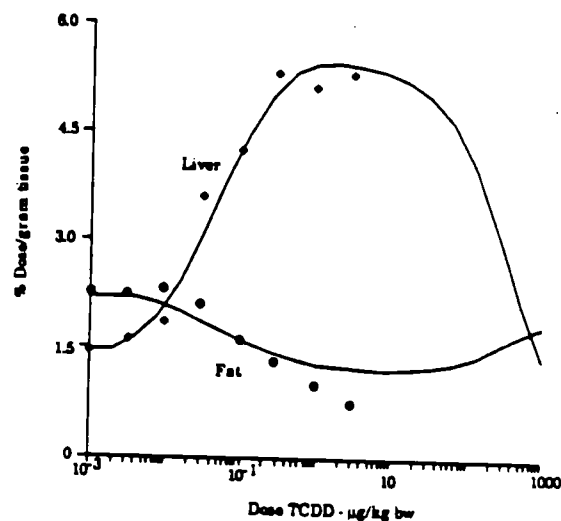


Fig. 3. Dose-dependence of dioxin tissue disposition in female Wistar rats 7 days following single subcutaneous doses. Tissue concentrations from Abraham *et al.*⁽¹⁰⁾ are expressed normalized to dose, as %dose/tissue. Model parameters are given in Table I.

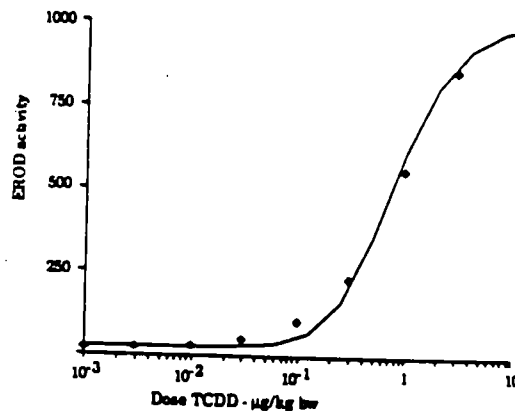


Fig. 4. Dose-dependence of cytochrome P4501A1 induction in female rats 7 days following single subcutaneous doses. Data from Abraham *et al.*⁽¹⁰⁾ for activity of EROD (Ethoxycresorufin-O-deethylase) in pmoles resorufin formed/min/mg protein.

eled as if it were instantaneously altered by the *Ah* receptor-dioxin-DNA interactions (Eq. 8). As data becomes available for the time course of P4501A2 induction, synthesis and degradation rate constants for this protein can also be directly included in this description. For both cytochrome P4501A1 and 1A2 induction, it was assumed that *Ah*-TCDD complex formation was equivalent (similar Kb_1 and Bm_1 values), but that the n value and K_d for the two responses differed (see Table I). Our model also allows an induction of dioxin metabolism following dioxin treatment. However, in contrast to the mouse, induction of metabolism in the rat, if present at all, was negligible. The constants employed assume at most only a doubling of the metabolic rate.

2.3. Data Analysis

The data used for this analysis were from two previously published studies with Wistar rats. The first study,⁽¹⁰⁾ with female rats, provides both dose-response characterization of liver concentrations and of liver P4501A1 activity and time-course characterization of dioxin tissue concentrations and enzyme activities. The second study⁽¹⁸⁾ examined liver and fat concentrations in male Wistar rats dosed weekly for periods up to 6 months.

Model simulations were performed using ACSL (Advanced Continuous Simulation Language, Mitchell and Gauthier Assoc., Concord, Massachusetts).

3. RESULTS

3.1. Dose-Response

In this study⁽¹⁰⁾ rats received a single subcutaneous dose of dioxin and were killed 7 days later. The disposition of dioxin in liver and fat was highly dose-dependent in the concentration range between 1 ng/kg and 10,000 ng/kg (Fig. 3). Normalized concentrations (% dose/g tissue) would be horizontal lines if disposition were dose-independent. The curvature appears to be due to the induction of a dioxin-binding protein, presumably cytochrome P4501A2. The smooth curves were obtained with the PB-PK model based on the parameters in Table I. For induction of the binding protein, n and K_d were estimated by fitting the data from Wistar rats⁽¹⁰⁾ and were, respectively, 1.0 and 50 pM. Using measured concentration estimates of basal and induced P4501A2,⁽¹⁷⁾ the affinity of the binding protein (Kb_2) was estimated from the curve fitting to be 6.5 nM. A value of n close

to 1.0 suggests little interaction among dioxin responsive DNA binding sites involved in expression of this particular gene. The complex behavior above 10 μ g/kg bw is commented on more fully in the Discussion.

The dose-response curve for cytochrome P4501A1 induction (Fig. 4) was described in a similar manner, but required a larger value for n_1 (2.3) to fit the data, indicating possible interactions among DNA binding sites for the *Ah* receptor-TCDD complex with this gene. The half-maximal induction response for P4501A1 occurred at about a 10-fold higher dose than the half-maximal response of the binding protein ($Kd_1 = 180$ pM).

The PB-PK model configured for these dose-response curves was also used to examine time-course elimination/induction after a single dose of 300 ng/kg (Figs. 5 and 6). Analysis of these results were especially useful for setting the rate constant for metabolism of dioxin and the *PA*, cross-products (i.e., the diffusion limitations) for liver and fat. The simulation of these curves requires inclusion of time-dependent growth parameters over the 100 days of the experiment. Growth rates and volumes of fat were estimated from growth curves for these rats available from suppliers.

3.2. Repeated Dosing

The primary health concerns with dioxin are associated with repeated low or chronic exposures. We analyzed data from a study in which liver and fat

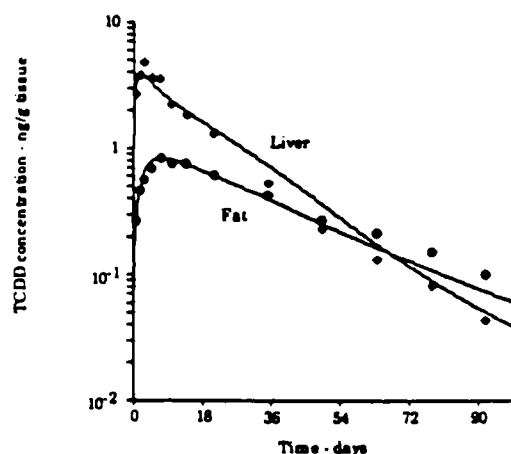


Fig. 5. Time course of liver and fat tissue dioxin concentrations following a single subcutaneous dose of 300 ng/kg in female Wistar rats. Concentrations are expressed as ng/g tissue, and the data are from Abraham *et al.*⁽¹⁰⁾ Parameters used were the same as in Figs. 3 and 4; however, growth of the rat and fat compartments were also included.

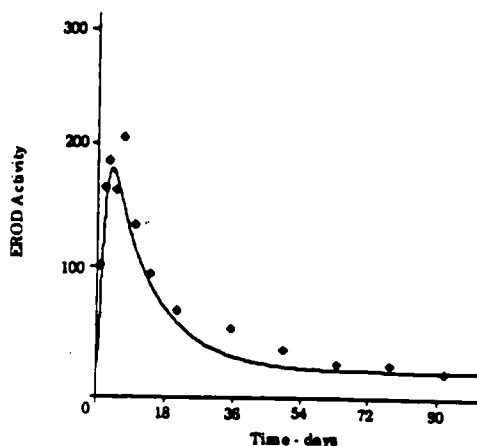


Fig. 6. Time course of cytochrome P4501A1 activity following a single dose of 300 ng dioxin/kg in female Wistar rats. Data are from Abraham *et al.*⁽¹⁰⁾ and parameters as in Fig. 5.

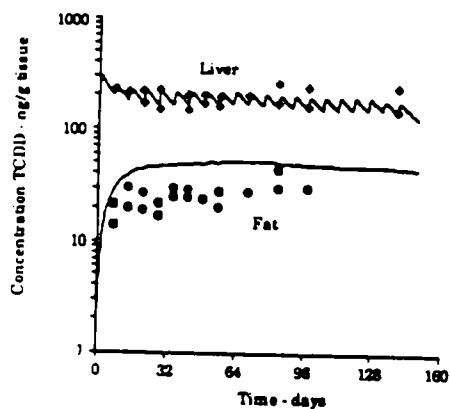


Fig. 7. Time course liver and fat concentrations in rats dosed weekly with 5 µg dioxin/kg starting 7 days after a loading dose of 25 µg/kg. These data, expressed as ng/g tissue, are from Krowke *et al.*⁽¹⁸⁾ The model parameters were as specified in other figures with several changes: P_f , P_s , P_{he} , and k_{fc} were, respectively 250, 50, 0.1 and 1.5. These contrast to values of 375, 30, 0.20, and 1.65.

concentrations were determined in male rats for up to 6 months during weekly dosing with dioxin.⁽¹⁸⁾ This study (Fig. 7) used a loading dose (25 µg/kg) followed by a fivefold smaller dose (5 µg/kg) every week. The simulations for this dosing scenario were conducted with model parameters very similar to those used in the single-dose exposures (Figs. 3–6). Small differences in fat and slowly perfused tissue compartment parameters are noted in the figure legends.

3.3. Correlation with Responses

The present EPA dioxin risk assessment is based on liver tumors in female rats.⁽¹⁾ Liver tumors are also increased in female and male mice.⁽²⁾ Liver initiation-promotion studies^(3,19,20) demonstrate that dioxin is a promoter and that the dose-response curve for its promoting action on altered hepatic foci closely follows the dose-response curve of its hepatocarcinogenic activity (Table II). Both of these dose-effect relationships are very steep.

Based on our preliminary repeated-dose PB-PK model for these Wistar rats, several measures of dose can be calculated for comparison with the promotional efficacy and carcinogenicity of dioxin in Sprague-Dawley rats (Table II). These include integrated total liver dioxin concentration during the treatment period, or integrated free liver dioxin concentration. In addition, measures of tissue dose related to enhanced expression of cytochrome P4501A1 and hepatic binding proteins can be calculated for the duration of the subchronic exposure. The measures of tissue dose associated with en-

Table II. Relationship Between Hepatic Exposure to Dioxin, Hepatic Protein Induction, and Hepatic Toxicity During Subchronic Exposures

Dose ^a	Altered foci ^b volume	Tumors ^c (liver)	AUCL-free ^d C_f	AUCL-total ^e C_t	AU-1A2 ^f ($BM2_t$)	AU-1A1 ^g (IND)
Control	0.8	1/86	0	0	0	0
0.0001	0.2	—	6.7×10^{-1}	3.5×10^1	7.0×10^3	2.3×10^1
0.001	0.3	0/50	6.4×10^0	5.9×10^2	5.7×10^4	3.9×10^3
0.01	0.7	2/50	5.3×10^1	1.1×10^4	2.1×10^5	2.7×10^4
0.1	2.8	11/49	4.2×10^2	1.2×10^5	3.2×10^6	2.0×10^5
1	—	—	3.8×10^3	1.0×10^6	3.5×10^7	3.0×10^6
10	—	—	3.8×10^4	5.4×10^6	3.5×10^8	3.1×10^7

^a Biweekly doses used; dose expressed as average dose per day in µg/kg.

^b Volume as percent of liver occupied by altered cells, from Pitot *et al.*⁽¹⁸⁾

^c Liver tumors observed in female rats in the bioassay study of Kociba *et al.*⁽¹¹⁾

^d Total area under the curve for free dioxin in the liver, units are nmoles-hr/liters.

^e Total area under the curve for total dioxin in the liver, units are nmoles-hr/liters.

^f Integrated exposure to induced levels of binding protein; units are nmoles-hr/liter; and the calculation integrates the third term on the right side of Eq. (6).

^g Integrated exposure to induced levels of cytochrome P4501A1; units are enzyme unit-hr; and the calculation only considers the enhanced level of activity due to induction.

hanced gene expression (assumed to be related to P4501A1 activity or the sequestration of dioxin in liver by cytochrome P4501A2) are the integrated level of these gene products (as protein concentration of 1A2, or activity of 1A1) over time. Enhanced expression refers to the increase over any basal levels of expression of these gene products. This correlative approach does not assume that there should be a direct relationship between induction of these cytochromes and the promotional action of dioxin at all dose levels.

The tumor promotional response of dioxin in the rat liver is most closely correlated with the integrated expression of the P4501A1 gene (Table II) under these exposure conditions. For instance, the responses (columns 2 and 3) increase rapidly with increasing dose between 0.01 and 0.1 $\mu\text{g/kg/day}$. The integrated level of cytochrome P4501A1 increases by a factor of about 10 in this range, while the integrated amounts of binding protein (P4501A2), whose induction is already nearly saturated by 0.01 $\mu\text{g/kg/day}$, only increases by 50% in this region of dose. Dioxin concentrations increase in this dose range, but dioxin itself is not believed to be responsible for toxic effects.

4. DISCUSSION

4.1. Pharmacokinetics

The half-life of dioxin in male rats was initially reported to be 20–30 days.⁽²¹⁾ These early studies were conducted at high, inducing doses; elimination curves were obtained for only about two half-lives; and analysis relied on chemical detection of dioxin in tissues. The half-lives in hamsters (10–15 days) and guinea pigs (30–40 days) were also estimated from time course data with a simple one-compartment model for dioxin kinetics but used radiochemical detection of labeled dioxin.^(22,23) These various studies were relatively insensitive to the dose-dependent effects apparent only when disposition is examined at much lower doses (Fig. 3) and most apparent when both fat and liver concentrations are measured. The alteration in the ratio of liver-to-fat concentration ratio is the most sensitive marker for this dose-dependence (compare Figs. 3 and 8b). These low dose effects can now be readily evaluated by use of high specific activity ^3H -labeled dioxin. Single doses of 1 ng/kg caused minimal induction of P4501A1 (Fig. 4) or of the dioxin binding protein (Fig. 3). Protein induction, however, becomes significant at a dose of 30–50 ng/kg dose and

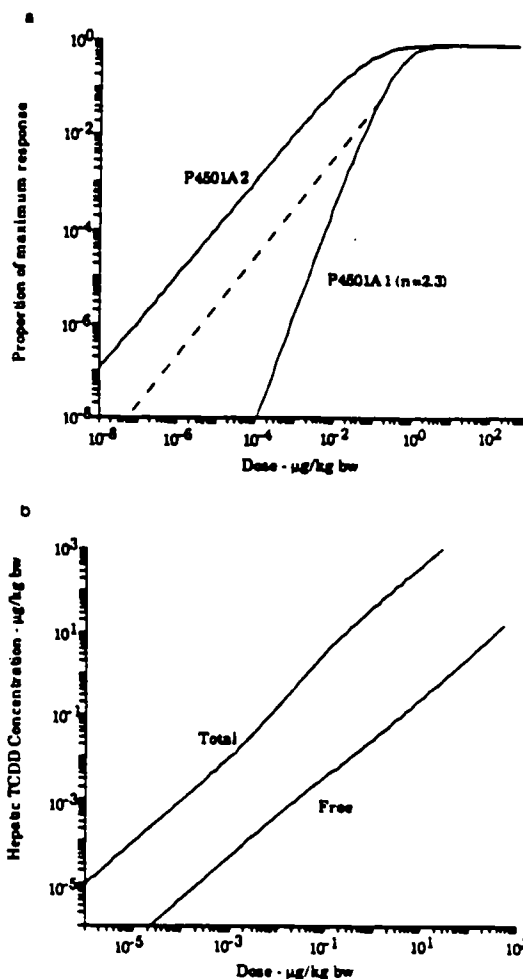


Fig. 8. Low-dose extrapolation of measures of tissue dose for dioxin in subchronic exposures. (A) Dose measures related to induction of cytochrome P4501A1 and the liver binding protein. The steeper line is for enhanced P4501A1 which has a cooperativity term of 2.3. Two extrapolations are depicted with cytochrome P4501A1; the case of a Hill coefficient of 2.3 throughout the dose range, and for the case of a linear relationship below several percent response (dashed line), the latter is more biologically realistic. (B) Dose measures for total liver dioxin and for free dioxin in liver. The total increases in a greater than linear fashion with dose in the region where induction of the binding protein occurs. In that same dose region the tissue dose of free dioxin does not increase in direct proportion to exposure level. These calculations used the model applied to data in Fig. 7 and calculated exposures for a 6-month period.

markedly alters dioxin disposition. Simple linear compartmental analyses of dioxin pharmacokinetics have completely ignored dose-dependent protein binding.

PB-PK models have been reported for polychlorinated biphenyls,⁽²⁴⁾ 2,3,7,8-tetrachlorodibenzofuran,⁽²⁵⁾

and, in a preliminary form, for dioxin itself⁽²⁶⁾. With the exception of the work by Leung and colleagues,^(13,14) none of these descriptions have included specific hepatic binding. In all these other models, chemicals were distributed simply by nonspecific, dose-invariant partitioning and focused on behavior at higher doses with substantial induction of binding proteins. Because of the limited dioxin-binding capacity of the liver, even in fully induced animals, the liver/fat concentration ratio reaches a maximum and is predicted to decrease at higher body burdens (Fig. 3), where hepatic binding to induced proteins approaches saturation. While this region of dose was not examined by Abraham *et al.*,⁽¹⁰⁾ the time course of elimination has been examined after a single dose of 600 $\mu\text{g/kg}$ in hamsters, a species which is more resistant to the acute toxic effects of dioxin than are rats. In this study, the liver/fat ratio increased from 1.0–2.7 as the body burden fell from 600 to 100 $\mu\text{g/kg}$ ⁽²²⁾, consistent with the predictions in Fig. 3 for the dose range above 10 $\mu\text{g/kg}$ bw.

Several people ingested relatively large amounts of dioxin-like polychlorinated dibenzofurans in the Yusho and Yu Cheng poisoning incidents in Japan and Taiwan, respectively. In several instances, liver and fat samples were obtained at autopsy, and the proportion of dose in the liver was found to be dependent on the total body burden. The % dose/liver increased from about 5 at ambient exposure levels to over 60 at a body burden of the furans toxicologically equivalent to a burden of 3–5 μg dioxin/kg body weight. The body burden for half-maximal sequestration in the liver in people was estimated by Carrier *et al.*⁽²⁷⁾ to be 0.5–1.5 μg dioxin/kg body weight. Thus, this dose-dependent hepatic distribution is common both to rats and people with very similar body burdens required for half-maximal sequestration. The liver/fat concentration ratio, however, varies for different isomers. Higher chlorinated isomers tend to have ratios higher than for TCDD itself. The reasons for these differences are unknown; however, one suggestion derived from modeling efforts is that KB_2 , the dioxin-binding protein dissociation constant, is lower for the higher-chlorinated analogs (i.e., they have higher affinities for the binding proteins than does dioxin itself).

4.2. Protein Binding/Induction

The challenge in providing a biologically realistic PB-PK model for dioxin is the need not only to account for the determinants of disposition (i.e., tissue partitioning, biotransformation rates, and protein binding constants), but also to describe the pharmacodynamic events

related to the induction of specific dioxin-binding proteins in the liver. Knowledge of the molecular mechanisms of the interactions of the dioxin-*Ah* receptor complex with regulatory regions of specific genes is growing rapidly^(28,29), and shows clearly a role for the ternary dioxin-*Ah* receptor-DNA complexes in regulating gene transcription. Our present model of disposition is based on a ternary complex being obligatory for the pharmacodynamic activity of dioxin at the genomic level. The description of these interactions requires estimates of binding constants between the *Ah* receptor and dioxin and between the *Ah* receptor-dioxin complex and sites on DNA. The ternary interactions with DNA are believed to enhance transcription of mRNA leading eventually to increased amounts of specific dioxin-binding proteins, particularly cytochrome P4501A2.

The binding maximum (BM_1) and binding affinity (KB_1) for TCDD binding to the *Ah* receptor have been previously reported. The values used in our model differ significantly: our estimate of BM_1 is only about 10% of the value determined by Gasiewicz and Rucci⁽³⁰⁾ and our KB_1 is also much lower than the value reported by Bradfield and Poland.⁽³¹⁾ The discrepancy in KB_1 is due simply to our decision to reference binding to the free dioxin in the liver. If we had used total partitioned dioxin, our number would increase by a factor of 20 and be more similar to the *in vitro* value.⁽³¹⁾ However, the lower BM_1 value represents a more fundamental discrepancy between *in vitro* measurements and *in vivo* pharmacokinetics.

In the present study, the binding parameters of the *Ah* receptor were largely estimated from the tissue concentrations of TCDD at noninducing levels, shown in Fig. 3. The liver concentration at doses below 0.01 $\mu\text{g/kg}$ is especially sensitive to BM_1 , the *Ah*-binding maximum. In addition, the dose at which hepatic sequestration is half-maximal is extremely sensitive to KB_1 , the *Ah*-binding affinity. If BM_1 were increased to the literature value, the liver concentration of TCDD in the low dose region (i.e., noninduced animals) would be greatly overestimated. One possibility to explain this discrepancy is that only a relatively small proportion of the available receptors participate in binding dioxin and maintaining induction at physiologically realistic concentrations of TCDD *in vivo*. In mouse hepatoma cell lines, the total receptor concentration decreases after exposure to dioxin.⁽³²⁾

The other important binding constant K_d , for the DNA interactions, also affects the placement of the induction curve, whether for 1A2 or 1A1, along the dose axis. The Hill-type coefficients control the steepness of the induction response. With the two responses exam-

ined, induction of liver sequestration (i.e., 1A2) shows higher affinity (lower K_d value) but lower cooperativity (smaller n value) than does induction of 1A1. This apparent cooperativity with 1A1 ($n_1 = 2.3$) is consistent with the observation that there are at least four dioxin responsive elements in the regulatory region of this gene.⁽²⁸⁾

The PB-PK model structure developed here assumes that a single type of *Ah* receptor-dioxin complex interacts with DNA binding sites of variable affinity to regulate different genes. More complex models with different *Ah* receptor-dioxin complexes (due to other protein interactions, for instance) might lead to different conclusions about cooperativity in these two responses, but such models do not seem warranted by available biological data at this time.

4.3. Pharmacokinetics of the *Ah* Receptor

The biological activity of dioxin is a consequence of both delivery of dioxin to target cells and the dynamics of the processes that regulate *Ah* receptor concentration and *Ah* receptor binding characteristics. The present model extends our understanding of the biological determinants of dioxin action *in vivo*; however, it does not account for time variant changes in the receptor. In completely induced animals, only a relatively small fraction of total basal receptor binds to nuclear sites, even with complete induction of P4501A1 activity.⁽³³⁾ Furthermore, in mouse hepatoma cells *in vitro*, the total cellular receptor concentration decreases after treatment of the cells with dioxin.⁽³²⁾ In contrast, there is an apparent increase in *Ah* receptor in the rat liver following TCDD treatment.⁽³⁴⁾ These complexities in *Ah*-receptor kinetics should have minimal effects on our analysis, which focuses on longer-term behaviors, after 7 days or over the 100-day excretion period. New experimental techniques with appropriate antibodies⁽³⁵⁾ should soon be available for measuring the total amount of cellular *Ah* receptor. With these techniques, *Ah* receptor kinetics can be more fully investigated by examining total amount of receptor, receptor distribution, tissue dioxin, mRNA, and protein for P4501A2 over the first several hours to days after intravenous administration of dioxin. Such studies will improve both our appreciation of the pharmacokinetics/pharmacodynamics of the *Ah* receptor, and of the dosimetry of dioxin itself. It bears repetition that the determinants of dioxin pharmacokinetics cannot be examined in the absence of additional information. The pharmacokinetics of dioxin, of the *Ah* receptor, and of hepatic binding proteins are inextricably coupled.⁽³⁶⁾ Continued

progress in physiological modeling of dioxin pharmacokinetics now relies on the progress in uncovering the biology of the *Ah* receptor,⁽³⁷⁾ especially regarding its ability to affect the expression of dioxin-binding proteins.

The current PB-PK model greatly simplifies the sequence of processes involved in activation of the receptor by dioxin. Normally, the receptor is complexed with heat shock protein(s) 90, which probably dissociate after the receptor binds dioxin.^(38,39) Another protein species is then required for the interaction of the complex with DNA sites.⁽⁴⁰⁾ In the future, it will be necessary to model these events if they are found to be critical control elements for gene regulation; however, they are not understood in sufficient detail to justify their inclusion in the dosimetry model at this time.

4.4. Risk Assessment Implications

Greenlee *et al.*,⁽²⁹⁾ in a recent perspective on biologically based risk assessment for dioxin, outlined the biological steps involved in receptor-mediated growth modulatory effects. They are recognition, transduction, and response (Fig. 1). Our PB-PK model includes elements of recognition (binding of dioxin to the *Ah* receptor), transduction (binding of the complex to DNA), and response (the changes in rate of transcription/translation of P4501A1 and the binding protein). The responses of these two proteins are not thought to be causally linked to the adverse effects of dioxin. Specific mitogenic and mito-inhibitory cellular processes altered by dioxin treatment are more likely causally related to toxicity.^(41,42) Even if we had modeled regulation of growth factor proteins, it is unclear what specific role they play, singly or in combination, in cell replication, cell differentiation, or toxicity. It will be of interest to examine the kinetic characteristics of induction (or repression) of these other proteins to see if they are controlled in a manner similar to P4501A1 or the sequestering protein. Since data on modulation of growth regulatory genes is not yet available, we have correlated various tissue dose measures from the present model with the promotional efficacy of dioxin. Of the several measures of tissue dose examined (Table II), the integrated exposure to P4501A1 is most closely correlated with promotion and with hepatic tumors. However, there is no expectation of causality between tumor responses and these induced proteins, and this correlation should be regarded cautiously.

In using the integrated P4501A1 exposure, the low dose extrapolation slope approaches the value of the Hill

coefficient ($n_1 = 2.3$), reflecting the cooperativity which appears to exist for induction of this gene by the ternary TCDD-*Ah* receptor-DNA interactions (Fig. 8a). The differences in the low dose extrapolation with measures of tissue dose related to protein induction (Fig. 8b) is related both to their respective slopes (n values) and the ease of induction by dioxin (the values of Kd and Kd_1). The mathematical formulation of these cooperativity interactions is based on a $(n + 1)$ order interaction. Such interactions are highly unlikely since the rate of these association processes fall sharply with decreasing ligand concentration. Normally, cooperative relationships arise due to enhanced affinity for a second ligand molecule after the binding of the first ligand. In this way, all events involved are bimolecular. The Hill equation is simply a convenient way to treat this more complex series of events. With these multiple bimolecular steps, the low dose induction behavior should have a slope of 1 and be characterized by the binding parameters of the first ligand. Dose measures related to free or total dioxin in liver are also complexly related to administered dose; neither curve is strictly linear with dose and the departures from linearity are in opposite directions.

Appropriate measures of tissue dose for risk assessment must focus on specific cellular events, such as cell proliferation rates with developing preneoplastic foci in the livers of dioxin-treated rodents. Among the important ongoing efforts to improve the biological basis of dioxin risk assessment are studies analyzing regulation of dioxin-responsive growth regulatory genes in liver⁽⁴³⁾ and the effect of these genes on replication and focal growth. Eventually, biological response models will have to predict the relationship between replication or differentiation and the tissue exposure to these growth factors. Only when this level of resolution is achieved will it be possible to more fully justify any particular approach to low-dose extrapolation with dioxin.

The basic behavior of dose-dependent hepatic sequestration is a characteristic of many congeners of dioxin⁽⁴⁴⁾ and is observed in several species including people.⁽⁴⁵⁾ This basic PB-PK model structure appears to be applicable to many chemicals that act *via* interactions with the *Ah* receptor and should prove, in time, useful for risk assessment applications with this broad group of important contaminants with common receptor-mediated mechanisms of toxicity. These dosimetry models will have to be combined with emerging quantitative descriptions of cell and tissue responses to develop a complete biologically motivated risk assessment model. Some of the challenges of this approach have been outlined in this paper.

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APPENDIX

There are two mass balance differential equations for each tissue, one for tissue blood (*tb*) and another for tissue (*t*). Respectively,

$$dA_{tb}/dt = Q_t(C_s - C_w) + PA_t(C_t/P_t - C_w) \quad (1)$$

$$dA_t/dt = PA_t(C_w - C_t/P_t) \quad (2)$$

For fat, slowly perfused, and richly perfused tissues, these equations are integrated for amount and tissue concentrations calculated by dividing amount (A_t) by volume (V_t).

$$A_t = \int_0^t dA_t \quad (3)$$

$$C_t = A_t/V_t \quad (4)$$

The tissue free (diffusible) concentration is calculated by dividing the tissue concentration (C_t) by the tissue partition coefficient (P_t)—Eq. (1) and (2). The liver equation [Eq. (5)] also contains a term for loss due to metabolism:

$$dA_l/dt = PA_l(C_w - C_{lf}) - V_l k_f(1 + \text{fold}(Cl_f/(C_{lf} + Kb_1)))Cl_f \quad (5)$$

In the development of the mathematical description used here, there is the possibility for inducing metabolism in direct proportion to the fractional occupancy of the *Ah* receptor by dioxin. The extent of maximal induction as increase over the basal rate is controlled by the fold term in the above equation. For rat, this value was set to 1.0; it may vary in other species, such as the mouse.⁽¹³⁾

The total mass in the liver is then apportioned between free dioxin (C_{lf}) and bound forms of dioxin.

$$A_i = P_i V_i C_{if} + [BM_1 C_{if} / (KB_1 + C_{if})] + [BM_2 C_{if} / (KB_2 + C_{if})] \quad (6)$$

The second term on the right of the equality is the concentration of the *Ah*-TCDD receptor complex (*Ah*-TCDD) and the third term is the concentration of the complex of TCDD and the induced binding protein.

The activity of P4501A1 at time, *t*, is calculated from the basal synthesis rate (K_0), the maximum increase in synthesis rate (K_{0max}), the *Ah* receptor-TCDD complex concentration, the complex-DNA dissociation constant (Kd_1), the appropriate Hill term (n_1), and the P4501A1 degradation rate constant (k_1).

$$d(P4501A1)/dt = K_0(1 + K_{0max}(Ah-TCDD)^{n_1}/((Ah-TCDD)^{n_1} + Kd_1^{n_1})) - k_1 P4501A1 \quad (7)$$

The concentration of P4501A2 at any time, BM_2 , was calculated assuming an instantaneous adjustment of binding protein concentrations and *Ah*-TCDD levels,

$$BM_2 = BM_{20} + BM_2(Ah-TCDD)^{n_2}/((Ah-TCDD)^{n_2} + Kd_2^{n_2}) \quad (8)$$

where BM_{20} is the maximum induction of the binding protein. This formulation assumes very rapid induction of the binding proteins in these studies.

For the longer duration studies, the changes in total body weight and proportion of weight as fat compartment volume were included via table functions available in the ACSL software package.

For the single-dose studies in female rats,

	Simulated time (hr)		
	0	840	1344
Body weight (kg)	0.215	0.3	0.34
Fat (%bw)	0.07	0.09	0.13

For the repeated dose studies in male rats,

	Simulated time (hr)		
	0	840	1344
Body weight (kg)	0.35	0.39	0.41
Fat (%bw)	0.10	0.12	0.13

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APPENDIX C

Under the assumptions of 8.2, when fitting the two-stage model to the Kociba et al. (1976) data, there is the potential to estimate as many as 24 parameters (four dose groups, each with its own two-stage model having six parameters). As discussed in Appendix C, the data given in this study make it impractical to estimate this many parameters with any degree of accuracy (Kopp-Schneider and Portier, 1992). To reduce the number of parameters in the model, β_N and δ_N were assumed to be known without error. Assuming that, on average and over finite time, the population of normal cells is effectively constant, it is reasonable to assume that $\beta_N = \delta_N$. This assumption may not hold true for TCDD since the labeling index for normal cells seems to increase with increasing exposure to TCDD (Lucier et al. (19??)). However, to illustrate the use of the two-stage model, this assumption will be employed. Labeling data suggest that normal hepatocytes undergo mitosis at an average rate of one mitotic event per 300 days (a 2 percent labeling index for a 6-day labeling experiment) suggesting that $\beta_N = 3.333 \times 10^{-3}$. This value was used in the analysis that follows. Other values for $\beta_N = \delta_N$ were tried and had no effect on the resulting model parameters as long as it was assumed that changes in β_N do not imply changes in μ_{N-I} (Portier and Kopp-Schneider, 1991). The number of normal hepatocytes was assumed to be 6×10^8 for the female rat.

There are 16 parameters to be estimated from the tumor incidence data. Table C-1 shows one set of parameter estimates resulting from this fitting exercise (labeled "Unrestricted" under the model heading). Multiple attempts at fitting this model to the tumor incidence data indicated that the model was not identifiable; this means that there are an infinite number of parameter estimates that give effectively the same answer for these data. The main problem seems to be estimating μ_{N-I} and μ_{I-M} simultaneously (for example, in the high-dose group, any model with $\mu_{N-I} \cdot \mu_{I-M}$ equal to $3.4 \cdot 10^{-15}$ seemed to yield equivalent fits). The main reason for including the "Unrestricted" model in Table C-1 is that this model represents the best fit we can possibly achieve, and the likelihood (Column 6) represents a

Table C-1. Fitting the two-stage model to the tumor incidence data of Kociba et al. (1976) using the pathology of Sauer et al. (1991)

Model	Dose (ng/kg/day)	Parameters				Likelihood
		μ_{N-1}	β_1	δ_1	μ_{1-M}	
1. Unrestricted	0	1.14×10^{-10}	6.90×10^{-2}	3.14×10^{-2}	1.20×10^{-12}	-58.61
	1	9.48×10^{-10}	4.75×10^{-2}	1.58×10^{-2}	$9/2 \times 10^{-15}$	
	10	9.85×10^{-10}	5.27×10^{-2}	2.44×10^{-2}	1.12×10^{-12}	
	100	3.33×10^{-5}	2.01×10^{-2}	1.61×10^{-2}	1.03×10^{-10}	
2. Unrestricted μ_{N-1}	0	2.55×10^{-8}	1.75×10^{-2}	1.06×10^{-2}	1.31×10^{-9}	-64.96
	1	1.37×10^{-8}	—	—	—	
	10	1.36×10^{-7}	—	—	—	
	100	5.80×10^{-7}	—	—	—	
3. Unrestricted μ_{1-M}	0	$1/31 \times 10^{-5}$	2.86×10^{-2}	2.16×10^{-2}	2.46×10^{-12}	64.96
	1	—	—	—	1.37×10^{-12}	
	10	—	—	—	1.53×10^{-11}	
	100	—	—	—	5.81×10^{-11}	
4. Unrestricted β_1	0	2.56×10^{-7}	1.24×10^{-2}	1.79×10^{-2}	3.02×10^{-9}	-67.57
	1	—	5.82×10^{-3}	—	—	
	10	—	2.15×10^{-2}	—	—	
	100	—	2.46×10^{-2}	—	—	
5. Unrestricted β_1 (β_1 in low-dose group set to zero)	0	2.56×10^{-7}	6.70×10^{-3}	1.23×10^{-2}	3.02×10^{-9}	67.57
	1	—	0.0	—	—	
	10	—	1.59×10^{-2}	—	—	
	100	—	1.90×10^{-2}	—	—	

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best possible measure of goodness-of-fit. We will compare the likelihood for other models with this target likelihood.

The first restricted model to be considered is a model in which the effect of TCDD is only on the mutation rate from normal cells to initiated cells in the two-stage model. This model is fit by forcing β_I , δ_I , and μ_{I-M} to be constant across all dose groups and allowing μ_{N-I} to vary freely as dose changes. The parameter estimates for this model are given in Table C-1 as model (2). There are nine fewer parameters in this model than in the unrestricted model. Comparing the two likelihoods, it is evident that this model fits the data significantly worse than does the unrestricted model ($X^2_9 = 12.71$, $p < 0.01$, as a technical note, since there is a problem with identifiability, the degrees-of-freedom, 9, for this X^2 random variable is inflated, which would inflate the p-value and the significance of the result would remain).

The problem with identifiability does not abate by restricting the parameters in the model. This is illustrated by the next model (3) in Table C-1. In this model, it is assumed the effect of TCDD is restricted to the rate of mutation from the initiated state to the malignant state. In this model, β_I , δ_I , and μ_{N-I} are held constant over all groups and μ_{I-M} changes as dose changes. Three points indicate the problem of nonidentifiability with this model. The first is that the likelihood for this model (3) and the model changing μ_{N-I} (2) are identical. This indicates that the two models explain the same amount of noise in the data. The second point is that, even though the magnitude of the birth rate β_I has changed, the difference $\beta_I - \delta_I$ has not ($\sim 0.7 \times 10^{-2}$). Further modeling with these data indicate this to be true over a wide range of β_I values. The third point is that, for each dose group, $\mu_{N-I} \cdot \mu_{I-M}$ is the same in the two models. Again, use of different fixed values for μ_{N-I} in model (3) and μ_{I-M} in model (2) support this result.

The net result is that, unlike the results seen by Moolgavkar and Lubeck (1992) when fitting colon cancer data to the two-stage model, the magnitude of the mutation rate μ_{N-I} relative to μ_{I-M} cannot be addressed for the TCDD data. However, the relative change in the

product of these two mutation rates as a function of dose can be studied. This will be done later in this document.

A second restricted model is to consider that dioxin's only effect is on the birth rate of initiated cells (β_I). This is in fact the modeling approach used by Thorslund (1987). This model assumes that μ_{N-I} , δ_I , and μ_{I-M} do not change as a function of exposure to dioxin. This model is given as model (4) in Table C-1. This model is also inconsistent with the data (likelihood = 67.57) and provides a fit that is worse than the mutational effect model given by (2). [Technical note: It is difficult to directly compare these two models since they constitute nonnested models (Becher and Wahrendorf, 19??)]. This implies that the effect of TCDD on tumor incidence in these rodents is more likely due to a mutational effect than a mitogenic effect on initiated cells. Caution must be taken when interpreting this result. First, no statistical confidence can be placed on this statement, so the observed difference in the two models may be due solely to random change. Second, this statement is only justified within the restricted context of this two-stage model of carcinogenesis. If any assumptions of the model are incorrect (independent cell action, constant rates, two stages, etc.), the interpretation based on this model could be biased.

The problem with identifiable parameters also remains with this formulation of the model. This is illustrated by the fifth model in Table C-1. In this model, the birth rate in the lowest dose group was fixed to be zero. The resulting likelihood and estimates of μ_{N-I} and μ_{I-M} remained the same. The estimated birth rates (β_I and death rate δ_I) changed, but the difference between the two in the various dose groups did not change. Repeated applications of this formulation of the model confirmed this problem of identifiability in this case.

With this problem of identifiability, there are basically only two parameters to be estimated for the two-stage model for each dose group from these data. These are the mutation parameter, given by $\mu = \mu_{N-I} \cdot \mu_{I-M}$, and the proliferation parameter, given by $\Pi = \beta_I - \delta_I$. Table C-2 gives the estimates of these parameters for the simple models considered here. The first model corresponds to the case where we allow both the mutation

Table C-2. Net parameter estimates from the two-stage model of carcinogenesis

Two-stage parameters changed	Doses				Other parameters
	Control	1 ng/kg/day	10 ng/kg/day	100 ng/kg/day	
Both	1.37×10^{-22} 0.0376	0.077×10^{-22} 0.0317	8.86×10^{-22} 0.0283	3.43×10^{-15} 0.0040	
Both (proliferation rate < 0.02)	2.80×10^{-20} 0.02	1.91×10^{-20} 0.0196	18.79×10^{-20} 0.0196	3.58×10^{-15} 0.00198	
Mutation rates	3.34×10^{-17}	1.79×10^{-17}	17.8×10^{-17}	76.0×10^{-17}	Proliferation rate = 6.90×10^{-3}
Proliferation rates	-0.0055	-0.0121	0.00362	0.00669	Mutation rate = 7.73×10^{-16}

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parameter and the proliferation parameter to change as a function of dose (model (1) in Table C-1). For this model, as dose increases, the mutation parameter drops to one-half the control value for the 1 ng/kg/day dose group and increases substantially in the remaining two dose groups (6.5-fold for 10 ng/kg/day and 25×10^6 -fold for 100 ng/kg/day). The hepatocyte replication parameter drops as a function of dose. In the 100 ng/kg/day dose group, the replication parameter is small relative to control (tenfold smaller) to adjust for the much larger mutation parameter. The reason for this particular pattern is clear if one studies the tumor incidence data from the Kociba et al. (19??) study. Over time, the tumor incidence in the highest dose group is larger than the others (the mutation parameter and the replication parameter combine to control the magnitude of the response) but climb less steeply with time (this is controlled by the replication parameter).

The replication rates in the control group and the two lowest dose groups are very high. Assuming the death rate is zero, this would correspond to a labeling index of 40.9 percent for a 7-day labeling experiment in the control animals. If the death rate is >0 , the labeling index would be even larger. Also, if one cell entered the initiated state on day 1, by 730 days (2 years), one would expect a clone of size 8×10^{11} , ~ 140 times the size of a normal rat liver. To control for this problem, the same model was fit to the data with the replication parameter constrained to be less than 0.02 (a first-day clone would be expected to have a size of 2×10^6 by study end). This resulted in the same pattern of mutation parameters but with a mutation parameter two orders of magnitude larger in the control and two lowest dose groups. For all three groups, the replication parameter was estimated to be at the boundary (0.02). The parameters for the high-dose group did not change.

The model in which the replication parameter is constant across dose groups and the mutation parameter changes for each new dose is given in row 3 ("Mutation Rates") of Table C-2. The pattern in this case is very clear and matches the observed cumulative tumor incidence well with the mutation rate dropping in the 1 ng/kg/day dose group then rising in the remaining groups. This observed drop is not statistically significant from the control mutation parameter (likelihood ratio test, $p > 0.05$). The constant replication rate (6.9×10^{-3})

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is reasonable and is not likely to produce unrealistically large clones of initiated cells. As mentioned before, this model provided a significantly worse fit to the data than did the unrestricted model.

Finally, the estimated parameters for the model in which the replication parameter changed with dose and the mutation parameter was constant over all doses is given by the last row ("Replication Rates") in Table C-2. The change in replication rates mirrors the tumor response in the same manner as was seen for the mutation rates. The replication rates are of reasonable size and should not produce impossibly large clones. As mentioned earlier, this model does not fit the data as well as the unrestricted model or as well as the model with fixed replication rate over dose and varying mutation rate over dose.

There is other evidence that can be used to examine the adequacy of this model for tumor incidence from exposure to TCDD. Lucier and colleagues recently conducted an initiation/promotion study in female Sprague-Dawley rats (Tritscher et al., 1992; Kohn et al., 1992; Portier et al., 1992; Sewall et al., 1992; Maronpot et al., 1992). In this study, they measured number and size of preneoplastic foci in liver sections. It has been suggested that the cells in these lesions correspond to the initiated cells in the two-stage model of carcinogenesis. If this is true, it is possible to apply the methods of DeWanji et al. (1990) to these data to analyze the growth characteristics of these cells (Moolgavkar et al., 1991). However, as before, we run into the problem of nonidentifiability. Since the data in these studies were collected at only one time point, it may not be possible to estimate all of the parameters in the first half of the two-stage model (i.e., μ_{N-I} , β_I , and δ_I) and get a unique solution. For each dose group, one of these parameters should come from outside information or continuous dose-response models should be assumed.

Maronpot et al. (1992) measured the rate of cell proliferation in these rodents (Tritscher et al., 1992) using immunocytochemical detection of cells that had undergone replicative DNA synthesis. The average labeling index by dose group is given in Table C-3 for the uninitiated animals (saline controls) in this study. It is seen there is a slight drop in mean labeling index ($p > 0.05$) from control to the group given 3.5 ng/kg/day. The labeling

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index then increases with increasing dose. Under the assumption of a linear birth death process, it is possible to convert these labeling indices into estimated birth rates for these cells using the formula:

$$\beta = \frac{1}{2t} \ln \left(\frac{1}{1 - LI} \right)$$

where β is the birth rate, t is the number of days over which labeling was done, and LI is the labeling index (Moolgavkar and Lubeck, 1992). This conversion is given in row 2 of Table C-3. These rates are in nonfocal hepatocytes (normal cells) and correspond to an average of one to two births per year per hepatocyte. TCDD seems to double the control birth rate for a dose of 125 ng/kg/day (a relative change of almost 100%--row 3 of Table C-3).

Maronpot et al. (1992) also calculated the labeling index in focal hepatocytes in the high-dose group from this study. The mean LI for animals in the initiated group exposed to 125 ng/kg/day of TCDD was 42.5 percent, which corresponds to a birth rate of 0.0395. The ratio of birth rate in nonfocal cells to birth rate in focal cells is $0.0395/0.00526 = 7.5$. Assuming this ratio is constant over all dose groups, we can rescale the birth rates in the remaining dose groups to correspond to birth rates for focal cells resulting in the rates shown as row 4 in Table C-3.

Using the same method as that used by Moolgavkar et al. (1991), given these birth rates, it is possible to estimate $\mu_{N,1}$ and δ_1 for the focal lesion data of Lucier et al. (1991). The resulting model did not fit these focal lesion data.

To get a handle on how large the labeling index would have to be to result in a reasonably good fit to the data, the model was again fit to the liver foci data, allowing β_1 to roam freely and the ratio δ_1/β_1 to be <0 . The resulting parameter estimates are given in Table C-4.

Table C-3. Results from two-stage model for hepatocarcinogenesis in rats						
Model	Parameter	TCDD Dose Administered				
		Control	3.5 ng/kg/day	10.7 ng/kg/day	35.7 ng/kg/day	125 ng/kg/day
Labeling index data and calculations	Labeling index	3.41	3.22	4.87	5.31	7.09
	Birth rates	2.75×10^{-3}	2.34×10^{-3}	3.56×10^{-3}	3.90×10^{-3}	5.26×10^{-3}
	Relative change (%)	0	-14.9	29.5	41.8	91.3
	Scaled birth rates	2.06×10^{-2}	1.76×10^{-2}	2.67×10^{-2}	2.93×10^{-2}	3.95×10^{-2}

SOURCE: Lucier et al., 1991; Tritscher et al., 1991; Maronpot et al., 1992.

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There is considerable instability in the estimated model parameters for the nonparametric model (A) in Table C-4, possibly due to the fact that there may not be sufficient data in each dose group to allow for the estimation of individual parameters. This is especially true for the low-dose groups in which there are few focal lesions and they are generally of small size. However, the model does fit the data rather well.

The first task is to test if the effect of dose, within the context of this model, is significant or not. This can be restated as the hypothesis that one set of model parameters will fit all five treatment groups. This is accomplished by fitting the same two-stage model to all five data sets, treating them as one large experimental group (this model is not shown in the table). Using a likelihood ratio test, there is a significant change in the two-stage model parameters with $p < 0.001$. The next step is to attempt to simplify the final model to include fewer parameters and provide a continuous description of the effect of dose on the various model parameters.

Based on the existing understanding of how TCDD induces PGST+foci in this biological two-stage model, it would be natural to assume that $X_1\mu_1$, the mutation rate from normal cells to initiated cells, does not change as a function of dose. Model B in Table C-4 addresses this basic model. In fitting a new model to these data, the first task is to assess, using a proper statistical test, if this model fits the data as well as the original model. Using the likelihoods, it is possible to test this hypothesis using a likelihood ratio test. For these data, this yields $p=0.673$, indicating that a model with a constant mutation rate fits the data as well as the fully nonparametric model and suggesting that TCDD at most causes a marginal increase in either X_0 , μ_1 , or both. Maronpot et al. (1992) noted some evidence of hepatotoxicity via histopathological changes, small increases in relative liver weight and alterations in serum chemistry. The increases in relative liver weight would suggest the effect may be on X_0 , but the hepatocytes were also enlarged making this determination difficult. As discussed in Kohn et al. (1992) and Portier et al. (1993), it is possible for TCDD to have a small effect on mutations in these animals via a secondary pathway. This finding is consistent with that conjecture.

Table 1: Estimated model parameters in the two-stage mathematical model of carcinogenesis (Figure 1) for the non-initiated (saline) animal

Dose ^a	$X_0\mu_1^b$ (mutation rate)	β^c (birth rate)	ρ^d (death/birth)	log-likelihood
A. Fully nonparametric in dose				
Control (0.0)	$3.851 \cdot 10^{-4}$	$3.041 \cdot 10^{-2}$	$6.077 \cdot 10^{-2}$	282.79
3.5	$4.346 \cdot 10^{-4}$	$3.548 \cdot 10^{-2}$	$6.062 \cdot 10^{-2}$	
10.7	$3.860 \cdot 10^{-4}$	$3.749 \cdot 10^{-2}$	$1.267 \cdot 10^{-1}$	
35.7	$9.964 \cdot 10^{-4}$	$3.576 \cdot 10^{-2}$	$3.354 \cdot 10^{-2}$	
125.0	$7.0435 \cdot 10^{-4}$	$3.664 \cdot 10^{-2}$	$6.210 \cdot 10^{-2}$	
B. Constant mutation rate, other parameters nonparametric in dose (pure promoter)				
Control (0.0)	$8.423 \cdot 10^{-4}$	$6.778 \cdot 10^{-2}$	$6.385 \cdot 10^{-1}$	281.62 ($p=0.673$)
3.5		$6.045 \cdot 10^{-2}$	$4.998 \cdot 10^{-1}$	
10.7		$9.510 \cdot 10^{-2}$	$6.988 \cdot 10^{-1}$	
35.7		$3.509 \cdot 10^{-2}$	$0.000 \cdot 10^{-1}$	
125.0		$3.372 \cdot 10^{-2}$	$0.000 \cdot 10^{-1}$	
C. Constant ratio (ρ) and birth rate (β), nonparametric for $X_0\mu_1$ (pure initiator)				
Control (0.0)	$0.241 \cdot 10^{-3}$	$3.578 \cdot 10^{-2}$	$5.386 \cdot 10^{-2}$	280.78 ($p=0.855$)
3.5	$0.415 \cdot 10^{-3}$			
10.7	$0.339 \cdot 10^{-3}$			
35.7	$1.066 \cdot 10^{-3}$			
125.0	$0.731 \cdot 10^{-3}$			
D. Constant ratio (ρ) and hyperbolic ^e birth rate (β), $X_0\mu_1$				
Intercept	$3.070 \cdot 10^{-4}$	$3.003 \cdot 10^{-2}$	$3.825 \cdot 10^{-3}$	276.73 ($p=0.146$)
$V_{\max}$	$5.909 \cdot 10^{-4}$	$4.948 \cdot 10^{-3}$		
k_d	18.17	2.20		

^a ng/kg body weight/day.

^b mutations per day per mm³ of liver.

^c births per cell per day.

^d deaths per birth.

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e. parametric form is $\text{rate}(\text{dose}) = \text{intercept} + E_{\max} \text{dose} / (D_{0.5} + \text{dose})$.

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Over the entire dose range, the ratio of death rate to birth rate follows an erratic pattern that varies over several orders of magnitude. Sensitivity analysis shows that the likelihood is insensitive to rather large changes in the ratio, suggesting it may be possible to get an adequate fit to these data with a constant value for ρ across all dose groups. Analyses done by Moolgavkar et al. () and Luebeck et al. () relied on this assumption. A model with ρ constant across all dose groups indicates that there is no effect of TCDD on the ratio of the death rate to the birth rate of PGST+foci in uninitiated animals.

Model C, in contrast to model B, tests the hypothesis that there is no effect of TCDD on growth rates of initiated cells. The results are not significant (xxx?) indicating a pure initiation model fits these data as well as a pure promotion model. Model C also provides a much clearer pattern to the effect of dose on the mutation rate and birth rate for the two-stage model. The mutation rate ($X_{O\mu_1}$) basically increases until the highest dose group where there is a slight decrease. This decrease may be due to a single animal in the 35.7 ng/kg/day dose group. This would suggest that we might model the mutation rate (and the birth rate) by the hyperbolic function; for the mutation rate, this would be given as

$$X_{O\mu_1}(\text{dose}) = \text{intercept} + V_{\max} \text{dose}/(k_d + \text{dose}) \quad [1]$$

A similar model is used for the birth rate. The parameter estimates for this model are presented in Table C-4 as Model D. A likelihood ratio test indicates that this model provides an adequate fit to these data ($p=0.146$).

Several other models were fit to these data. These models were various combinations of constant, nonparametric, linear, and hyperbolic dose-response functions for the three rate functions ($X_{O\mu_1}$, β , and ρ) for the two-stage model. The model given in Table C-4 as Model D seems to be the best parameterization that agrees with the data and the biological understanding of the carcinogenicity of TCDD.

The DEN-initiated animals must be handled differently than the noninitiated group. It is believed that initiation with DEN results in a number of the normal cells undergoing

mutation. This process is assumed to occur in a very short period. To accommodate initiation, Luebeck et al. () introduced a second mutation rate process at the start of the experiment. These data were fit using the methods of Luebeck et al (). The resulting model agrees very closely with the model for the saline-treated animals, the main difference being a larger mutation rate.

The calculations have hidden assumptions that could have a bearing on the interpretation of the results. First, we have ignored the changes in labeling index in the normal hepatocytes. This could change the size of the pool of normal hepatocytes, which is assumed constant for these calculations. This might alter the parameter estimates. In addition, these changes in the rate of replication in nonfocal tissue could alter the mutation rate μ_{N-1} (Portier and Kopp-Schneider, 1991). As for tumor incidence, the model assumes constant rates and independent cell kinetics. Finally, the large estimated percentage of cells that are initiated can only imply most of these initiated cells are single cells and cannot be observed on histopathological examination. This has not been verified. The analysis is very sensitive to the choice of values for the radius of a cell (Moolgavkar et al., 1991) and to the minimum size of a detectable focus. These issues cannot be discussed fully in this brief document.

APPENDIX D

It is possible to compare the parameters estimated for the two-stage model to predictions from the PB-PK models to try to locate a reasonable mechanistic link between the two classes of models, to aid in species extrapolation and to help guide us in choosing the most appropriate curvature for low-dose extrapolation. The two-stage model parameters that were estimated from the tumor incidence data are compared to 14 dose surrogates from the 3 main PB-PK models reviewed earlier. Leung et al. (1990²) suggested using occupancy of the cytosolic (Ah) receptor, averaged over the length of the study. They also suggested using average binding to the microsomal proteins. These predictions are shown in Table D-1 for the study of Kociba et al. (1978), along with the predicted final concentration of TCDD in the liver of these animals. The correlation of these parameters with the two-stage model parameters is shown in the last 3 columns of Table D-1 in rows 2 ("Both"), 3 ("Mut. Rates") and 4 ("Prol. Rates"). It is clear that these three surrogates correlate well with all but the two-stage model in which the effects of TCDD were treated as pure promotional effects. The high correlations and low p-values for these correlations is driven by the order of magnitude differences in the parameters and the dose surrogates. (Caution should be used in judging these correlations to carry any weight of scientific evidence.)

1990² Andersen et al. (1992) suggested four dose surrogates to be used in any risk assessment for TCDD. These are based upon free TCDD in the liver, total TCDD in the liver, amount of induced CYP1A1, and amount of induced CYP1A2. As for Leung et al. (1990²), these are integrated over the lifespan of the animal (in this case, total integrated rather than average, but this will not affect the correlations). As for Leung et al. (1990²), the correlation with all two-stage model formulations/parameters is significantly different from zero for three of the surrogate doses (excluding CYP1A2) and all but the promotion-only TCDD model. The correlation of induced CYP1A2 with the two-stage model parameters is not significantly different from zero for all two-stage models. Note that the highly nonlinear

Table D-1. A comparison of dose-surrogates with parameters estimates from the two-stage model of carcinogenesis

Dose surrogate	Dose				Correlation ^a		
	Control	1 ng/kg/day	10 ng/kg/day	100 ng/kg/day	Both ^b	Mut. Rates	Prol. Rates
Loung et al. (1988)							
Concentration ^c	0.0	0.3	4.4	69.4	1.00 ⁺⁺ -1.00 ⁺⁺	0.99 ⁺	0.70
Ah-receptor occupancy	0	2	17	61	0.96 ⁺ -0.96 ⁺	1.00 ⁺⁺	0.81
Microsomal binding ^d	25	29	132	132	0.96 ⁺ -0.96 ⁺	1.00 ⁺⁺	0.81
Andersen et al. (1992)							
Free TCDD in liver ^f	0	6.4	53	420	0.99 ⁺⁺ -0.99 ⁺⁺	1.00 ⁺⁺	0.73
Total TCDD in liver ^g	0	590	11,000	120,000	1.00 ⁺⁺ -1.00 ⁺⁺	1.00 ⁺⁺	0.72
Induced CYP1A1 ^h	0	3900	270,000	200,000	0.99 ⁺⁺ -0.99 ⁺	1.00 ⁺⁺	0.74
Induced CYP1A2 ⁱ	0	57,000	210,000	320,000	0.79 -0.79	0.89	0.87
Kohn et al. (1992)							
Free TCDD in liver ^j	0	0.1237	0.7663	5.0522	0.99 ⁺ -0.99 ⁺	1.00 ⁺⁺	0.74
Ah-receptor/TCDD complex ^k	0	1.0202	8.8617	49.352	0.99 ⁺ -0.98 ⁺	1.00 ⁺⁺	0.76
CYP1A2/TCDD complex ^l	0	1.6857	27.3142	464.85	1.00 ⁺⁺ -1.00 ⁺⁺	0.99 ⁺	0.69
TGF- α ^m	0	0.005899	0.05408	0.3788	0.99 ⁺⁺ -0.98 ⁺	1.00 ⁺⁺	0.74
Internalized EGF receptor ⁿ	0	0.4829	3.7213	13.668	0.97 ⁺ -0.96	1.00 ⁺⁺	0.83
Total CYP1A1 ^o	190	2198	14,213	42,196	0.95 -0.95	0.99 ⁺⁺	0.83
Total CYP1A2 ^p	4517	5999	15,747	44,579	0.96 ⁺ -0.96 ⁺	1.00 ⁺⁺	0.81

^aPearson correlation coefficient - + indicates statistical significance at the 0.05 level, ++ indicates significance at the 0.01 level (caution should be used in applying the p values too literally due to the small sample sizes involved).

CYP1A1 curve correlates well with the first two two-stage models. If this were used as a dose surrogate for TCDD toxicity (in the independent framework chosen by Andersen et al. [1993]), the resulting low-dose risks would be substantially smaller than those of any other dose surrogate. Induced CYP1A2 fails to correlate due to the strong saturation seen at the two highest doses; an effect not observed in the two-stage model fits.

Kohn et al. (1993) did not suggest specific dose surrogates but did suggest mechanisms that naturally lead to the choice of certain surrogates. Seven such surrogates are given in Table D-1: free liver TCDD, Ah-receptor bound TCDD, CYP1A2-bound TCDD, integrated TGF- α , internalized EGF receptor, integrated CYP1A1, and integrated CYP1A2. With the exception of integrated CYP1A1, all dose surrogates correlated as well with the two-stage parameters as the dose surrogates from the other two PB-PK models. None of the surrogates resulted in correlations with the promotion model, which were significantly >0 . The internalized EGF receptor correlates well with the promotion model but not significantly better than 0 and not better than induced CYP1A2 in the Andersen et al. (1993) model. Total CYP1A2, which Kohn et al. (1993) suggest could be tied to secondary mutagenic effects of TCDD, correlates well with the mutation model. All of the dose surrogates predicted by the Kohn et al. (1993) model have positive slope at dose 0 and would behave, in the low-dose region, as a linear model. Risk estimates utilizing these dose surrogates and the two-stage model would likely result in a $<$ tenfold change in risk over what would be estimated by applying a one-stage model to the Kociba et al. (1978) data.

It should be possible to correlate these same dose surrogates with the model parameters arising from the liver foci analysis. However, none of the authors calculated dose surrogates for this design situation. This can be done at a later time.

One final point on dose surrogates. The choice of which measure of exposure you decide to correlate with which measure of effect is a somewhat arbitrary decision. The choices above were mostly chosen because they have traditionally been used in this context. However, there is no pressing mechanistic reason why these particular choices are best. The

major two variables in this decision are which endpoints(s) and how to consider time factors. For example, we could also have considered induced amount of Ah receptor as a dose surrogate; there is no a priori reason to exclude it. It is also possible to integrate over shorter periods of time. This is not really mechanistically justifiable for TCDD, but for other compounds, which show a short mitogenic effect or get rapidly metabolized into toxic compounds of brief duration, shorter integration periods would be more appropriate. Thus, some thought should be given to a choice of dose surrogate based on mechanistic considerations. The paper by Kohn et al. (1993) discussing their mechanistic model can be used to provide considerable direction on this topic (see Appendix A).

TETRACHLORODIBENZO-P-DIOXIN EMPIRICAL RELATIONSHIPS FOR NON-CANCER ENDPOINTS

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I. INTRODUCTION

Dose-response assessment is an essential element in determining health risks associated with environmental contaminants. The evaluation of dose-response requires information on a range of quantifiable doses or exposures and corresponding measured responses. The study of adverse effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the use of dioxins as tools to elucidate underlying biochemical and physiological mechanisms of cellular action are pivotal in the overall evaluation of dioxin exposure. The determination of the shape, slope and of the dose-response functions of TCDD for several endpoints, over multiple time points and in multiple species, is critical to the understanding of the potential human health risks and exposure to dioxin.

Dose-response relationships for several endpoints can be established using human data (Poole, this document) however quantification of exposure can be difficult. The assessment of TCDD presented here primarily employs controlled animal studies in which administered dose is known and delivered dose is either measured or can be estimated. The endpoints described were those found to have some consistency across species and strains. This extensive review of dose-response relationships has been completed to better elucidate key biomarkers or surrogates for cancer and other life threatening diseases.

TCDD and members of this family of compounds are exogenous ligands for the Ah receptor(AhR). This receptor had originally been hypothesized to be a member of the nuclear

hormone receptor family. The overall hypothesis of TCDD action, put forth by several groups, (see Whitlock chapter this document) can be simplified as follows: TCDD and the other members of the family (ligands) enter the cell by mass action, bind to the AhR (receptor), the receptor-ligand complex translocates to the nucleus via specific transport proteins and subsequently binds to specific sequences on the gene. This in turn evokes the production (or perhaps the suppression) of several mRNA species. Recent results have identified a specific consensus sequence on the gene which has been named the xenobiotic response element (XRE)(Whitlock,1991, Denison, 1991). In addition to the DNA sequence identification, the structure and amino acid sequence of the AhR protein was reported by Burbach et al., (1992). Both the XRE(s) and the structure of the AhR are analogous to the steroid receptors and their respective genomic response elements. This similarity is important in regard to biological models of TCDD action and risk assessment.

Dose-response relationships have been established for several endpoints in intact and surgically altered animals. In vitro experiments have been used to determine critical concentration and structural relationships for TCDD effects at the cellular and molecular level. In the vast majority of these studies the role of the AhR-ligand interaction has been essential but not necessarily sufficient to evoke a detectable biological response.

Although all toxic endpoints for TCDD may not be Ah locus mediated the evaluation of dose response allows us to conclude that the critically sensitive events are Ah receptor mediated. The multiple steps in receptor mediated processes suggest that the dose-response relationships are not linear. This series of cellular events can be best modeled as a simple series of log-differential equations which basically take the form of Michaelis-Menten hypotheses, which include Michaelis-Menten and Hill functions. This format allows for slope estimates, as

well as inhibition and saturation phenomena. Hence, if curvilinearity of the dose-response exists, it should be possible to model using kinetic equations. Indeed, this may well be the case for some end effects of TCDD, however, recent rodent experiments suggest the induction of cytochrome P-450IA1 (CypIA1) is linear through zero (curve fitting with a Hill function) using long term exposure and very low doses (Lucier et al., 1991a).

Low dose phenomenon like thymic atrophy and immunotoxicity have also been described and are the result of multistep processes in which many of the steps are unknown, and others are non-specific. Non-cancer endpoints have generally not been subject to dose-response modeling such as that used for assessment of cancer endpoints although many have been consistently reported after TCDD administration. The focus of this dose-response assessment is on toxic endpoints for which a mechanism has been hypothesized and on the description of low dose effects. In addition, there is the practical criteria of using a satisfactory number of dose points.

To improve our understanding of non-cancer endpoints and to elucidate possible mechanistic relationships between endpoints, several simple functional forms were used in the empirical analysis of relationships between TCDD dose and non-cancer endpoints. Throughout the document datasets have been selected for a more rigorous dose-response assessment. The criteria used for inclusion is endpoint and dataset specific, but generally it depends on the number and range of dose points. Specific rationale for selection is described with the specific endpoint. Because of the exploratory nature of this analysis the number of assumptions regarding dose-response relationships were kept to a minimum. Each functional form used will be briefly discussed with comments on the nature of these assumptions. For a more extensive mathematically detailed explanation of these functions see Appendix.

Initially a x-y plot of the data was made to provide a visual examination of dose-response. Attempts to use the Linear Multistage Model for fitting the dose response did not give satisfactory correlation coefficients. In addition the underlying biological assumptions for this model would not be appropriately applied to the non-cancer endpoints described in this document. Although these graphs are not included in this document they were used as a first step in the analysis. The first and simplest model used to describe selected data sets was the linear function.

The Linear Model

$$y = mx + b$$

(x = dose; y = response; m = slope; b = Background response)

Many dose-response relationships in pharmacology and toxicology are known to fit the linear model. For example, the elimination of some chemicals (ie., chloroform) from plasma obeys 1st order kinetics and as such is modeled as a linear function. In many cases the actual dose-response relationships are nonlinear but appropriate transformations of the independent and dependent variables are used to obtain a linear relationship. For example, Scatchard and Lineweaver-Burke plots represent ligand-receptor binding using equations in a linear form however, the binding phenomenon is non-linear (Tallarida and Jacob, 1979). The success of the linear fit is typically evaluated via least squares criteria resulting in an R^2 value which quantifies the "goodness of fit". However, in the case of a nonlinear phenomenon that has been transformed into a linear relationship, the transformation introduces a bias in the R^2 estimate. (These R^2 values are not directly compared to those derived from linear functions).

The second functional form used to evaluate the TCDD dose-response relationships was the Michaelis-Menten equation. This is a hyperbolic function often used to fit the relationship between enzyme-substrate or receptor ligand interactions.

The Michaelis-Menten Model

$$y = ax / (b+x)$$

(x = dose; y = response; a = maximum response; b = concentration of x at 1/2 maximum response)

The assumption inherent when plotting the ligand-receptor binding relationship is that there is a single rate function that represents the relationship between the free receptor and the bound receptor over specified doses, and that a finite number of binding sites must be occupied to initiate a response and that saturation of the receptors occurs, and that this maximum interaction is reached at some finite dose (Goldstein et al., 1974).

The third function used was the Power Law formalism. The relevant equation is nonlinear and includes a power (not necessarily an integer) as a term in the function.

Power Law Function

$$y = ax^g$$

(x = dose; y = response; g = kinetic order; a = limiting response)

This functional form has been historically used to represent a variety of nonlinear biological phenomenon including the saturation of, or Synergy in, enzyme systems; thus the name "S-Systems" (Savageau, 1991). This formalism has been used to describe processes obeying power laws which provides adequate descriptions of many nonlinear interactions that occur in nature. It is not a surrogate for Michaelis-Menten function, but provides an approximation of individual

biological interactions or associations. The exponent in the Power Law function ("g") is related to the pharmacological kinetic order (rate of response) of the observed process. If the "g" value is greater than 1 the response is superlinear and positive cooperativity is assumed, whereas if the response is less than 1 and sublinear, then negative cooperativity is assumed. The Power Law has fewer degrees of freedom than the Hill function (see below) and it can fit subsets of doses allowing for different reaction rates to be determined. Although it has not been specifically applied to toxicological results for TCDD, the generalized approach of the Power Law formalism is compatible with the pleiotropic responses seen after TCDD administration (Savageau, 1991). The Hill and Michaelis-Menten functions describe a response over an entire dose range. However, the Power Law, when applied to subsets of a data, may provide a mechanism for describing changes in underlying biology from low to high doses.

The fourth function used in this analysis was the Hill function. This nonlinear function is a generalization of the Michaelis-Menten equation as it incorporates in it the flexibility of the Power Law approach.

The Hill Function

$$y = (ax^g) / (b + x^g)$$

(x = dose; y = response; g = kinetic order; b = concentration of x at 1/2 maximum response; a = maximum response)

It has been used to model receptor-ligand interactions, and because of its wide acceptance and flexibility an attempt to fit this formalism was made for selected datasets (Boeynaems and Dumont, 1975). As in the Power Law formalism, the value of the "g" exponent in the Hill plot determines the behavior of dose response: if greater than 1 the response is superlinear, if the

exponent is less than 1 it is sublinear¹. The Hill plot is not as simple as the Power Law function because of the greater number of variables. The Hill plot assumes saturation at the high doses, as well as an "inertial"² type of response at low doses. If the underlying biology of a system indicates that saturation occurs at high doses, then the Power Law function, because it is exponential, does not accurately describe the high dose phenomenon.

In the analysis of empirical relationships selected data sets were tested using various functional fits. The Michaelis-Menten and Hill functions were adopted for use because it is the most widely used model that assumed receptor-ligand or substrate-enzyme interactions. The nonlinear model used to fit all data sets was the Power Law function because of its simplicity. Initially these functions were used to fit the percent-response form of data which was used as a relative normalization procedure. Subsequently, these functions were used to fit raw data. The Power Law function, because it was not constrained by underlying assumptions, was then used to fit ranges of doses to look for changes in the exponent possibly detecting rate changes. Analysis of the relationships for specific endpoints are discussed in detail throughout this chapter with the descriptions of the specific endpoints.

Because many of the data sets have few values much of the available statistical analysis (mean square error of fit, analysis of co-linearity) were not performed. Hypothesis testing to evaluate curve fits was also limited by the number of data points. These data could be made

¹ A superlinear dose-response could generally suggest positive cooperativity or synergy through the measured dose-response range, whereas, a sublinear dose response could imply negative cooperativity or an inhibitory effect.

² The definition of inertia is " a property of matter by which it remains at rest or in some uniform motion in the same direction unless acted upon by a net external force" Merkin, 1985

more robust by the analysis of raw data. Since rigorous statistical analysis was not applied to the data three criteria were used to conclude a "poor fit" existed. First a low R^2 or a high chi square value, second, visual observation of the curve fit, and third the inability to fit the data to a particular function.

Table I: Summary of Dose Response Functions		
LINEAR MODEL	Assumes biological response is linear.	$R^2 > 0.8$ typically assumes good fit
MICHAELIS-MENTEN FUNCTION	1. Assumes a non-linear biological phenomenon. 2. Assumes minimal or no response at low doses. 3. Assumes maximum activity is reached in dose range.	Chi Square "goodness of fit" dependent on degrees of freedom
HILL FUNCTION	1. Assumes a non-linear biological phenomenon. 2. Assumes low dose response is negligible or "inertial". 3. Assumes saturation or maximal activity is reached at high doses.	Chi Square "goodness of fit" dependent on degrees of freedom
POWER LAW FUNCTION	1. Assumes a non-linear biological phenomenon. 2. Assumes synergy or cooperativity.	$R^2 > 0.8$ typically suggests a good fit; however it should be taken into account that the R^2 estimate is logarithmically biased.

In assessing potential health risks, an analysis of dose-response provides an estimate of the severity of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin. The focus of this dose-response assessment is on studies using multiple doses and on evaluating multiple endpoints in several species. The hope, in evaluating responses in a variety of species, is that consistency will add confidence to the estimates of human health risks subsequently made. This report emphasizes reevaluation of effects that occur at low doses; these being the most relevant to environmental health. Responses at higher doses are also included to demonstrate species differences and estimations of what might occur under incidental high exposures (such as industrial accidents). Experimental evidence indicates that TCDD is extremely toxic in some systems at low doses. However, although qualitatively similar, the magnitude of response is species and strain specific.

II. ACUTE TOXICITY

A large body of data exists describing the acute toxicity of TCDD in a variety of species. Acute data on lethality is briefly described here and will be referred to throughout the document. Acute responses are generally considered Ah receptor mediated segregating with the Ah locus. In this analysis of non-cancer endpoints this acute toxicity information is used as a basis of comparing tissue, species and strain sensitivity. Sensitivities that differ from those listed in Table II will be probed for endpoint specific factors that may modify the response.

The acute responses listed in Table II are LD_{50} 's which vary almost 10,000 fold. The accumulated levels of TCDD causing lethality after continuous administration are consistent with the levels causing effects after single exposure which strongly suggests that over short periods of time the total body burden is the rate limiting factor in morbidity. It is generally recognized (Poland and Knutson, 1982) that the guinea pig is most sensitive to the lethal effects of TCDD followed by the rat (sensitive strain), the mouse (sensitive strains), rat and mice (non-sensitive strains) and by the hamster. However, the rhesus monkey (see below) may be more sensitive than the guinea pig. Because of the variety of protocols used to assess acute toxicity, several studies are reviewed here.

In a continuous feeding study, rhesus monkeys were administered 500 ppt TCDD for 9 months (Allen et. al. 1977). Four months after this treatment period 4 of the 8 animals died after receiving an estimated total dose of 18.6 ug/animal (approximately 3.5 ug TCDD/kg or 0.013 ug TCDD/kg/day). Based on these data the LD_{50} may be considerably less than the 70 ug TCDD/kg (lowest dose tested) reported by McConnell et al., (1978) (Table II). In a continuous feeding study in Hartley guinea pigs, 5 of 10 males died after a total dose administration of 0.6 ug TCDD/kg to 1.17 ug TCDD/kg. Four of 10 females died after a total dose administration

Table II
Comparative Acute Toxicity of TCDD
LD50 Values

Species	Sex		LD50 ug TCDD/kg	Time to Death	Reference
Hartley guinea pig	Male	oral	0.6	5-34 days	Schwetz, 1973
Hartley guinea pig	female	oral	2.5	42 days	Silkworth et al., 1982
Hartley guinea pig	male	oral	2.1	19-42 days	Schwetz et al., 1973
Golden syrian Hamster	male	i.p.	>3000	50 days	Olson, et al., 1980
Golden syrian Hamster		oral	1157	50 days	Olson et al., 1980
Golden syrian Hamster	male	oral	5051	26-43 days	Henck et al., 1981
Sherman (Spartan) rat	male	oral	22	9-27 days	Schwetz et al., 1973
Sherman (Spartan) rat	female	oral	45	13-43 days	Schwetz et al., 1973
Han/Wistar rat Long Evans	male	i.p.	>3000 10		Pohjanvirta et al., 1990
Fischer rat Charles River Fischer rat Harlan Fischer rat Fredrick Cancer Center CD rat Charles River	male	oral	164 340 303 297	24.8 ± 0.6 28.3 ± 0.5 25.9 ± 0.8 24.5 ± 1.0	Walden, 1985
New Zealand albino rabbit	male and female	oral	115	6-39 days	Schwetz et al., 1973
New Zealand albino rabbit	male and female	dermal	275	12-22 days	Schwetz et al., 1973
Rhesus monkey	female	oral	<70 ug TCDD/kg	approx 4 weeks	McConnell, et al., 1978
Rhesus monkey	female	oral	3.5 ug TCDD/kg 0.013 ug TCDD/kg/day	28-44 weeks	Allen, et al., 1977
DBA/2J mice	male	oral	2570	30 days	Chapman and Schiller, 1985
B6D2 ₁ /J mice	male	oral	296	30 days	Chapman and Schiller, 1985
C57BL/6J mice	male	oral	182	30 days	Chapman and Schiller, 1985
C57BL/6J (Ah ^{bb})	male	oral	159	22 days	Birnbaum, et al., 1990
C57BL/6J (Ah ^{dd})	male	oral	3351	22 days	Birnbaum, et al., 1990
C57BL mice	male	oral	114	15-24 days	Vos et al., 1973

of 1.3 to 1.8 ug TCDD/kg (DeCaprio et al., 1986). Although this phenomenon has not been fully evaluated, female animals in general appear to be less sensitive to the acute toxicity of TCDD than do the males. This is exemplified by female guinea pigs and Sherman rats which were found to be less sensitive to the lethal effects of TCDD than the male animals (Table II). The magnitude of the response may vary across or within species, but there are a number of consistent lesions. At the measured LD₅₀ in all animals examined, there is a species specific delay in onset of the appearance of toxicity, generally about three weeks. Animals begin to lose weight immediately and continue to lose up to 50% of their body weight, depleting their adipose tissue until their death. This is characterized as the wasting syndrome. Body weight loss was also observed after a single TCDD treatment in male Fischer F-344 rats (po 100 ug TCDD/kg), male albino guinea pigs (ip 2 ug TCDD/kg) and male C57BL/6 mice (po 360 ug TCDD/kg). Based on observations of food consumption of these animals and pair fed animals, Kelling et al. (1985) concluded that hypophagia appeared to cause some loss of adipose tissue accounting for weight loss. The precise mechanism underlying the wasting syndrome is not known however, body weight loss appears to contribute more to lethality in the some species and strains (ie., guinea pig and the Sprague Dawley rats than in others (ie., Fischer rats and C57BL/6 mice) (Kelling et al., 1985; Peterson, et al., 1984).

III. SYSTEMIC EFFECTS

A. INTRODUCTION

Administration of TCDD has consistently been shown to cause a variety of toxic effects. Most pathological changes were found to affect epithelial tissue; however, specific dermal lesions are observed in some species. In man, exposure to TCDD results in chloracne, a skin

lesion, which is characterized as "follicular hyperkeratosis in the absence of inflammatory comedones"³ In monkeys there is hair loss along with thickening and keratinization of glandular tissue. Oral administration in monkeys, rabbits, and hairless mice results in dermal lesions. Gastrointestinal lesions characterized as hyperplasia of the gastric mucous or intestinal epithelium have been found in monkeys, cows and hamsters but not in rats, and guinea pigs. Urinary tract hyperplasia has been reported in the guinea pig, monkey and cow. Liver toxicity, immunotoxicity, and thymic atrophy occur in several species at very low doses. In addition, tissue specific fetal development is affected by TCDD in several species (Poland and Knutson, 1982). These effects occur at low doses and some dose-response relationships have been established for a few of these endpoints. The comparison of dose response of several endpoints is the focus of this report. In all mammalian species tested thymic atrophy is one of the most sensitive clinical indicators of toxicity.

B. THYMIC ATROPHY

Guinea pigs are most sensitive to the acute lethal effects and to many of the systemic effects of TCDD. Harris et al. (1973) administered eight weekly doses of 0.0, 0.008, 0.04, or 0.2 ug TCDD/kg to female guinea pigs which resulted in a statistically significant⁴ decrease in thymus weight in the 0.04 ug TCDD/kg dose group (LOAEL = 0.0057 ug TCDD/kg/day equivalent dose, NOAEL = 0.0011 ug TCDD/kg/day). The Michaelis-Menten functional fit of this data (Graph A1)⁵ is poor primarily because of the minimal number of data points.

³Zugerman, C. (1990) Chloracne Clinical Manifestation and Etiology, *Dermatologic Clinics* 8(1), 209.

⁴All statistical significance described in this report was determined by the study investigators.

⁵ All graphs discussed in text are located at the end of the document.

Additional dose-response analysis was not performed on this data set. In A 90 day study, Decaprio et al. (1986) administered 2, 10, 76, and 430 ppt TCDD in the feed. Daily intake was calculated as 0.00012, 0.00061, 0.0049, and 0.026 ug TCDD/kg/day in males and 0.00012, 0.00068, 0.0049, and 0.031 ug TCDD/kg/day in females. In males, the more sensitive species in this study, the absolute and relative thymus weight was significantly different from controls at the 0.0049 ug TCDD/kg/day (See Table III). This level is comparable to that seen in the Harris et al. (1973) study.

In female rats, 31 daily doses of 0.0, 0.1, 1.0, and 10 ug TCDD/kg resulted in a decrease thymus weight after 24 days in the 0.1 ug TCDD/kg/day dose group (cumulative dose 0.24 ug/kg)(Harris, et al., 1973). In a 13 week study Kociba et. al., (1976) demonstrated a dose related decrease in thymus weight in male and female Sprague-Dawley (S-D) rats administered 0.1 ug TCDD/kg/day orally 5 days/week. Graphic depiction of this percent decrease in thymic weight in male and females (Graph A2) demonstrates a "good fit" to the Michaelis-Menten type response. The female Sprague Dawley rats appeared slightly more sensitive to this response, although the doses where a decrease in thymic weight was observed was the same. Microscopic examination revealed complete involution of the cortical region of the thymus of rats administered 1.0 ug/kg/day and slight involution in those administered 0.1 ug TCDD/kg/day. The statistically significant decrease in thymus weight appeared to be a sensitive indicator of microscopic changes in the thymus. A dose-dependent decrease in absolute thymus weight was reported in Rhesus monkeys administered acute oral doses of 70 ug TCDD/kg (lowest dose administered) or greater (McConnell, 1978). However, this dose may not accurately reflect the sensitivity of this endpoint in the monkey, but does provide information on the consistency of this response.

Thymic atrophy has been reported in rats, guinea pigs, monkeys, hamsters and there are suggestions of thymic effects in humans. Effects on thymic hormones have been observed in humans exposed (not quantified) to TCDD. A statistically significant lower mean serum thymosin_{alpha-1} was observed in a study that compared 94 people allegedly exposed to TCDD in contaminated residential areas to a nominally unexposed control population. The study found thymosin levels were inversely associated with years of living in the contaminated area (up to 7 years) when controlling for age and socioeconomic factors. Thymosin is produced in the epithelioid cells of the thymus and functions to modulate the maturation of prothrombocytes to mature thrombocytes. Decreased serum thymosin_{alpha-1} suggests an effect on the thymus in this human population exposed to environmental levels of TCDD. Unfortunately concentrations of TCDD in these areas not serum levels (a more accurate estimate of TCDD exposure) were reported (Stehr-Green et al., 1989).

In six studies a range of single doses of TCDD were administered orally to C57BL/6J mice. Four of these studies (McConnell et al., 1978a, Birnbaum, et al., 1990, Chapman and Schiller, 1985, and Bombick et al., 1988) tested doses that were greater than 30 ug TCDD/kg and found a statistically significant decrease in thymic weight or thymic atrophy at all doses tested. Vos, et al., (1974) administered single oral doses of 0, 0.2, 1.0, 5.0, and 25 ug TCDD/kg/day. At 1.0 there was a statistically significant decrease in thymus/bwt. with a NOAEL at 0.2 ug TCDD/kg. Kerkvliet and Brauner (1990) tested similar doses: 0, 0.2, 1, 2.0, and 5.0 ug TCDD/kg, in female C57BL/6J mice reporting a dose-dependent decrease in relative thymic weight. Graphic comparison of these two studies (Graph B1) demonstrates similar responses in the 1 to 10 ug/kg dose range, although the male mice of a different strain appear more sensitive. Since the linear range of the dose response curve lies between 0.2-10 ug

TCDD/kg a linear model was also fit to this range therefore data sets could be directly compared. [Graph B2 demonstrates that the slope of both these dose-response curves are similar (Vos, male mice = 0.57 and Kerkvliet, female mice = 0.46)]. It becomes apparent when comparing this Graph B2-(A) to Graph B2-(B) that the range of doses used for comparison is important. Although many studies describe thymic atrophy, few have enough data to plot comparative dose-response curves, therefore removing data points for this type of comparison will generally be problematic.

Poland and Glover (1980) found a dose-dependent decrease (graphically depicted) in thymic atrophy in C57BL/6J and DBA mice after a single i.p. injection. The ED_{50} was 10^{-5} mol/kg (3.22 ug TCDD/kg) in the C57BL/6J mice and the DBA/2J mice were 10 X less sensitive to this effect. The ED_{50} for induction of the Aryl Hydrocarbon Hydroxylase (AHH) in these animals was one-tenth the ED_{50} for thymic atrophy in both DBA and C57BL/6J mice.

In a review of the dose-response literature it is critical to note that the intra- and inter-species sensitivities are linked to the Ah locus (Whitlock, 1987). Different responses for TCDD in a variety of strains of mice were investigated to determine if the toxicity segregated with the Ah responsive mice. Thymic atrophy was observed after a single i.p. injection of 20 ug TCDD/kg TCDD was administered to sensitive species C57BL, C3H, and BALB mice. The same dose produced no thymic atrophy in DDD, AKR, and DBA mice (less sensitive species). These effects corresponded well with the induction of aryl hydrocarbon hydroxylase (AHH) activity in the livers of these strains of mice. The toxicity of TCDD to the thymus segregates with the Ah locus. (Nagayama, et al., 1989) Intraspecies differences do not appear to be related to different levels of cytosolic Ah receptor, but to a different cellular response to the ligand-receptor complex. Bombick et. al., (1988) administered multiple doses of TCDD in a single i.p.

injection to DBA and C57BL/6J mice. The LOAEL for thymic atrophy in the DBA (115 ug TCDD/kg) was four times greater than the LOAEL (30 ug TCDD/kg lowest dose tested) in the C57BL/6J mice. A comparison of thymic atrophy in wild type (sensitive) and congenic strains of C57BL/6J by Birnbaum, et al., (1990) indicated that the sensitivity of these strains is associated with the Ah locus.

Four strains of mice with differing susceptibility to Ah receptor mediated toxicity were used to investigate the role of this receptor in thymic atrophy and related immunotoxicity. C57BL/6, DBA, C3H/HEN, B6D2F1 male mice were administered a single i.p. injection of 0, 1.2, 6.0, and 30 ug TCDD/kg TCDD. All but the DBA mice showed a statistically significant decrease in thymic weight at the two highest doses. The dose-response relationships using a "Michaelis-Menten" type fit (See table III and Graph B3) demonstrate a good correlation between receptor induction and thymic atrophy (Vecchi-et al., 1983). The fit to some of the data sets was not satisfactory for the C57BL/6 and DBA mice, therefore the use of other functions was explored. These strain sensitivities were also demonstrated with the Power law function (Graph B4) with good correlation coefficients in the responsive strains, 0.84 to 0.97. Linear fits to these data can also be used to demonstrate relative sensitivity, however correlation coefficients are generally lower (Graph B5) resulting in a poor fit.

The comparison of Long Evans (LE) and Han/Wistar (H/W) rats provide a unique opportunity to observe intraspecies differences. Long Evans rats are more sensitive to the acute toxic effects of TCDD than Han/Wistar rats, differences which not are believed to correspond with the Ah locus. L-E and H/W rats were administered a single i.p. dose of 5, 50, and H/W rats were additionally administered 500 ug TCDD/kg. Tissues were weighed at day 1, 4, 8, 16, and 32. A decrease in thymus to body weight ratio was observed 8 days post dosing in H/W

rats at a dose of 5 ug TCDD/kg. No effect was seen at this dose on day 1 or 4 post dosing. In L-E rats this same dose produced a decreased thymus to body weight ratio, but was observed earlier at 4 days post dosing. (Pohjanvirta, et al., 1989, 1990)

The distribution of TCDD in these two strains of rats was also examined by Pohjanvirta, et al., (1990). Both strains were administered a single i.p. injection of 5 ug/kg ¹⁴C-TCDD. Although many tissues were examined, liver, thyroid, kidney, spleen, and lung had very similar distribution patterns across tissues, but the distribution of TCDD to the thymus was very different. In Long Evans rats there is a continuous increase in TCDD concentration from 4 hrs to 16 days to a maximum of 0.18% of total dose concentrated in the thymus, followed by a decrease at the 32 day endpoint. In the Han/Wistar rat the concentration of TCDD was about half the level in the L-E rat (0.07%), and remained constant from day 1 to day 8, followed by a decrease (Pohjanvirta et al., 1990). Further studies are needed to explain the differences in tissue distribution and TCDD sensitivity in these two strains of rats.

Olson et al., (1980) evaluated the toxicity of TCDD in the Golden Syrian Hamster, a species not highly sensitive to the effects of TCDD having a 50 day LD₅₀ of greater than 3000 ug TCDD/kg, ip ; and an oral LD₅₀ of 1157 ug TCDD/kg. Olson et al. (1980) also evaluated the thymic response to TCDD after a single ip injection of 0, 5.0, 25, 100, 500, 1000, 2000, and 3000 ug TCDD/kg. Because of the number and range of data points, these data were evaluated more extensively using all the functional forms described in the introduction. Graph B6 depicts a "Michaelis-Menten" dose-response relationship, and a dose-dependent decrease in thymic weight was noted at 100 ug TCDD/kg which becomes significant (see Table III) at 500 ug TCDD/kg/day and greater. Reduced thymus weight was the most sensitive alteration noted in gross and histopathological examination. Olson et. al.(1980a) measured tissue distribution of

radioactive TCDD in the hamster after a single oral dose of 650 ug TCDD/kg. The distribution to the thymus reached a maximum at day 1 post injection and by day 3 TCDD content (measured as percent dose TCDD/g tissue) had begun to decrease from the thymus. The peak concentration in other tissues varied from day 1 to day 10 (liver was day 3) and had decreased substantially in all tissues by day 20. An important factor in tissue specificity may be excretion and distribution rates.

The Olson et al. (1980) data set (Table III) was used for additional dose-response analysis because of the number of data points in this study. In this data set, hamsters were administered single oral doses from 5 to 3000 ug TCDD/kg and thymus weight was measured at 50 days post-dosing. The percent response data were evaluated using the Linear, Michaelis-Menten and Hill functional forms described in the introduction. These data normalized as percent response provided a better fit to the functions than raw data. This could be due to the imprecision of changes in tissue weight as a toxic endpoint, or that normalized tissue weight is a more biologically relevant indicator of toxicity. Because negative values of percent response are not compatible with the Power Law function, raw data was used when evaluating this function. The linear model of normalized response (Graph B6-(A) did not fit well ($R^2 = 0.65$) presumably because it not reflect the biology of receptor-ligand interactions. The graphic representation of these data using the Michaelis-Menten function does begin to show a maximum response through the top dose of 3000 ug TCDD/kg (Graph B6-(B) and appears to reflect biological phenomenon. The third functional form used was the Hill plot (graph B6-(C) which also incorporated the data at the high doses. The log-linear plot of the Hill function depicts an "inertial" type of response at low doses with a "g" value over the entire dose range of 1.09, a linear/superlinear dose response. The Power Law function (Graph B-7(A) graphed

over the entire dose range demonstrated a sublinear ($g = -0.39$, $R^2 = 0.76$) decrease in absolute thymus weight. The Power Law function was subsequently applied systematically to subsets of doses to obtain optimal R^2 values. The Michaelis-Menten and Hill plots could not be used for this additional analysis because of the restrictions within these functions. The result of this exercise was to graphically observe a change in kinetic order from the low dose, 5.0 to 500 ug TCDD/kg, (Graph B-7(B) with a "g" value of -0.17 ($R^2 = .85$) to the high dose data, 500 to 2000 ug TCDD/kg (Graph B7-(C) with a "g" value of -1.57 ($R^2 = .99$). This increase in kinetic order with increasing doses suggests an inertial response at low doses and a synergistic response occurring at high doses suggesting that an inhibitory protein may be saturated at higher doses or induction of additional factors may occur at high doses to enhance the response. However, this is very speculative due to the uncertainties inherent in the use of clinical endpoints and reported means values, the constraints of using % response normalized data, and the limitations of evaluating a single species.

Several factors including tissue distribution, nuclear uptake, receptor concentration, and receptor affinity, could also be responsible for the differences in toxicity observed in hamsters, rats guinea pigs and mice. Pohjanvirta et al., (1990) identified strain differences in tissue distribution, Lund et al., (1982) found that nuclear uptake of TCDD in the thymus is only 6% of the uptake in the liver, and Carstedt-Duke (1979) studied the distribution of the TCDD receptor in various tissues in the Sprague-Dawley rat. The highest concentration of receptor, in tissues examined, was found in the thymus 25.2 fmol/mg protein. Cultures of several tissues from the Sprague Dawley rat were incubated with 10 nM TCDD to determine cytosolic Ah receptor concentrations. In the thymus, receptor concentrations remained relatively constant between day 2 and day 70 (Gasiewicz, ?). Although species differences in these factors are not

well characterized the differences seen in tissues provide evidence that these factors are also involved in species differences.

Studies indicate that thymus cells have a high concentration of Ah receptors, and that TCDD induces receptor mediated enzyme induction in this tissue (Cook et. al., 1987). Dose related enzyme induction (7-ethoxycoumarin O-deethylase activity) has been observed in human thymic epithelial (HTE) cultures with a NOAEL of 0.1 nM and LOAEL of 1 nM. In HTE cultures it was observed that individual cultures differ in their sensitivity and that the Ah receptor concentration is not an accurate predictor of enzyme activity. In addition, in HTE cultures there is an Ah receptor mediated decrease in thymocyte maturation. This was determined by co-culturing thymocytes with HTE and examining the responsiveness of these cultures to mitogens (concanavlin A and phytohemagglutinin). Concentrations of TCDD suppressing thymocyte maturation ranged from 0.1 nM to 1.0 nM. (Cook et. al., 1987)

The pathology of TCDD induced thymic atrophy has been characterized as a depletion of small cortical cells in the thymus, thinning cortex and an increase in macrophage in the cortex characteristic of cell damage (McConnell, et al., 1978a). Kociba found in a 13-week repeated dose study in rats, a pronounced decrease in thymocyte number in the cortical region of the thymus (Kociba, et al., 1976)

Thymocytes develop into mature T-lymphocytes, and apoptosis occurs as a natural part of the selection process in this maturation. TCDD and glucocorticoids both increase thymocyte apoptosis. This could have a dramatic effect on the immunological status of the animal (Nebert et al., 1990). McConkey and Orrenius (1989) proposed that the decreased number of thymocytes seen in vivo experiments results from an increase in Ca^{2+} -mediated endonucleases, normally seen as part of the thymocyte maturation process. It has been suggested that this

effect is similar to the effect seen with exogenous glucocorticoid treatment. Analogies in the mechanisms of action have been noted between glucocorticoid and TCDD nuclear receptor mediated mechanisms of action. Similarity of their action on thymocyte Ca^{+2} endonucleases as measured by % DNA fragmentation led researchers to propose a receptor mediated mode of action on thymocytes. Additional evidence that TCDD and glucocorticoids act in a similar manner to increase thymic atrophy through depletion of thymocytes includes the findings (McConkey and Orrenius 1989) that inhibitors of glucocorticoid induced thymic suicide, reduced the effects of TCDD, and reduced the glucocorticoid sensitivity in thymocytes. This does not preclude the possibility of different mechanisms of action with shared modulators of receptor-ligand interaction.

An alternative mechanism to induce thymic atrophy was proposed by Bombick, et al., 1988. After administration of TCDD to sensitive mice an increase in protein-tyrosine kinase was observed. Previous reports (Bombick et al., 1988) found that protein tyrosine kinases are associated with lymphocytes, and that they may be involved in lymphocyte maturation. This seems very plausible especially since the protein kinase p60 activates oncogenes and may play a role in mitogen signal transduction. Hence, any inhibitor of mitogenesis and signal transduction initiated by TCDD on the DNA would stop maturation and produce thymic atrophy. A dose-dependent increase in protein kinase was observed following *in vitro* exposure of 2-10 nM TCDD to B lymphocytes. The increase occurred rapidly (increases observed 5 minutes after exposure) and was significant at approximately 2.5 nM (Clark et al., 1991). The significance of this observation to TCDD mediated immune alterations *in vivo* has not been determined.

Table III Species Comparison of Thymus Weight Changes After TCDD Administration

Species	Dosing Regimen	Effect	Dose ug TCDD/kg	Response % change Thymus Weight		NOAEL	LOAEL	Reference
DBA/2J	single ip (9:1 corn oil/acetone)	decrease thymus weight (day 2)	0 30 60 115	0 4.7% 10.6% 40.1%*			115 ug /kg	Bombick, et al., 1988
C57Bl/6J female mice	single oral	decrease thymic/bwt 7 days post dosing and absolute thymus weight	0 0.2 1.0 2.0 5.0	0 thymus/bwt +3% 6.6% -16.7% 23.4%	0 +4.5% 5.1% -12.9% -19.9%			Kerkvliet and Brauner, 1990
C57Bl/6J	single ip (9:1 corn oil/acetone)	decrease thymus weight (day 2)	0 30 60 115	0% 27.2%* 35.0%* 51.1%*			30 ug/kg = 0.92 nM/kg	Bombick, et al., 1988
C57Bl/6 Sch. st. male	single oral	decrease thymus weight (week 2 and week 6)	0 0.2 1.0 5.0 25.0	0% 9.3% 12.5%* 33.1%* 79.9%*	0% 3.5% 18.4%* 45.6%* 79.8%*		1.0 ug/kg = 3.1 nM/kg	Vos, et al., 1974
C57Bl/6 DBA C3H/HeN B6D2F1 male mice	single ip injection measurements 12 days post dosing	% decrease thymus weight	0 ug/kg 1.2 6.0 30.0	C57Bl/6 0% 11% 44%* 54%*	DBA/2 0% 19% 16% 27% C3H/HeN 0% 20% 40%* 27%* B6D2F1 0% 8% 24%* 44%*	1.2 ug/kg C57Bl/6 C3H/HeN B6D2F1	6.0 ug/kg C57Bl/6 C3H/HeN B6D2F1	Vecchi et al., 1984
Long Evans rats males	single ip	decrease thymus weight	0 5.0 50.0	0 Day 4 20%* 20%*	0 Day 8 40%* 60%*	Day 16		Pojhanvuta, et al., 1989
Sprague Dawley rats males	oral 5 X week 13 weeks	decreased thymus weight	0 (0) (tot. dose) 0.001 (0.065) 0.01 (0.65) 0.1 (6.5) 1.0 (65)	0 1.9% 3.8% 21.0% 75.0%				Kociba, et al., 1976
Sprague Dawley rats female	oral 5 X week 13 weeks	decreased thymus weight	0 (0) (tot. dose) 0.001 (0.065) 0.01 (0.65) 0.1 (6.5) 1.0 (65)	0 7.5% 15.0% 42.5% 77.5%				Kociba, et al., 1976
Golden Syrian Hamster male	single ip injection measure 50 days post dosing	decrease thymus weight	0 5 25 100 500 1000 2000 3000	0.0 1.9% 2.9% increase 32.7% 53.5%* 84.2%* 94.0%* 81.2%		0.101 g (raw data) 0.099 g 0.104 0.068 0.047* 0.016 0.006 0.017	500 ug/kg = 155.0 nM/kg	Olson, et al., 1980
Guinea pigs Hartley	continuous dose in feed 90 days	decrease relative thymus weight	Male Female 0 ng/kg/day 0 0.12 0.12 0.61 0.68 4.90 4.90 26 31	Males 0% 15% 13% 24%*		Females 0% 6% 16% 12%		DeCaprio, et al., 1986
Guinea pigs Hartley female	8 X weekly oral	decrease thymus weight (day 56)	ug/kg/week (total dose) 0.0 0.008 (0.064 total) 0.04 (0.32 total) 0.2 (1.6 total)	0 17.8% 25.4%* 47.2%*			0.04 ug/kg = 0.12 nM/kg (0.32 total = 0.96 nM/kg)	Harris, et al., 1973

2.2

The development of mature thymocytes involves the passage of cells through a variety of subpopulations with unique phenotypes. Antigenic determinants can be used to identify these subpopulations. Kerkvliet and Brauner (1990) used flow cytometric analysis to quantify these alteration in thymocyte subpopulations after p.o. administration of 2 ug TCDD/kg in C57BL/6J female mice. There was a significant TCDD dependent increase in the percentage of CD4⁺CD8⁻ double negative (DN) thymocyte phenotypes and a decrease in the percentage of double positive (DP) CD4⁺CD8⁺ thymocytes. These changes were not observed in the lymphocyte population of the spleen indicating that the T-cell population of the thymus is uniquely sensitive to TCDD toxicity. Although dose-response trends were not reported in this study, the alteration in the percent of thymocyte subpopulations was a sensitive indicator of TCDD immunotoxicity. The alteration of these subpopulations has also been demonstrated *in vitro* further supporting the hypothesis that an imbalance in percent of cell types may be responsible for TCDD induced alterations in immunocompetence (Lundberg et al. 1990).

Fine, et al.(1990) investigated the effects of TCDD on the intrathymic development of T-cells by quantifying the effects of a single i.p. injection TCDD on subpopulations of cells in BALB male mice. All populations of thymocytes were reduced substantially, 50-76% decrease in total number of cells of each type, in addition the total cell population as measured by thymic cellularity was decreased approximately 90%. Although the total number and type of cells decreased the percent of cells in each population changed. This suggests that TCDD is working through two different mechanisms. There was an increase in the percent of CD4-CD8- double negative (DN) , CD4+CD8-, and CD8+CD4- single positive (SP) thymocytes cell types, and a decrease in CD4+CD8+ double positive (DP) cell types. These data are supported by

Baylock et al. (1992) who found that fetal thymic cell population percentages varied in a similar manner. They suggested that TCDD altered the maturation of fetal thymocytes affecting the transition from DN to DP. Holladay, et al. 1991) found the effect of TCDD on fetal thymocyte maturation was more significant at gestation day (GD)18 than at day 6 or day 14 after birth, indicating a sensitivity in fetal thymocytes that decreases with age (Blaylock et al., 1992).

Decreased thymus weight in the perinatal period is related to a decrease in immune system function, and as such may be of critical importance in the understanding of the toxicity of TCDD. Pregnant Wistar rats were administered a single oral dose of 0.0, 0.005, 0.5, 0.125, 0.25, and 0.35 ug TCDD/kg on gestation day (GD) 16. A dose related decrease in pup thymus weight was reported. In addition, there was a dose related increase in 7-ethoxycoumarin deethylase activity, and on biphenyl 2-hydroxylase activity at the above administered doses, with a NOAEL for enzyme induction of 0.005 ug TCDD/kg (Madsen, et al., 1989).

TCDD produces a consistent response of thymic atrophy in the guinea pig, rat, hamster, monkey and in the sensitive mice. This effect is receptor mediated and although the mechanism is not completely understood, several plausible mechanisms have been proposed. Because the thymus is a key organ in immunological responses it is not surprising that TCDD affects the immune system.

C. Immunotoxicity

TCDD-mediated immunotoxicity and the implications of enhanced and suppressed immune function are described in detail in the Immunotoxicity chapter of this document (Kerkvleit and Burleson 1992). In addition, detailed evidence that the immunotoxicity of TCDD and related halogenated aromatic hydrocarbons is, at least in part, Ah-receptor mediated is

presented. Studies with multiple doses and protocols that most resembled other studies (similar species, strains and dosing regimens) were evaluated for comparison of immunological endpoints. Because of the variety of protocols tested to evaluate TCDD immunotoxicity some endpoints have not shown clear dose-response relationships, however there are some that do. The premise that immunotoxicity is at some level Ah receptor mediated leads to the assumption that the response of some immunotoxicity endpoints may be related to the response of other Ah receptor mediated events.

Exposure to TCDD results in thymic atrophy, alterations in bone marrow and general immunosuppression in almost every species examined. (Luster et al., 1979,1980) Suppression of cell-mediated immunity, (CMI), measured as a response to phytohemagglutinin (PHA) and concanavalin A (Con A), may be observed in the neonate during thymic organogenesis: the most sensitive time for this type of immunotoxicity (Luster, et al., 1987). Vos (1974a) investigated the role of maternal treatment of (0, 1.0, 5.0 ug TCDD/kg) administered prenatally GD 11 and GD 18, and/or postnatally day 7 or 14 on cell-mediated immunity (CMI) of Fischer-344 rat pups. The response of spleen cells to PHA was significantly decreased in 25 day old pups after maternal postnatal exposure only at the 5 ug TCDD/kg level. However, after pre and postnatal maternal exposure there was a significant decrease in the PHA response at 1.0 ug TCDD/kg indicating that prenatal exposure is a more sensitive period for immunotoxicity. There was significant fetal toxicity associated with immunosuppression at the 5 ug TCDD/kg level. The susceptibility of pups is believed to be greater than in adult rats because of the role the thymus plays in CMI. Although the relevance of the thymic atrophy seen in animals as it relates to the adult human population may be questioned, the thymus is an important organ in the development and function of the human immune system. Similar results were reported by

Faith and Moore (1977) in thymus cells from 25 day old female Fischer rat pups exposed postnatally to TCDD. There was a significant decrease in responsiveness to PHA at the 5 ug TCDD/kg level, but not to Con A. The impact of pup age on CMI is assessed by comparing the responsiveness to PHA in 1 and 4 month old mice. The responsiveness to PHA was significantly reduced in 1 month old mice that were administered 4 weekly doses of 25 ug TCDD/kg TCDD. Four month old mice administered the same doses (0, 1.0, 5.0, and 25 ug TCDD/kg) weekly for 6 weeks had no differences in PHA responsiveness when compared to controls although all doses showed significant thymic atrophy. The suppression of cell mediated immunity in sexually mature (young adult) rats and mice was demonstrated to be a relatively insensitive endpoint which differed significantly from controls at the 25 ug TCDD/kg dosage level only. The difference in sensitivity based on age is consistent with what is known about the development of the thymus (Vos and Moore 1974a).

Thomas and Hindsill (1979) described a dose-related decrease in relative thymus weight in offspring of adult female Swiss mice exposed to TCDD in feed at concentrations of 1, 2.5, 5 and 10 ppb for 4 weeks prior to mating. A statistically significant decrease in relative thymus weight and number of SRBC (sheep red blood cells) plaque forming cells per gram of spleen (both indicators of immunotoxicity) were observed in the offspring of animals exposed to 2.5 or 5 ppb TCDD. Although food consumption data was not reported in this study and daily intake and fetal dose can not be determined, the spectrum of toxic effects presented are relevant. Both endpoints are indicators of immunotoxicity. No overt signs of maternal toxicity were observed at doses producing immunotoxicity.

Another effect observed after prenatal exposure was that allograft rejection times were prolonged in a dose-dependent manner in 23 day old C57BL/6J mice exposed pre and postnatally (GD 14 and GD 17) to 2.0 and 5.0 ug TCDD/kg levels of TCDD (see Table IV) and in rats exposed postnatally on day 1, 8, and 15 to the 5.0 ug TCDD/kg level (Vos and Moore 1974a).

Vecchi et al. (1980) examined the response of C57BL/6J adult mice splenocytes to Con A and lipopolysaccharide (LPS) after TCDD treatment to assess cell-mediated immunity. Male mice were administered 0, 1.2, 6 and 30 ug TCDD/kg. At 7 and 14 days post dosing, splenocytes from these animals were tested for their response to Con A and LPS. At 30 ug TCDD/kg a significant increase in mitogenic response was observed at 1.6 and 3.2 ug/sample Con A. NOAEL=6.0 ug TCDD/kg. There was no splenocyte response to LPS in mice administered 30 ug TCDD/kg. This study indicated that relatively high doses (30 ug TCDD/kg) are required to affect cell-mediated immunity in adults.

Vos et al. (1973) also examined cell-mediated immunity in female Hartley guinea pigs receiving 8 weekly doses of 0, 0.008, 0.04, 0.2, and 1.0 ug TCDD/kg and in female CD rats administered 6 weekly doses of 0, 0.2, 1.0 and 5.0 ug TCDD/kg (Table IV). Vos et al. (1973) selected delayed hypersensitivity to tuberculin toxin in guinea pigs and rats to assess CMI. The results are reported as diameter of skin reaction. In guinea pigs administered TCDD, sensitized with tuberculin on day 35 post dosing and challenged on day 47 and 54, there was a dose dependent decrease in diameter of skin reaction which was significant at the 0.04 ug TCDD/kg/week level (0.32 ug TCDD total dose). TCDD inhibited the delayed hypersensitivity response in these animals. There was no significant change in diameter of skin reaction in rats

sensitized on day 28 and challenged on day 42 (see Table IV). This response is consistent with results of acute toxicity data that found guinea pigs are more sensitive to the toxic effects of TCDD than rats. However, rats are generally not used for delayed hypersensitivity testing. Hence, the guinea pig results stand on their own.

Another method Vos et al. (1973) used to assess cell-mediated immunity is to examine local graft vs. host reactions (GVH). B6D2F1 recipient mice administered 0.0, 0.02, 1.0, and 5.0 ug TCDD/kg were injected with C57BL/6J mice spleen cells. To assess the GVH response the ratio of the weight of the right and left popliteal lymph nodes was compared. A dose dependent decrease in ratio was observed and was significant at 5.0 ug TCDD/kg (Table IV)

Rosenthal et al. (1989) assessed the response to endotoxin (*E. coli* LPS, 0.25, 5 and 25 mg/kg) administered 7 days post TCDD treatment to female B6C3F1 at single oral doses 0, 50, 100, and 200 ug TCDD/kg. Effects observed included an increase in mortality from 5 mg/kg endotoxin at the 200 ug TCDD/kg level and a decrease in the ability of mice administered 50 ug TCDD/kg to clear 2.5 mg/kg endotoxin from their blood. The sensitivity of this endpoint was not tested, as all administered doses resulted in an some effect (Table IV). The increased responsiveness to endotoxin segregated with the $Ah^{b/b}$ locus (sensitive strain) and was not seen in the $Ah^{d/d}$ (less sensitive strain) congenic mice. Furthermore, the response to endotoxin and TCDD was reversed by administration of methyl prednisolone leading the authors to conclude that an inflammatory component is involved in the response.

The most sensitive indicator of cell-mediated immunity is the reduction in cytotoxic T-lymphocytes (CTL). Nagarkatti et al.(1984) demonstrated a decrease in CTL response at a cumulative dose of 4 ng/kg. Kerkvliet et al. (1990a) noted a dose-related decrease in CTL at 10

and 20 ug TCDD/kg (single oral dose TCDD). Graphic representation of there data indicated a dramatic decrease in the CTL response in C57BL/6J mice and a minimal decrease at these doses in non-responsive congenic mice. A controversial study by Clark et al. (1981) demonstrated a dose-related suppression of CTL in C57BL/6J mice. Doses as low as 0.004 ug TCDD/kg to 40 ug TCDD/kg resulted in a decrease in CTL activity. It has also been reported by Clark et al. (1981) that TCDD does not deplete the number of CTL precursors, but induces a population of suppressor T-cells. Clark's data demonstrate suppression at extremely low levels, however, the protocol used is very difficult to precisely replicate (Holsapple et al. 1991, 1991a).

Cell mediated immunity is only one type of response the body employs as a defense mechanism. Humoral immunity may be assessed by measuring the serum level of induced anti-toxin in response to toxoid challenge. Vos et al. (1973) measured the response to the tetanus toxoid in guinea pigs administered 8 weekly doses of 0, 0.008, 0.04, and 0.2 ug TCDD/kg. Guinea pigs were injected with toxoid on day 28 post TCDD administration and anti-toxin levels were measured 7, 21, and 28 days after initial injection. There was a significant increase in serum anti-toxin levels at 0.008 and 0.04 ug TCDD/kg levels 7 days after toxoid injection, however, not at 21 and 28 day. There was a significant decrease in anti-toxin levels in the 0.2 ug TCDD/kg dose at these two time points suggesting a dose-dependent response (Table V).

Another method of assessing humoral immunity is to quantify the production of specific antibodies in response to an antigen characterized as thymus-dependent and thymus-independent antigens. The sheep red blood cell (SRBC) antigen is the most common antigen used to assess immune competence after TCDD treatment. This assay has produced consistent

and reproducible results while using multiple doses. Therefore this endpoint will be evaluated more empirically. Vecchi (1980) assessed the immune response in C57BL/6J male mice after i.p. treatment with 0, 1.2, 6, or 30 ug TCDD/kg. 4×10^5 SRBC, a T-dependent antigen, and 0.5 ug of type III pneumococcal polysaccharide, a T-independent antigen, were used to assess humoral immunity. At all doses, with both antigens, and at all time periods assessed, TCDD significantly decreased the plaque forming cells/ 10^6 splenocytes (a measure of immune response) in a dose-dependent manner (Table V). The antibody-mediated immunity was suppressed in C57BL/6J mice at the lowest dose tested, 1.2 ug TCDD/kg, a dose lower than that found to suppress CMI.

Davis and Safe (1988) measured plaque forming cells (PFC)/ 10^6 splenocytes) in C57BL/6J mice after administration of 0.322, 0.422, 1.2, and 3.1 ug TCDD/kg followed by an i.p. injection of 4×10^5 SRBC. Although statistical significance was not assessed in this study, there was a dose-dependent decrease (Table VI) in PFC/ 10^6 viable cells seen in all dose levels. These authors also evaluated the response of other polychlorinated dioxin compounds to examine structure activity for Ah receptor affinity and concluded that the humoral response to T-dependent antigens is Ah receptor mediated. In addition the authors suggest that TCDD-receptor complex may modulate early B-cell differentiation.

Narasimhan et al. (1993) assessed the same endpoint in B6C3F1 mice and found a statistically significant decrease in PFC/ 10^6 viable cells at 0.05 ug TCDD/kg (Table VI). Analysis of dose-response for immunotoxicity is complicated by the variety of endpoints and dose ranges reported in the literature. However, the use of PFC/spleen was a consistent measure of humoral immunity in three studies. The Kerkvliet and Brauner (1990); Narasimhan et al. (1993) unpublished); Silkworth et al. (1989); and the Davis and Safe (1988) data sets were selected for

additional dose-response analysis. These data sets normalized to percent response, were fitted to the Michaelis-Menten function (graph C1) using doses in the same range⁶. All but the Kerkvliet and Brauner (1990) data set fit this function poorly. The fit to the Power Law function for all four data sets (graph C2) shows a consistent response between the Silkworth (DBA2) and the Kerkvliet data, ie. both fit the function well ($R^2 = 0.99$ and 0.98 respectively), and had comparable exponents in the function (" g " = 0.32, 0.30). The Davis and Safe (1988), Silkworth et al. (1989) and Narasimhan et al. (1993) data sets all had higher " g " exponents and poorer fits. A plausible explanation for these variation could be that test methodologies differed (ie. use of different control solutions) or there are genuine species and sex differences that modulate this response. It is interesting to note that the shape of the dose-response curves for the DBA and BALB mice used in the Silkworth et al. (1989) experiment are very different (Graph C2) and this suggests that the dose values used are capturing different segments of the dose-response curves for these two strains of mice.

The Narasimhan et al. (1993) data set was used for additional analysis because of the number and range of doses reported. The functions that best fit the raw data points were the Michaelis-Menten and the Hill functions (Graph C3-A, C3-B). The Power Law Function (Graph C4-A) fit all raw data values for PFC's/spleen with an R^2 value of 0.89 and a " g " value of -0.43. A Power Law functional fit of the entire data set using normalized percent response data could performed because at very low doses there was a negative value which is not

⁶The entire data set from Safe, 1992 was not used for this analysis. Direct comparison to Kerkvliet and Brauner (1990) and Davis and Safe (1988) used data that from a similar dose range.

mathematically consistent with this function. Systematic selection and analysis of data subsets using % response normalized data (excluding extremely low values) resulted in values of "g" = 2.2; and $R^2 = 0.99$, in the low dose range (0.025 to 0.1 $\mu\text{g TCDD/kg}$) (Graph C4-B) suggesting superlinearity. In contrast, the high dose range (0.5-2.5 $\mu\text{g TCDD/kg}$) resulted in "g" = 0.23 and $R^2 = 0.87$, suggesting sublinearity or a lower rate of response (Graph C4-B). The higher exponent at the low dose may represent synergy or positive cooperativity. However it is also consistent with the existence of non-nuclear receptor-mediated interactions at low doses, or may suggest induction of additional inhibitory factor at higher doses. When an analysis of subsets of this data was applied to raw data a different pattern of dose-response was observed. At the low administered doses the absolute response resulted in a sub-linear fit with a "g" value of -0.57, $R^2 = 0.89$, whereas at higher doses a steeper linear fit was observed, "g" = -0.99, $R^2 = 0.98$ (depicted in graph C4-C). Whether raw data of percent response normalized data is a better representation of low dose phenomenon can not be concluded from this analysis.

Kerkvliet et al. (1990) demonstrated differences in humoral immunity related to the Ah responsiveness of C57BL/6J congenic mice after acute exposure to TCDD. The dose-dependent decrease in splenic antibody response to the T-cell independent antigen TNP-LPS revealed differences in sensitivity which were consistent with strain Ah responsiveness. The response of these strains to T-cell dependent antigen was more complex and therefore could not be directly related to Ah receptor responsiveness (See Table V). Graph C5-A depicts the different sensitivities of the two strains using a Michaelis-Menten function with a reasonably good fit. The Power Law function also depicts strain sensitivity (Graph C5-B) although the fit is poor.

Vecchi et al. (1983) compared multiple doses (3) in three strains of mice to demonstrate

that the decrease in antibody production as measured by PFC's/ 10^6 spleen cells segregated with the Ah receptor (See Table IV, Graph D1-A, D1-B). The Michaelis-Menten and the Power Law function are both good representations of the species differences in sensitivity. Because of the differences in the correlation and the "g" value in the Power Law, it appears that the best fit to this functional form is at the doses where a change in response is measured.

In an acute study (Morris et al. 1992) a dose-dependent decrease in anti-SRBC IgM AFC (antibody forming cells)/spleen was observed in all doses (4.2, 14, and 42 ug TCDD/kg) administered orally to the B6C3F1 mouse (Ah responsive strain) however, only at the 42 ug TCDD/kg dose in DBA/2J mice (less responsive). Subchronic administration of the same cumulative dose revealed statistically significant decrease (not clearly dose dependent) at all doses in both strains. This study clearly shows (Table V) that exposure conditions can alter response. The acute exposure study demonstrates that B6C3F1 mice are approximately 10 times more sensitive than the DBA mice, however the results of the subchronic study suggest that there may be a modification of the Ah mediated strain sensitivity. These results support what is known about Ah receptor mediated toxicity that cumulative doses will establish an equilibrium between receptor-ligand formation, receptor biosynthesis, and receptor-mediated effect (EROD induction, or suppressed immune response). A lower dose can maintain this equilibrium whereas a higher dose would be required to induce the same effects with a single dose.⁷ Clearly Morris et al. (1992) has demonstrated this phenomenon with humoral immunity.

Kramer et al. (1987) using highly purified murine B-cells suggested early events may be

⁷In comparing Birnbaum and DeVito's data (Table X) and Safe's data (Table IX) the dose to induce a statistically significant increase in EROD after acute administration was 0.5 ug/kg whereas administration of 0.0975 cumulative dose in a subchronic protocol induced the same level of EROD induction.

the target of TCDD in these cells since TCDD increased basal kinase activity in the presence of added phospholipid, but did not increase protein kinase C (PKC) activity. The authors proposed that TCDD affects basal kinase activity at the level of protein phosphorylation. Hence, protein kinase involved in phosphorylation and early activation may be the target of TCDD and result in altered B-cell differentiation. Changes in basal protein kinase activity, although Ah receptor mediated, may not require translocation to the nucleus and therefore this endpoint may respond very differently than other events mediated through XRE activation. It has been hypothesized that B-cells are a direct target of TCDD toxicity (Dooley and Holsapple 1988). The effect of TCDD on T- and B- cell interactions in the antibody response was investigated using a T-dependent antigen SRBC's and two T-independent antigens DNP-Ficoll (dinitrophenol-Ficoll) and TNP-LPS (trinitrophenol-lipopolysaccharide). The antibody response was assessed by enumeration of antibody forming cells (AFC's) in spleens of female B6C3F1 mice administered 0, 0.1, 1.0 and 10 ug TCDD/kg daily for 5 days (Table V). Five ug TCDD/kg total dose produced a decrease in AFC induced by all three antigens. Their findings that the response to T-dependent and T-independent antigens was decreased at the same dose level of TCDD led the authors to conclude that B-cells were the primary target of TCDD and that this immunosuppression is at the level of B-cell differentiation. These doses are high doses for immunomodulating effects (100 X the LOAEL in the Narasimhan et al. (1993) study) and therefore may not represent the lowest dose effect.

To assess the suppression of humoral immunity by TCDD and the role of the Ah receptor in that suppression, Holsapple et al. (1986), and House et al. (1990) administered a single p.o. dose of 0, 0.05, 0.1, 0.5, 1.0, and 2.0 ug TCDD/kg to female B6C3F1 mice and subsequently administered SRBC a T-dependent antigen. The response was measured by the

Table IV

Cell-Mediated Immunity

Species	Dosing regimen	Effect	Dose (ug TCDD/kg)		Response (%change)				NOAEL	LOAEL	Reference
C57BL/6J 23 day old mice pups	0, 2.0, 5.0 ug/kg pre and postnatally (GD 17 and 17 and day 18-15)	increase rejection time of tail skin graft	0 2.0 5.0	0 19%* 25%*							Vos and Moore, 1974a
F-344 28 day old rat pups	0 and 5 ug/kg maternal postnatally on days 0, 7,14	increase rejection time of tail skin grafts	0.0 5.0	0 +18%*							Vos and Moore, 1974a
Female albino rats CD	0, 0.2, 1.0, 5.0 ug/kg weekly for 6 weeks. Day 28 rats were injected with M. tuberculosis and day 42 injected with 5 ug tuberculin PPD	Thickness of skin reaction on 24 and 48 hrs after final injection	0 0.2 1.0 5.0	Skin diameter 24 hrs 0 +3.4% +5.1% +0.7%	Skin diameter 48 hrs 0 10.5% 5.9% 4.6%	Skin Thickness 24 hrs 0 9.5% +6.62% 10.5%	Skin Thickness 48 hrs 0 2.7% -8.2% -13%	N/A	N/A	Vos et al., 1973	
Male B6D2F1 mice	0, 0.2, 1.0 and 5.0 ug/kg weekly for 4 weeks. C57BL mice. Recipient mice received graft of spleen cells in right hind foot pad.	Response measured as difference between right and left popliteal lymph node.	ug/kg (total dose ug/kg) 0 0 0.2 0.8 1.0 4.0 5.0 20.0	Right-left Popliteal lymph nodes % differs from control 0 0.2% 35% 66%*				N/A	N/A	Vos et al., 1973	
Female B6C3F1 mice	Single oral dose of 0, 50, 100 or 200 ug/kg followed by 7 day iv treatment with E. coli ET5 endotoxin 5 or 25 ug/kg	Response mortality at 48 hrs	ug/kg 0 50 100 200	Mortality 5 ug/kg endotoxin 0% 0% 87.5%*		Mortality 25 ug/kg endotoxin 0% 50%* 100%* 100%*				Rosenthal et al., 1989	
C57BL/6 male mice	weekly ip injection X 4 wks. At week 5 sensitized painting ears with 3% oxazalone challenged with 1% oxazalone	ear thickness 10 ³ mM measured	Dose 0 0.4 ug/kg 4 ug/kg 40 ug/kg	Increment in ear thickness 24 hrs after challenge 0 +54% 38%* 32%*				0.4 ug/kg	4 ug/kg	Clark et al., 1981	
Hartley guinea pigs	8 X weekly oral administration	day 35 0.05 ml injection of tuberculin bacillus hypersensitivity tested day 47 and 54 with tuberculin injections (1.25 ug) skin reactions measured 24 and 48 hrs. after tuberculin injections skin diameter measurements % difference from control	Dose ug/kg/wk (total dose) 0 0.008(0.064) 0.04 (0.32) 0.2 (1.6)	skin diameter at 47d 24hrs 0 0 4% 8% -29%*	diameter skin 47d - 48 hrs 0 -9% 15%* 42%*	skin diameter 54d - 24 hrs 0 -5% -9%* 22%*	skin diameter 54d - 48 hrs 0 9% 12%* 41%*			Vos, 1973	

Table V Humoral (Antibody-Mediated) Immunity

Species	Dosing regimen	Effect	Dose (ug/kg)	Response (%change)				NOAEL	LOAEL	Reference
male C57Bl/6J 6-8 wks	ip with 1CDD followed by (SRBC) injection. PFC measured 7, 14, 21 and 42 d after 1CDD	measured % decrease from control PFC/10 ⁶ splenocytes	0 ug/kg 1.2 6 30	0 7d 54%* 86%* 96%*	0 14d 42%* 70%* 89%*	0 21d 60%* 77%* 89%*	0 42d 55%* 85%* 88%*	N/A	1.2 ug/kg	Vecchi, 1980
Hartley Guinea pigs female	8 X weekly oral administration	day 28 and day 42 0.1 ml tetanus toxoid injected into hind footpad Serum antitoxin measured day 35, 49, and 56	0 dose ug/kg/wk 0.008 (0.64) 0.04 (0.32) 0.2 (1.6)	0 Day 25 +16%* +21%* 5%	0 Day 49 -13% -22% -41%*	0 Day 56 +8% -7% -29%*	Day 35 N/A Day 49 & 56 0.04	Day 35 0.008 Day 49 0.2 Day 56 0.2		Vos, 1973
B6C3F1 mice	oral administration 1 acute oral dose 2 14 day cumulative dose	decrease formation of anti-SRBC IgM Antibody forming cells	0 ug/kg 4.2 14 42	1 0 64% 67.5% 83%	2 0 78% 75% 88%					Morris et al, 1992
DBA 2J mice	oral administration 1 acute oral dose 2 14 day cumulative dose	decrease formation of anti-SRBC IgM antibody forming cells	0 ug/kg 4.2 14 42	1 0 16% 9.5% 33%	2 0 56% 83% 88%					Morris et al, 1992
Female B6C3F1 mice	oral administration of 1CDD daily for 5 days 48 hrs after last treatment sensitized with antigen	Antibody forming cells measured after sensitizing with SRBC DNP Ficoll INP LPS in vivo assay	0 ug/kg/day 0.1 1.0 10.0	0 Response SRBC 32% 72%* 94%*	0 Response DNP Ficoll 60% 86%* 87%*	0 Response INP LPS N/A 64%* ND				Dawley and Holsapple, 1988
Female B6C3F1 mice	single p.o. 1CDD, day 8 post dosing immunized with SRBC	Spleen cell IgM anti SRBC AIC response measured as change in IgM AIC 10 ⁶ recovered cells	0 ug/kg 0.05 0.1 0.5 1.0 2.0	0% change from control IgM AIC +4.2% +5% +6% +8%* +6%*	0 weight % change from control +18% +1% +12% +1% 6%		IgM AIC = 0.5	IgM AIC = 1.0		Holsapple et al, 1986
C57Bl/6J male mice	ip injection of 1CDD weekly for 4 weeks bacterial and viral challenge 2 d after dosing	mortality rates reported	ug/kg/week (ug/kg/day) 0 (0) 0.5 (0.07) 1.0 (0.14) 5.0 (0.7) 10 (1.4) 20 (2.8)	Bacterial challenge mortality 25% 25% 65% 70% 65% 95%	Viral Challenge 45% mortality ND ND 15% 0% 10%					Huguenot et al, 1975
C57Bl/6J male mice Ab ^{0.1} and Ab ^{0.2}	single oral dose 1CDD prior to ip injection of 25 ug INP LPS	% decrease number of nucleated spleen cells	0 ug/kg 1 5 10 20 50	0% (Ab ^{0.1}) 10.6% 17% 28.5% 24% not tested	0% (Ab ^{0.2}) not tested 12.6% 22.1% 15.1% 25.2%		1 ug/kg Ab ^{0.1} 5 ug/kg Ab ^{0.2}	5 ug/kg Ab ^{0.1} 10 ug/kg Ab ^{0.2}		Kerkhofs et al, 1990a
B6C3F1 mice	single ip injection of 0, 0.1, 1.0, 10 ug/kg	Spleen cell IgM, IgG, INP LPS, and INP Ficoll AIC response 10 ⁶ recovered cells	0 ug/kg 0.1 1.0 10	0 IgM AIC 3% 57%* 77%*	0 IgG AIC -14% -22% -42%*	0 INP Ficoll 28% 66%* 91%*	0 INP LPS N/A N/A +6%	IgM 0.1 IgG 0.1 INP Ficoll 0.1	IgM, IgG, and INP Ficoll 1.0	Housser et al, 1990
Male C57Bl/6 male mice	weekly ip injection X 4 wks week 5 injected with 1.2 X 10 ⁶ 5 days later mg 2x10 ⁶ SRBC	measured footpad 24 hrs after challenge	0 0.4 ug/kg 1CDD 4 ug/kg 1CDD 40 ug/kg 1CDD	0 Response footpad swelling 3% 14% 38%						Clark et al, 1981
Female B6C3F1 mice	14 daily doses p.o. 1CDD	1. Serum complement activity CH50 representing the amount of serum necessary to lyse 50% of the target cells 2. Serum level of complement component C3	0 Total dose ug/kg/day 0.01 0.05 0.1 0.5 1.0 2.0	0 CH50 %decrease 41%* 11%* 38%* 54%* 65%* 81%*	0 serum C3 %decrease 3% 6% 2% 8%* 19%* 26%*		CH50 = N/A C3 = 0.1	CH50 = 0.01 C3 = 0.5 ug/kg/day		White, 1986

Table VI
Dose - Response Effects of TCDD on PFC response in C57BL/2N and B6C3F1 Mice

Species	Dosing Regimen	Effect	Dose	% Response		NOAEL	LOAEL	Reference
C57BL/6N mice male	Single i.p. injection with TCDD followed 5 days by a single i.p. injection of 4×10^6 SRBC	% decrease in splenic plaque forming cells/ 10^6 spleen and per viable cells 4 days after SRBC treatment	0 ug/kg 0.322 0.644 1.19 3.09	0% 6.3% 43.6% 84.8% 91.6%	PFC's/ 10^6 spleen 777 731 438 118 65	N/A	N/A	Davis and Safe, 1988
C57BL/6J Female	single oral dose TCDD 2 days prior to single i.p. injection 2.5×10^6 SRBC	% decrease in splenic plaque forming cells/ 10^6 spleen cells	0 ug/kg 0.2 1 2 5	0 % 30.4 % 52.6 % 67.4 % 78.3 %				Kerkvliet and Brauner, 1990
Male B6C3F1 mice	single oral dose TCDD administered orally 2 days prior to injection with SRBC	% decrease PFC/ 10^6 spleen cells	0 ug/kg 0.25 1.0 4.0 16.0	0 % 6.6 % 18.2% 79.6% 98.6%		1.0 ug/kg	4.0 ug/kg (all doses were decrease)	Silkworth et al., 1989
DBA/2J male mice	single oral dose TCDD administered orally 2 days prior to injection with SRBC	% decrease PFC/ 10^6 spleen cells	0 ug/kg 0.25 1.0 4.0 16.0	0 % 23.5% 32.6% 57.1% 87.9 %			at all doses significant decrease observed	Silkworth et al., 1989
B6C3F1 Mice female		% decrease in splenic plaque forming cells/ 10^6 spleen and per viable cells 4 days after SRBC treatment	0 ug/kg 0.005 0.010 0.025 0.050 0.1 0.5 1.0 2.5	0% -7.8% -18.6% 2.3% 13.1% 55.7% 62.7% 84.5% 92.5%	939 1012 1114 917 816* 416* 350* 146* 70*	0.025 ug/kg	0.05 ug/kg	Narasimhan et al., 1993

Complement is another important factor in the immune response. White et al. (1986) investigated the effect of TCDD on complement levels and activity in female B6C3F1 mice administered 0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 ug TCDD/kg total dose administered over 14 day period. As a measure of complement activity White estimated CH_{50} which is the amount of treated mouse serum required to lyse 50% of the cells. There was a significant decrease in (CH_{50}) activity at all TCDD doses tested (Table V). A significantly reduced level of the C3 complement component was found at 0.5 ug TCDD/kg. However, the C3 component was the only component measured. It is concluded that several components may be affected, hence the decrease in CH_{50} activity is a more sensitive indicator of toxicity to the complement system than individual components.

In summary, immune system may be profoundly affected by TCDD administration, and may prove to be the most sensitive indicator of TCDD toxicity. A broad spectrum of immunotoxic effects have been reported after TCDD administration in adult animals. Significant among these are the increases in mortality in TCDD treated mice challenged with influenza (House et al. 1990) and the decrease in complement (CH_{50}) activity in which the LOAELs were both less than 0.01 ug TCDD/kg. In addition the PFC assay has been found to be an extremely sensitive indicator of toxicity (Narasimhan et al. (1993) found LOAEL = 0.05 ug TCDD/kg). Without studies comparing similar endpoints across species the implications of this toxicity is difficult to assess. It has been suggested that the susceptibility of the developing immune system is even greater than that of adult animals. This and other effects on the developing fetus are very sensitive to TCDD administration.

D. Teratogenic/Developmental Toxicity

The effects of TCDD on proliferative cellular events make it a particular concern in the developing fetus. Although only 0.0005% of maternally administered TCDD reaches affected tissues in the fetus of the C57BL mouse (Abbott and Birnbaum 1989), many effects including thymic atrophy, cleft palate, and hydronephrosis are observed (Couture et al. 1990). The responses to maternally administered TCDD are dose dependent, and based on studies of sensitive and resistant strains of mice, appear to segregate with the Ah receptor locus (Poland and Glover 1980; Silkworth et al. 1989).

D.1. Cleft Palate

Couture et al. (1990) summarized 23 studies which described the teratogenic and developmental toxicity of TCDD. Of the 14 studies summarizing toxicity in mice, 13 listed cleft palate and 8 listed hydronephrosis or renal congestion as toxic endpoints. None of the remaining 9 studies, performed in other species, list cleft palate as an endpoint, and only one study in hamsters described hydronephrosis. All species have not been studied, yet it is believed that cleft palate is unique to mice. Although hydronephrosis is believed to occur via a mechanism related to cleft palate it has been reported to occur in other species including rat and hamster. Various protocols have been used to evaluate cleft palate and hydronephrosis, the two most common fetal abnormalities, and generally the response is dose dependent.

Mice, because of their unique sensitivity to cleft palate, have been the focus of the research on the developmental and teratogenic effects of TCDD. Coutney and Moore (1971) observed an increase in the incidence of cleft palate and kidney anomalies in three strains of mice DBA/2J, C57BL/6J and CD-1 after dams were administered 3 ug TCDD/kg/day s.c for 10 days (GD 6-15). In the CD-1 mice a dose dependent increase in cleft palate incidence and

kidney anomalies was observed in offspring administered 1 and 3 ug TCDD/kg/day. Courtney and Moore (1971) also observed that CD rats administered 0.5 ug TCDD/kg/day did not have offspring with an increase in cleft palate. The authors concluded that the increase in cleft palate was specific to the mouse, however, there was an increased incidence of kidney anomalies (not characterized) in these rats. In addition, no significant maternal toxicity (characterized as decreased maternal weight gain or increase relative liver weight) was observed in these CD rat or CD-1 mice. The effects of TCDD administered orally 0, 0.001, 0.01, 0.1, 1.0 and 3.0 ug TCDD/kg/day on gestation days (GD) 6-15 on fetal development in Carworth CF-1 mice was investigated by Smith et al.(1976) (Table V). At the 1.0 and 3.0 ug TCDD/kg/day dose a statistically significant increase in cleft palate was observed. At the highest dose an increase in dilated renal pelvis was observed. Since there was a response at only two doses it is difficult to conclude if there is a dose-response relationship using this protocol. The NOAEL for developmental /teratologic effects was reported as 0.1 ug TCDD/kg/day (1.0 ug/kg total dose), and LOAEL was 1.0 ug TCDD/kg/day (10 ug/kg total dose). Doses comparable to that of Courtney and Moore (1971).

Courtney (1976) also investigated the teratogenic effects of TCDD using oral and subcutaneous routes of administration. Pregnant CD-1 mice were administered 0, 25, 50, 100, and 200 ug TCDD/kg/day using both routes of administration, and additional dose of 400 ug TCDD/kg P.O. for 10 days (GD 6-15). Maternal toxicity, evident in all dose groups, was characterized by a decrease in maternal body weight gain, an increase in relative liver weight, and in some cases marked edema. Parameters used to assess fetotoxicity were number of live fetuses/litter, number of abnormal fetus/litter, percent cleft palate, percent kidney anomalies (characterized as hydronephrotic kidneys), and percent pups with a club foot. A dose-

dependent increase in percent cleft palate and percent hydronephrosis was detected in all groups administered TCDD orally, except at the highest dose where it was observed that the percent kidney anomalies decreased probably because of the extent of maternal toxicity at this dose. (Table VII). The mice administered TCDD s.c. had a response (increase cleft palate) close to maximal at the lowest dose tested, although the same dose administered orally resulted in a minimal but detectable response. Additionally, the comparison of 2,3,7,8-TCDD to 1,2,3,4 TCDD and OCDD shows 2,3,7,8 TCDD to be the most potent fetotoxin of these congeners tested. The doses in this study were extremely high and do not reflect the sensitivity of cleft palate and hydronephrosis as toxic endpoints (Courtney 1976). The CD-1, CF-1, C57BL/6J and the DBA mice clearly have different sensitivity to TCDD induced cleft palate. An incidence of 19% cleft palate was observed in CD-1 mice administered 25 ug TCDD/kg/day orally GD 7-15, while only 1.0 ug TCDD/kg/day in the CF-1 mouse (the most sensitive of the species tested) resulted in 21% cleft palate in offspring (Smith et al. 1976). C57BL/6J administered 4 ug TCDD/kg/day and DBA administered 8 ug TCDD/kg/day had 47% and 27% cleft palate incidence respectively (Table VII) (Silkworth et al. 1989a). After the initial reports that TCDD was teratogenic in mice, the focus of research was to characterize these fetal anomalies and on finding a sensitive period during gestation when these effects occurred. Unfortunately, because of the study designs, fetal tissue dose and distribution was not obtained, therefore, quantitative assessment of dose response relationships can not be done.

To determine if there is a sensitive period that cleft palate or hydronephrosis occurred, Birnbaum et al. (1989) and Weber et al. (1985) administered TCDD to mice on GD 10 or 12. In the offspring of pregnant C57BL/6N mice administered a single oral dose on GD 10 of 0.

12, 17, and 22 ug TCDD/kg, a significant and dose-dependent increase in percent of fetuses with cleft palate was observed. Maternal toxicity characterized as decreased weight gain and increased relative liver weight was significant at the 17 ug TCDD/kg dosage level.(Weber, et al., 1985). However, kidney damage which was significant at all levels, was not clearly dose dependent because of a near maximal response at the lowest dose. These doses are equivalent to the doses administered for 10 days to the CF-1 (Smith, et al. 1976) mice which produced cleft palate and hydronephrosis. Similar doses were also used by Birnbaum et al. (1989) to assess the teratogenic effects of TCDD in C57BL/6N mice. Pregnant mice were administered a single oral dose of 0, 6, 12, 15, or 18 ug TCDD/kg on GD 10, or doses of 0, 6, 9, 12, 15 ug TCDD/kg on GD 12 (Table VII). On GD 10 and GD 12 maternal toxicity characterized as increase relative liver weight was observed at all doses. A statistically significant dose-related increase in cleft palate was observed at 12 ug TCDD/kg on GD 10 and at 9 ug TCDD/kg on GD 12. Maximal cleft palate induction was observed in C57BL/6N mice administered TCDD on gestation day 12. The observed incidence was about 60% greater than the incidence observed at the same dose (15 ug TCDD/kg) on GD 10. Couture et al. (1990a) reported, in an experiment using single dose administration in C57BL/6J mice, that on GD 10 or 12 a LOAEL of 12 ug TCDD/kg for cleft palate, and a LOAEL of 3 ug TCDD/kg on GD 6, 8, 10, 12 or 14 for hydronephrosis.

To determine when palate formation was most sensitive to TCDD, Pratt et al.(1984) administered a single s.c. dose of 100 ug TCDD/kg to C57BL/6J mice on days 3-13 of gestation. Greater than 95% of the fetuses from dams administered TCDD on days 8,9, and 10 had cleft palate. The maximum response was observed on day 10. Histological examination of palate

formation in controls revealed significant cell death of medial epithelial cells resulting in palate closure. In the TCDD treated mice this cell death was not observed. This apoptosis (programmed cell death) is apparently inhibited by an Ah receptor mediated mechanism (Pratt et al. 1984). The role of the Ah receptor in the teratogenic effects of TCDD after repeat dose administration was investigated by Silkworth et al. (1989a). By comparing C57BL/6J and DBA/2J mice Silkworth determined that the incidence of cleft palate segregated with the Ah receptor. Pregnant C57BL/6J mice were administered 0, 0.5, 1.0, 2.0, and 4.0 ug TCDD/kg/day during gestation day 6-15 (total dose of 0, 5, 10, 20, and 40 ug TCDD/kg). DBA/2J were administered up to 8.0 ug TCDD/kg/day. (Table V) The most sensitive indicators of maternal effects in C57BL/6J mice was an increase in relative liver weight and a decrease in relative thymus weight seen at all doses. A significant increase in relative liver weight and a decrease in the relative thymus weight was seen in the DBA mice at the 2.0 ug TCDD/kg/day and 8.0 ug TCDD/kg/day dose level, respectively, indicating a 4-8 fold decrease in TCDD sensitivity in the DBA/2J mice consistent with other studies. In both strains of mice there was a statistically significant increase in percent cleft palate/dam at the highest doses 4 and 8 ug TCDD/kg/day. These doses were 4-8 times higher than the LOAEL for maternal effects. This study indicated that the fetal abnormality (cleft palate) seen in DBA and C57BL/6J mice was a less sensitive indicator of TCDD's effects than the measures of maternal effects, but that percent of offspring with hydronephrosis was more sensitive being significantly increased in both strains at 0.5 ug TCDD/kg/day. This is not true with all mice strains. In the CF-1 mice studied by Smith et al. (1976) using a similar protocol, the teratogenic effects (cleft palate) of TCDD proved to be a more sensitive indicator than maternal effects characterized as increase relative liver weight.

Most importantly this comparison of C57BL/6J and DBA/2J points out that in these two mouse strains a statistically significant increase in hydronephrosis is observed at the same dose suggesting this may be an equitoxic measure of maternal dosing of TCDD, however the magnitude of this response is not equivalent at the LOAEL, 5 ug/kg.

Identifying the window in which maximal cleft palate occurred enabled Abbott and Birnbaum (1989) to investigate the mechanism by which TCDD disrupted medial epithelial cell terminal differentiation. Levels of EGF receptor were found to be a critical difference in the development of the embryonic palate in the presence of TCDD. Using the C57BL/6N, Abbott and Birnbaum, administered a single oral dose of TCDD on GD 10 or GD 12. Four days post dosing measurements of thymidine incorporation, concentration of EGF receptor, and degree of palate closure was shown to be markedly increased. It was concluded that the characteristic TCDD mediated cleft-palate formation is due to a defect in the fusion of the palatal shelves. Although the shelves meet, the medial cells which usually die during palate closure continue to proliferate under the influence of TCDD. The mechanism by which this occurs is shown to be related to the increase expression of the EGF receptor in the medial epithelium on GD 10 and 12, which coincides with the sensitive time for cleft palate formation (Abbott et al. 1989a).

The incidence of cleft palate in mice correlates well with their sensitivity to Ah receptor mediated toxicity. Poland and Glover (1980) administered a single s.c. dose of 30 ug TCDD/kg to pregnant dams of 10 different mice strains. Four of the five TCDD sensitive strains had a 54-95% incidence of cleft palate, whereas all the strains with a low sensitivity to Ah receptor mediated toxic effects had a < 4% incidence of cleft palate. Poland and Glover also investigated the strain differences using C57BL/6J, DBA/2J and their hybrid cross B6D2F1/J

mice. Mice were administered a single s.c. dose of 0, 3, 10, or 30 ugTCDD/kg on GD 10. A dose-dependent increase in cleft palate-live fetus was seen in the C57BL/2J mice (Table VII). In the other two strains cleft palate was only seen at the highest dose indicating a segregation with the Ah receptor.

Dencker and Pratt (1981) investigated the role the tissue concentration of the Ah receptor plays in cleft palate formation using two strains of mice C57BL/6J, and AKR/J (a TCDD resistant strain). The results of this comparison indicate that the concentration of Ah receptor is greater in the maxillary process (the region containing palate shelves) of the C57BL/6J mice than in the AKR/J mice. In addition, the embryonic C57BL mice maxillary process contained a higher concentration of Ah receptor (measured on GD 13) than the embryonic brain, liver, limb buds or skin. The Ah receptor without doubt has some role in cleft palate formation probably involving the altered transcription of a protein involved in palate epithelial cell division or apoptosis (Dencker and Pratt 1981).

Experiments using cultured embryonic palate tissue have enabled researchers to observe palate development more closely and to compare very sensitive endpoints in mouse, rat and human tissue. Abbott and Birnbaum (1989) established this methodology using C57BL/6N mice palate shelves. An extensive database of in vivo data has made this comparison possible. GD 12 mice palate shelves were exposed to 0, 10^{-13} , 10^{-12} , 10^{-11} , 5×10^{-11} , 7.5×10^{-11} , 10^{-10} , and 10^{-9} M TCDD (Table VIII for dose equivalents). A dose-response was observed in percent of palates not undergoing apoptosis (an endpoint that indicates the medial epithelial cells continue to grow resulting in cleft palate formation). The response increases until reaching the cytotoxic concentration of 7.5×10^{-11} M. The EGF receptor, measured by immunochemistry, increased

in medial epithelial cells at 10^{-11} , 5×10^{-11} , 10^{-10} , and 10^{-9} M (only doses reported). An increase in cell proliferation (thymidine incorporation) was also found to increase significantly. Table VIII points out the relationship between in vitro culture concentrations and administered dose. These calculations are based on the observation that 0.0003% of a 30 ug TCDD/kg dose administered to a pregnant C57Bl/6N mouse is distributed to the palate tissue (Abbott and Birnbaum 1989). The concentrations that produce cleft palate in vitro correlate well with the doses that are found in vivo to produce cleft palate. For example, Birnbaum et al. (1989) found an increase cleft palate after a single dose administration of 12 ug TCDD/kg. Abbott and Birnbaum (1989) found a significant increase in EGF receptor levels in medial epithelial cells at all dose above 10^{-11} (equivalent to 20 ug TCDD/kg). Decreased program cell death was observed at dose equivalents of 0.2 ug TCDD/kg to 200 ug TCDD/kg.

Rats are not as sensitive to cleft palate formation as are mice. This appears to be because TCDD is fetotoxic and maternally toxic. Therefore, fetal death may occur at lower doses than cleft palate formation. The pups of Holtzman rats administered 0, 1.5, 3, 6, and 18 ug TCDD/kg on GD 10 developed cleft palate only at the 18 ug TCDD/kg dose. A dose-dependent increase in percent fetal mortality and decrease in fetal thymus weight was observed in these rats. Although cleft palate was not observed at the low doses, the percent of fetuses per litter affected by intestinal hemorrhage was significantly increased in a dose-dependent manner at all doses (Olson 1989, 1990). Other endpoints were also found in other animal species, guinea pigs were treated orally with 0, 0.15 and 1.5 ug TCDD/kg on GD 9 and a significant increase in percent fetal mortality occurred at the 1.5 ug TCDD/kg dose (Olson 1990). The developmental toxicity of Golden Syrian Hamsters was investigated (Olson 1990).

after administration of 0, 1.5, 3, 6, or 18 ug TCDD/kg on GD 9. A significant dose related increase in relative liver weight was observed in dams at all doses. In addition, a significant dose-dependent increase in neutrophilic metamyelocytes, neutrophilic band cells, total liver P450 activity, and EROD activity was observed at all doses. A dose-dependent decrease in liver estradiol 2-hydroxylase activity was observed in the dams, which was significant at the 3 ug TCDD/kg dose level. Fetal liver EROD and total P450 also increased at all doses, but statistical significance was not determined due to small sample size. Fetal thymus area decrease and kidney congestion was observed at all dose levels. A dose-dependent increase in the incidence of hydronephrosis was observed and was statistically significant at the 3.0 ug TCDD/kg (maternally administered) dose level (Olson et al. 1990). The hamster is the least sensitive species tested to the acute toxic effects of TCDD (see Table II), but it is extremely sensitive to the developmental abnormalities observed. Kidney congestion and hydronephrosis are observed after a single dose of 1.5 ug TCDD/kg which is equivalent to the toxicity seen in the CF-1 mouse (Table V). This observation raises questions regarding the ability of acute toxicity to predict tissue specific sensitivity.

The in vitro palate culture provides a method to examine palate development in rats without the associated maternal or fetotoxicity of TCDD. Abbott and Birnbaum (1990) culturing GD 14 palates of F344 rats with TCDD, found a dose-dependent increase in the number of palates in which the medial peridermal cells did not go through programmed cell death (Table VIII). Since data on distribution of TCDD to the rat fetal palate has apparently not been reported it is not possible to determine the comparable administered dose. It is valuable to compare the tissue dose response in several species. The mouse is about 100-1000

Table VII TERATOGENIC EFFECTS: DOSE RESPONSE

Species	Dosing Regimen	Effect	Dose		Response			NOAEL	LOAEL	Reference
Carworth C1-1 mice	oral dose administered daily GD 6-15	percent litters with cleft palate	0 ug/kg/day 0.001 0.01 0.1 1 3	0 ug/kg tot. dose 0.01 0.1 1.0 10 30	0% palate 5% 0% 0% 6% 21%* 71%*	0% kidney 0% 0% 0% 5% 28%*	0.1 ug/kg/day	1.0 ug/kg/day 10 ug/kg	Smith et al. 1976	
CD-1 mice	1. daily oral dose administration 2. daily s.c. dose administration GD 7-16	% cleft palate /total fetuses	0 ug/kg/day 25 50 100 200 400	0 ug/kg tot. dose 250 500 1000 2000 4000	0. Total 3% 19% 66% 100% 100%	0. 2. s.c. 82% 79% 85% 100% 100%	N/A	N/A	Comtine, 1976	
CD-1 mice	1. daily oral dose administration 2. daily s.c. dose administration GD 7-16	% kidney anomalies/total fetuses	0 ug/kg/day 25 50 100 200 400	0 ug/kg tot. dose 250 500 1000 2000 4000	0. Total 34% 72% 71% 100% 50%	0. 2. s.c. 53% 58% 95% 38%	N/A	N/A	Comtine, 1976	
C57BL/6J mice	single oral dose administered on GD 10	1. percent litters with cleft palate 2. percent fetuses with cleft palate	0 ug/kg 12 ug/kg 17 ug/kg 22 ug/kg		1. 0% 40% 82% 100%	2. 0% 8.7%* 43.9%* 77.6%*		12 ug/kg	Wolber et al 1985	
C57BL/6J mice	daily administration GD 6-15	1. % cleft palate per dam 2. % hydronephrosis	0 ug/kg/day 0.5 1.0 2.0 4.0	0 ug/kg Total dose 5.0 10 20 40	1. 0% 0% 2.31% 2.33% 47.23%*	2. 0% 39.92%* 72.79%* 74.23%* 91.83%*	1. 20 ug/kg 2. 5 ug/kg	4 ug/kg/day	Silkworth et al. 1989a	
DBA/2J mice	daily administration GD 6-15	1. % cleft palate per dam 2. % hydronephrosis	0 ug/kg/day 0.5 2.0 4.0 8.0	0 ug/kg Total dose 5 20 40 80	1. 0% 0% 3.51% 3.47% 27.24%*	2. 0% 4.95% 13.16% 36.89% 60.47%*	1. 40 ug/kg 2. 5 ug/kg	8 ug/kg	Silkworth et al. 1989a	
C57BL/6N mice	single oral administration on GD 6,8,10,12	1. % fetuses/litter with hydronephrosis 2. % fetuses/litter with cleft palate	Dose 0 ug/kg 3 6 9 12 24	1. GD 6 25.7% 96.2% 100.0% 100.0% 100.0% 2. GD 6 0.0% 0.0% 1.0% 0.0% 3.5% 40.3%*	1. GD 8 2. GD 8 27.0 0.3 90.0 0.0 - - 100.0%* 9.7% 100.0%* 93.4%*	1. GD 10 2. GD 10 18.6 0.0 89.2 0.0 - - 100.0%* 53.9% 100.0%* 98.8%*	1. GD 12 2. GD 12 6.3%* 0.0 97.9%* 1.3 - - 100.0%* 76.0%* 100.0%* 100%*	Comtine et al 1990a		
1. C57BL/6J 2. B6D2F1 3. DBA/2J mice	single s.c. administration GD 10	% cleft palate live fetuses	0 ug/kg 3 ug/kg 10 ug/kg 30 ug/kg		1. 0% 0 3 54	2. 0% 0 0 13	3. 0% 0 0 2			Poland and Gikwer, 1980
Golden Syrian Hamsters	single oral dose GD 10	1. % kidney congestion 2. % hydronephrosis 3. % fetal mortality	0 ug/kg 1.5 3.0 6 18	1. 0 9.7% 28.5% 83.6% 91.1%*	2. 1.6% 11.2% 15.3%* 34.5% 0	3. 7% 10.8% 7.7% 8.7% 58.2%			Olson, 1990	
C57BL/6N Mice	single oral dose administered to dam on GD 10 or GD 12	increase cleft palate incidence	0 ug/kg GD 10 6 12 15 18	0 ug/kg GD 12 6 9 12 15	0 GD 10 incidence 0 20.86% 49.30% 70.42%*	0 GD 12 incidence 1.43 19.91% 51.45% 77.91%*	GD 10 = 6 ug/kg GD 12 = 6 ug/kg	GD 10 = 12 ug/kg GD 12 = 9 ug/kg	Birnbaum, et al. 1989	

times more sensitive when examining the endpoint of programmed cell death. Hence, it may be concluded that tissue distribution of TCDD is not the rate limiting factor for species sensitivity. Human effects of TCDD relating to pregnancy are difficult to determine, primarily because of the confounding effects of multiple potential etiologic agents, a relatively high background of birth defects, and a lack of robust exponential data. The cultured human palate allows direct comparisons between similar endpoints that have been evaluated in the rat and mouse species. Abbott and Birnbaum (1991) cultured human palatine tissues GD 52, 53, and 54 for 3-4 days. Evaluation of palate growth and development revealed that 100% of the palate medial epithelial cells continued to proliferate and scheduled programmed cell death did not occur at 10^{-8} and 10^{-9} M TCDD concentrations. Since only three concentrations were reported, a dose-response relationship is difficult to evaluate although there was an increased response observed between the 10^{-11} and the 10^{-8} M concentrations. Direct comparison between the human and mouse palate demonstrates that the concentrations at which 25% or 100% of palates were affected (programmed cell death did not occur) were 500 times more sensitive in the mouse than in the human palate (See Table VIII). These in vitro observations found that the human palate is sensitive to cleft palate formation through the same mechanism seen in mice; changes in growth factors (ie: EGF, and TGF's) that are involved in the mechanism of altering programmed cell death in the medial epithelial cells of the palate. The effects of TCDD on regulation of these growth factors causes epithelial cells to continue to proliferate resulting in palatine shelves that do not fuse normally.

Table VIII: Comparison of Effects of TCDD on In Vitro Palate Cultures				
Species	Gestation Days	Concentrations	Response	Reference
C57BL/6N mice	GD 12	Control 1×10^{-11} M 1×10^{-10} M 5×10^{-10} M 1×10^{-9} M 5×10^{-9} M 7.5×10^{-9} M 1×10^{-8} M	6.7% without Programmed Cell Death 25.7% without PCD 58.7% without PCD 66.7% without PCD 66.7% without PCD 100% without PCD 83.7% without PCD 50.7% without PCD	Abbott et al., 1989a
F344 rats	GD 14-15	Control 5×10^{-11} M 1×10^{-10} M 1×10^{-9} M 1×10^{-8} M	5.7% without Peridermal Cell Death 0.7% without Peridermal Cell Death 20.7% without Peridermal Cell Death 36.7% without Peridermal Cell Death* 60.7% without Peridermal Cell Death*	Abbott and Birnbaum, 1990
Human Palate Cultures	GD 52, 53 and 54	Control 5×10^{-11} M 1×10^{-10} M 1×10^{-9} M	0.7% without Peridermal Cell Death 25.7% without Peridermal Cell Death 100% without Peridermal Cell Death 100% without Peridermal Cell Death	Abbott and Birnbaum, 1991

Alteration of EGF has been reported in other cell systems. Hudson et al. (1985) found a concentration-dependent decrease in EGF receptor binding after treatment with TCDD in human keratinocyte cell line. These binding assays demonstrated two binding components for EGF: high and low affinity. Cells treated with TCDD have a loss of the high affinity binding component. TCDD does not directly bind to the EGF receptor and the loss of binding the high affinity receptor may be due to an alteration in other binding factors. The decreased binding affinity in human keratinocytes is associated with a decrease in DNA synthesis. In contrast, palate epithelial cells treated with TCDD in culture were found to have an increase in EGF receptor level and a concomitant increase in cell proliferation. The responses in the human keratinocyte and human palatine cells appears to be very different. This may indicate an intrinsic difference in cellular response to cell specific growth factors and subsequent differences in regulation of cell growth.

D.2 Hydronephrosis

Hydronephrosis is the second well characterized fetal anomaly observed commonly in TCDD treated mice. In contrast to cleft palate which is species and tissue specific for TCDD treated mice, kidney toxicity occurs after pre and postnatal treatment, and occurs in several species. The kidney lesions have been characterized as an increase cell proliferation of the epithelial cells of the uterine lumen. Cell proliferation causes obstruction/constriction of the ureter resulting in decreased urine outflow and "true hydronephrosis" (Abbott et al. 1987). Uterine epithelial hyperplasia has been observed in mice administered TCDD both pre and postnatally, in adult guinea pigs (McConnell et al. 1978a) in Rhesus monkeys (Gupta et al. 1973) and reported as kidney anomalies, but not further characterized in CD-rats (Courtney and Moore 1971).

TCDD administered to pregnant C57BL/6 mice (GD 10-13) at 1.0, 3.0 and 10.0 ug TCDD/kg/day (total dose 4, 12, 40 ug/kg) resulted in a dose dependent increase in the incidence of early stages of hydronephrosis and "renal papilla which are markedly reduced in size, or non-existent, in a few cases resulting in an enlarged renal pelvis." These lesions were found predominantly in the right kidney. The lesions were also found in pups exposed postnatally and were qualitatively similar to lesions found in fetuses exposed in utero (Moore et al. 1973). Fetal hydronephrosis is a very sensitive indicator of TCDD toxicity with an incidence of 89-98% observed after administration of a single dose of 3 ug TCDD/kg during GD 6, 8, 10, or 12 (Couture et al. 1990a).

In mice hydronephrosis appears to be the most sensitive indicator of fetotoxicity. Silkworth et al. (1989a) demonstrated this by administering C57BL/6J and DBA/2J mice 0, 0.5, 1, 2, and 0, 0.5, 2, 4, 8 ug TCDD/kg/day respectively during GD 6-15. A statistically significant

increase in hydronephrosis was observed at the lowest dose tested 0.5 ug TCDD/kg/day (total dose 5 ug TCDD/kg) in both strains of mice with no NOAEL observed for this endpoint. In contrast cleft palate incidence was observed to be statistically significant (Table V) at the 4 ug TCDD/kg/day (40 ug/kg total dose) in C57Bl/6J mice and at 8 ug TCDD/kg/day (80 ug/kg total dose) in the DBA/2J mice. The sensitivity of these endpoints may be related to the window of sensitivity in fetal development that these anomalies can occur. Studies determining the mechanism of each of these lesions have helped to understand these differences.

Couture et al. (1990a) administered a single oral dose to C57BL/6N mice on GD 6, 8, 10, 12 or 14 to investigate the point in gestation that TCDD produced maximal toxicity. Unfortunately, all administered doses (3, 6, 9, 12, 24 ug TCDD/kg produced a statistically significant increase in the incidence of hydronephrosis from GD 6-14 so a sensitive time period was not determined (Table VII). The incidence appeared to decrease after day 14. Couture-Haws et al. (1991, 1991a) examined sensitivity to TCDD administered postnatally in dams administered 0, 6, 9, 12 ug TCDD/kg on postnatal day 1, 4, 8 and 14. All doses significantly increased the incidence of hydronephrosis on day 1 and 4. No increase was seen on PND 8 or 14. No maternal toxicity was observed at any dose. These results indicate that the neonatal kidney is affected by the level of TCDD available in milk.

The mechanism is similar to that for cleft palate since altered regulation of cell growth and death are responsible for cleft palate formation in the mouse. This is mediated through a sustained level of EGF production not found in control animals. TCDD induced hydronephrosis in the mouse is characterized as an increase in uterine epithelial cells causing closure of the ureter. The continued proliferation of these epithelial cells as measured by thymidine incorporation is found to correlate with a sustained level of EGF receptors (Abbott

and Birnbaum 1990a).

Cultured ureteric epithelial cells derived from GD 10 or 12 mouse fetuses had an incidence of 91-100% hyperplasia after exposure to 1×10^{-10} M (32 pg/ml) TCDD, a 4-fold increase over control. In addition, intense EGF receptor staining (immunohistochemistry) and increased thymidine uptake (indicating cell growth and proliferation) was observed in many of these sections (Abbott and Birnbaum 1990a). Based on the LOAEL of hydronephrosis in C57BL/6N mice in vivo (3 ug TCDD/kg) the tissue dose seen in the ureter of these mice is equivalent to 0.3 pg (Abbott and Birnbaum 1990a).

When comparing TCDD toxic endpoints, the effects on fetal development do not appear to be the most sensitive in all of the species studied. However, without information on the distribution in all species this conclusion is somewhat limited.

Hydronephrosis is a more sensitive fetotoxic endpoint in the mouse and in the hamster, occurring at doses that are not overtly toxic to dams. Cleft palate on the other hand appears to be a TCDD-related teratogenic effect unique to the mouse, but analysis of the mechanism reveals that the interference with programmed cell death by TCDD is not unique to the mouse. Cleft palate is found at doses where maternal effects are observed (characterized as decreased weight gain or increase relative liver weight) in C57BL and DBA mice, but cleft palate is not accompanied by maternal effects in the CD-1 or CF-1 mice, although these strains have been thoroughly examined.

D.3 Other

Giavani et al. (1982, 1982a, 1982b) attempted to characterize cleft palate and other abnormalities in rabbits and rats. The most frequently observed lesion among litter-bearing pregnant rats administered 0, 0.125, 0.5 and 2 ug TCDD/kg/day during GD 1-3 was

kidneys. This was not statistically significant and was observed at doses that were fetotoxic and maternally toxic, but it does demonstrate that kidney development is a target of TCDD toxicity when administered early in gestation (Giavini et al. 1982a). Pregnant rabbits administered 0, 0.1, 0.25, 0.5 and 1 ug TCDD/kg/day GD 6-15, TCDD had a significant increase in post-implantation losses reaching 100% at the highest dose. At maternally toxic and fetotoxic doses, a small increase in kidney abnormalities characterized as hydronephrosis and double kidney formation was observed (Giavini et al. 1982b). These observations indicate that the development of kidney anomalies, although found, are not the most sensitive indicator of fetotoxicity in the rabbit or the rat.

Giavini et al. 1982 investigated the effects of TCDD on the implantation characterized as mean corpus lutea, mean number of implantation, and percent preimplantation losses in Sprague-Dawley rats. Dams were administered 0, 0.125, 0.5, and 2.0 ug TCDD/kg/day for 3 days GD 1-3 (total doses 0, 0.375, 1.5, and 6 ug/kg). Fetotoxicity measured as fetal weight on GD 21, and maternal toxicity measured as weight gain was also evaluated. Maternal toxicity was observed at the highest dose only, and fetotoxicity was observed in the 0.5 and the 2 ug TCDD/kg/day dose group. The results indicate that no significant relationship between TCDD at doses up to 2 ug TCDD/kg/day and pre or post implantation losses, mean corpus lutea, or number or implantation sites. Fetotoxicity was found to be related to maternal toxicity (Giavini et al. 1982) as suggested by Khera (1987), who postulated that maternal toxicity may be responsible for the fetotoxicity found after administration of certain chemicals. In Sprague-Dawley rat (Sparschu et al. 1971) administered 0.03, 0.125, 0.5, 2.0 and 8.0 ug TCDD/kg GD 6-15 the most sensitive indicator of fetotoxicity was decrease fetal weight and gastrointestinal hemorrhage LOAEL = 0.5 ug TCDD/kg/day. This occurred at a dose that was not maternally

toxic although decrease fetal weight may be associated with undetected maternal toxicity. Gastrointestinal hemorrhage was also the most sensitive indicator of fetal toxicity observed in Holtzman rats by Olson (1990). Dams administered 0, 1.5, 3, 6, and 15 ug TCDD/kg single p.o. dose on GD 10 had a statistically significant increase in percent fetuses per litter affected at all doses administered.

Evaluation and comparison of reproductive endpoints are complicated by the predominance of species specific effects and the unique problems of fetal/tissue distribution. Understanding the underlying biochemical mechanisms responsible for adverse reproductive effects is essential to proceeding with comparative analysis. For cleft palate these mechanisms have been thoroughly investigated and any risk assessment will be enhanced by incorporating this information.

E. Hormone Effects

In the classic TCDD carcinogenicity study (Kociba et al. 1978) it was observed that female rats had a dose-dependent increase in liver cancer and a dose-dependent decrease in the incidence of spontaneous tumors in the uterus, breast and pituitary. These observations, coupled with reports suggesting that the mechanism of TCDD toxicity was mediated through a nuclear hormone-like receptor mechanism (Poland and Knutsen 1982) led researchers to investigate the role of hormones in modulating TCDD toxicity. Early work focused on thyroid hormone and the role of the thyroid in the toxicity of TCDD (McKinney et al. 1985; Lucier, this document). The possible role of estrogens and estrogen receptor in the toxicity of TCDD was recognized by several research teams (Gallo and Umbreit 1988). The estrogen receptor and the Ah receptor are both ligand-specific and response element specific (Denison 1991, Green and Chambon, 1991). Experimental evidence (Poland and Glover, 1980; Romkes and Safe,

1988) indicates that TCDD does not bind the estrogen receptor(ER) nor does estradiol(E2) bind to the Ah receptor. However, there may be direct competition for factors that modulate ligand-receptor binding, translocation, and response element binding i.e., heat-shock protein 90 (Perdew 1988, Green and Chambon, 1991). It may be the effect of TCDD on the concentration of uterine and hepatic ER that results in modification of estrogens sensitive tissues. In addition, subsequent alteration in estradiol levels from induction of CYP1A1 and Cyp1A2 may induce organ specific changes (DeVito et al. 1992a). Altered estradiol levels have also been implicated in a reduced immune response (Luster et al. 1985), and both an increase and decrease in tissue specific cancers. The relationship between TCDD, estradiol and cancer detailed in the Carcinogenicity Chapter of this document(Lucier) is still not fully understood.

The dose response relationships between TCDD and altered Estrogen Receptor(ER), and/or changes in serum estradiol can result in a variety of responses. Evaluation of TCDD in non-human primates revealed a decrease in ability to conceive and an increase in aborted fetus. Rhesus monkeys administered a continuous dose of 50 ppt TCDD in the feed (total dose 1.8 ug TCDD approx equivalent to 8.6 ng TCDD/kg/day) had increases in serum estradiol and progesterone. Abnormal hormone levels may have been responsible for only 2/8 treated animals conceiving versus 8/8 in the control group (Allen et al. 1979). McNulty 1985 reported a dose-related increase in fetal loss at much higher levels: 1.0 ug TCDD/kg and 5.0 ug TCDD/kg total dose (administration divided into 9 intragastric doses GD 20-40) which was accompanied by maternal toxicity. No hormone levels were measured in these Rhesus monkeys so its relationship to fetal loss can not be determined.

The effects of TCDD mediated alteration in hormone levels is not restricted to the

reduced ability to conceive or fetal loss. A decrease in uterine and body weight was observed in prepubescent female guinea pigs, rats and hamsters administered a single 4, 50, 400 ug TCDD/kg (an equitoxic) dose of TCDD (Hruska and Olson 1989). A statistically significant decrease in uterine weight occurred in guinea pigs and Sprague Dawley rats. Only a slight (not significant) decrease in uterine weight was observed in the hamster. In addition to uterine weight changes, increases in the uterine estrogen receptor density (measured using saturation analysis, followed by estimation of receptor density [Bmax] from Scatchard plot) were observed at above administered concentrations. The largest change in uterine ER density (Bmax) was in the rat, followed by the hamster, then the guinea pig. Neither the change in uterine weight nor the constitutive levels of ER (Bmax) predicted the increase in uterine receptor density. At these doses TCDD decreased the hepatic ER concentration significantly in rats and guinea pigs but not in hamsters. The species differences in TCDD mediated changes in hepatic ER may be partially explained by the constitutive levels of these receptors. In the three species studies, the constitutive level of ER in the liver predicted the magnitude of the decrease in hepatic ER. The differences in uterine and hepatic ER responses suggests that there may be other factors not found in the liver, but present in the uterus that modulate this response.

Species differences in many endpoints can be directly related to their differences in hepatic enzyme (P450) induction. Lin et al. (1991) investigated this relationship as it pertained to ER using C57BL/6 responsive strain (Ah^h) and the non-responsive strain (Ah^u). Mice were administered 0, 0.3, 1, 3, 10, 30, 100, and 300 ug TCDD/kg single oral dose. The study found that the magnitude of the decrease in hepatic ER in the Ah^h mice was significantly more than that of the non-responsive mice. Although the dose (LOAEL) at which the decrease in hepatic

ER was observed was similar in both strains, approximately 0.06 ug TCDD/kg.

In intact and ovariectomized Long Evans rats (Romkes et al. 1987) a dose dependent decrease in hepatic and uterine ER was observed after administration of 20 ug TCDD/kg or 80 ug TCDD/kg TCDD. This study also showed that the administration of estradiol with the TCDD further decreased the ER in the uterus, but did not have an effect on liver ER. This again indicates other factors (primarily hormonal) are responsible for the increase in sensitivity of the uterus to TCDD.

The receptor mediated toxicity of TCDD has been discussed in detail in this document (Whitlock chapter). Clearly TCDD's effective concentration and mechanism of toxicity are comparable to that of nuclear hormones. This does not imply that there is a direct interaction between TCDD and hormone receptors. In a study in which female Long Evans rats were administered a single i.p injection of 0, 20, 40, and 80 ug TCDD/kg a dose-dependent decrease in the hepatic and uterine ER and progesterone receptors (PR) was observed (Romkes and Safe 1988). This study also demonstrated that TCDD has no binding affinity for the ER and PR which suggests that TCDD does not exert its antiestrogenic properties through competition with the ER or PR.

DeVito et al. (1992a) has recently demonstrated that down-regulation of the hepatic and uterine estrogen receptor (ER) occurs at doses that increase Cyp1A1 (Table IX) but there are no accompanying changes of serum estradiol or increases in 2-OH estradiol (DeVito et al., 1991). Graph E (using a Michaelis-Menten and a Power Law function) depicts the increase in hepatic Ah activity at doses that decrease hepatic estrogen receptor. Although the Michaelis-Menten function can be fit to this data it is the Power Law function that provides insight into

this relationship. The similarity in the apparent kinetic order ("g" value) for the percent decrease in hepatic estrogen receptor and percent increase in AHH activity suggests a common mechanism of action. Supporting this is the information that this TCDD induced down-regulation of the ER segregates with Ah receptor (Lin et al. 1991). Other investigators have suggested that, in the MCF-7 breast cancer cell line, that TCDD induced increases in P450 enzymes increase estrogen metabolism resulting in a decrease in the ER (Gierthy et al. 1988). This is significant because it demonstrates that TCDD may induce decreases in estradiol which are Ah receptor mediated, and suggests that clinical effects could result from a decrease in estradiol not directly from TCDD.

It has been shown that *c-fos* is a nuclear regulatory protein that plays a role in the control of estradiol induced cell proliferation (Astroff et al 1991). At very low i.p. administered doses (0.064, 0.128 $\mu\text{g TCDD/kg}$), reduced constitutive levels of uterine *c-fos* mRNA were observed in Sprague-Dawley rats, however coadministration of exogenous estradiol increased the levels of *c-fos mRNA* (Graph F-A). Although TCDD does not interact directly with ER, it may compete with the activated estrogen receptor at the level of transcription. The power law function applied to the TCDD induced decrease in *c-fos* resulted in a functional fit with $R^2 = 0.99$, "g" = 0.66 (Graph F-B). This is consistent with the "g" values described later in this chapter that were computed for the TCDD induced increase in EROD activity (Abraham et al. 1988, "g" = 0.66) and benzo(a)pyrene hydroxylase (Kitchin and Woods, 1979, "g" = 0.77), suggesting the mechanisms by which TCDD alters *c-fos mRNA* may be, in part, common with those mechanisms involved in P450 induction.

Table IX HORMONE EFFECTS: DOSE RESPONSE

Species	Dosing Regimen	Effect	Dose	Response				NOAEL	LOAEL	Reference
Female Long Evans Rats	single ip	Uterine % decrease cytosolic ER % decrease nuclear ER % decrease cytosolic PR % decrease nuclear PR	0 ug/kg 20 50 80	C-ER 0 % 16.4 % 23.6 % 46.2 %	N-ER 0 % 12.4 % 32.0 % 68.3 %	C-PR 0 % 14 % 20 % 25 %	N-PR 0 % 3.5 % 17.9 % 22.9 %			Romkes and Safe, 1988
Female Long Evans Rats	single ip	Hepatic % decrease cytosolic ER % decrease nuclear ER % decrease cytosolic PR % decrease nuclear PR	0 ug/kg 20 50 80	C-ER 0 % 4.0 % 7.5 % 54.3 %	N-ER 0 % 12.3 % 45.5 % 26.9 %	C-PR 0 % 3.5 % 18.1 % 63.4 %	N-PR 0 % 21.7 % 27.5 % 64 %			Romkes and Safe, 1988
Female S-D rats	single ip	% decrease in c-fos mRNA units	0 ug/kg 0.016 0.064 0.128	0 % 20 % 44 % 82 %				0.016 ug/kg	0.064 ug/kg	Astolfi et al., 1991
Mice CD-1	single ip dose	% decrease hepatic ER % decrease uterine ER % increase hepatic AHH	0 ug/kg 1 3 10 30	H-ER 0 % 9.4 % 14.1 % 23.4 % 46.9 %	U-ER 0 % 4.5 % 0 % 40.9 % 36.5 %	H-Ahh 0 % 116.6 % 333 % 467 % 400 %				DeVito et al., 1992a

Profound changes occur in biological systems as a consequence of minute changes in hormone levels. Much evidence exists that TCDD acts like a hormone, primarily because of receptor action and the low concentrations that are associated with effects, but also because of the types of systems affected. Many qualitative changes that occur as a consequence of TCDD administration are well documented; however, the dose-response relationships for many of these endpoints have not been evaluated. Dose-response assessments and an increase understanding of the interaction with other hormone systems is important and should be a focus of future research.

F. Male Reproductive Toxicity

The male reproductive system is adversely affected by TCDD and related compounds. A detailed review of TCDD induced male reproductive toxicity is presented in the Reproductive and Developmental Toxicity chapter of this document (Peterson). Some of the effects are described here to help understand their dose-response relationships and compare the specificity and sensitivity of this response to other endpoints.

Overtly toxic doses of TCDD that decrease body weight and feed consumption decrease plasma androgen concentrations, testis and accessory sex organ weights and alters spermatogenesis and testicular morphology when administered to postweanling male rats (Moore et al. 1985, 1989, 1991; Mebus et al. 1987; Morrissey and Schewtz 1989; Kleeman et al. 1990; Bookstaff et al. 1990). The decreases in circulating androgens by TCDD are mediated through at least two different pathways. TCDD inhibits testicular steroidogenesis (Kleeman et al. 1990) and alters the regulation of pituitary luteinizing hormone secretion (Bookstaff et al.

1990). The ED_{50} for the androgenic deficiency is approximately 15 ug TCDD/kg (Moore et al. 1985). In this study Moore et al. administered single oral doses of 0, 6.25, 12.5, 25, 50 and 100 ug TCDD/kg to male Sprague-Dawley rats. Serum testosterone was measured 7 days post administration. Graphic representation (Graph G) depicts a dose-dependent decrease in serum testosterone. The Power Law function fit this data ($R^2 = 0.92$). When the power of the curve fit was compared to the power of the curve fit of other endpoints (Benzo(a)pyrene hydroxylase induction Graph K and EROD induction Graph M) it was found that the kinetic order ($g = 0.60$) was consistent with these other endpoints. There are no studies that can directly compare biochemical endpoints quantitatively to male reproductive endpoints because protocols similar to those designed by Moore et al. (1985) have not been used. However, the similarity of the curve fit using the power law function to biochemical endpoints (see biochemical endpoints discussion, section G) suggests a common mechanism may be responsible for the decrease in serum testosterone. These data indicate that the adult male reproductive system toxicity may be mediated by similar mechanism but is not as sensitive to the actions of TCDD in that androgen deficiency occurs at doses that induce overt toxicity.

While the adult male rat requires overtly toxic doses of TCDD to produce an anti-androgenic effect, the developing male reproductive system is markedly sensitive to TCDD. Maternal doses as low as 0.16 ug TCDD/kg decrease anogenital distance of 1 and 4 day old male rats, delays testicular descent and decreases seminal vesicle and ventral prostate weights in rats. The organ weights were consistently decreased from the fetal stage into adulthood (Mably et al. 1992a) Table X. Decreases in masculine sexual behavior and increases in feminization of male rats also occurred at maternal doses of 0.16 ug TCDD/kg (Mably et al.

1992b), in addition spermatogenesis was altered at maternal doses as low as 0.064 ug TCDD/kg. The single administration of 0.064 ug TCDD/kg to pregnant rats on gestational day 15 consistently decreased epididymis and cauda epididymis weights, cauda epididymal sperm counts and daily sperm production (Mably et al. 1992c). None of the doses of TCDD resulted in any demonstrable overt maternal toxicity (Mably et al. 1992a,b,c). These data indicate that in utero and lactational exposure to TCDD permanently impaired the development of the male reproductive system, as well as possibly impairing sexual differentiation of the CNS. Hence, the developing male rat reproductive system is one of the most sensitive to the toxic effects of TCDD.

The mechanism(s) by which in utero and lactational exposure to TCDD alters the development of the male reproductive system is not completely understood. Decreases in circulating androgens are mediated in part by reductions in testicular responsiveness to luteinizing hormone (LH) stimulation (Mably et al. 1992a). Decreases in accessory sex organ weights may be due to a combination of decreased androgen levels and decreased responsiveness of these organs to androgens (Mably et al. 1992a). Future studies are indicated to determine the relative importance of these effects, and of the possible role of altered testosterone metabolism in response to TCDD induction of phase I and phase II enzyme systems.

The alterations in sexual behavior in male rats appears to be due to impairment of sexual differentiation of the CNS which requires the presence of testosterone during perinatal development for male differentiation (Mably et al. 1992b). Once in the CNS, testosterone is converted to estradiol and both of these steroids must bind to their respective receptors for the

Table X Male Reproductive Toxicity

Species/Strain	Dosing Regimen	Effect	Dose	Response % decrease			NOAEL	LOAEL	Reference
Holtzman Rats	single dose GD 15	seminal vesicle wt male offspring after birth	0.0 ug/kg 0.064 0.16 0.4 1.0	0% day 32 0% 14% 27% 32%	0% day 49 9% 13% 48% 65%	0% day 63 13% 28% 38% 44%			Mably, 1992a
Holtzman Rats	single dose GD 15	ventral prostate weight percent of control	0.0 ug/kg 0.064 0.16 0.4 1.0	0% day 32 21% 29% 43% 57%	0% day 49 49% 46% 25% 50%	0% day 63 19% 31% 44% 47%			Mably, 1992a
Holtzman Rats	single dose GD 15	Caudal epididymus wt in male offspring	0.0 ug/kg 0.064 0.16 0.4 1.0	0% day 32 17% 21% 42% 46%	0% day 120 7% 10% 20% 25%				Mably, 1992a
Holtzman rats	single dose GD 15	anogenital distance/crown rump length day 1 or 4 after birth	0.0 ug/kg 0.064 0.16 0.4 1.0	0% day 1 6% 18% 29% 29%	0% day 4 9% 18% 27% 23%				Mably, et al., 1992a
Holtzman Rats	single dose GD 15	Caudal epididymal sperm in offspring	0.0 ug/kg 0.064 0.16 0.4 1.0	0% day 63 41% 53% 65% 71%	0% day 120 33% 37% 48% 58%				Mably, 1992c
Sprague Dawley rats	single po dose	serum testosterone level reported as % decrease	0.0 ug/kg 6.25 12.5 25 50 100	0% 18.8% 37.5% 43.8% 93.8% 93.8%					Moore, et al., 1985

proper differentiation of the CNS. Maternal doses of 1 ug TCDD/kg decreased plasma testosterone levels in male offspring on days 18 through 21 of gestation (Mably et al., 1992b). The reduction in plasma testosterone may account for the demasculinization and feminization of male rats seen in adulthood. However, it is possible that TCDD alters the sensitivity of the developing pituitary to androgens and estrogens (Mably et al. 1992b) perhaps by altering the respective hormone receptors (Umbreit and Gallo 1988; Devito et al 1990). The mechanism of the altered sexual behavior of male rats exposed in utero to TCDD remains undetermined. Quantitative dose response analysis of these very sensitive effects in male offspring are complicated by the incomplete understanding of the toxicokinetics of TCDD in the fetus. Additional research that focuses on understanding tissue distribution and metabolism in the fetus will aid in comparative analyses and help to better understand the implications of these findings for a human population.

G. Liver Toxicity

Hepatotoxicity is consistently found in animals across species, administered TCDD. The liver is one of the major sites for metabolism of exogenous and endogenous chemicals. Although clinical and morphological changes are seen in the liver, it is the induction of hepatic cytochrome P450IA1 family of metabolizing enzymes that is one of the most sensitive indicators in many species for receptor mediated responses.

Guinea pigs are most sensitive to the acute lethal effects of TCDD. Harris et al.(1973) administered 0, 0.008, 0.04, 0.2, and 1.0 ug TCDD/kg weekly for 8 weeks (equivalent to 0, .001 .006, .029, and 0.143 ug TCDD/kg/day) (Table XI). Absolute liver weight was not a sensitive indicator of toxicity, although liver to body weight ratios calculated from graphic data do show an increase

at the highest dose. DeCaprio et al. (1986) described the effect of subchronic exposure to TCDD on guinea pigs. Animals were administered 0, 2, 10, 76, and 430 ppt TCDD in feed for 90 days. Equivalent total doses calculated by author were for males 0.0, 0.12, 0.61, 0.4.9 and 26 ng/kg/day and for females 0.0, 0.12, 0.68, 4.9, and 31 ng/kg/day. At the high dose 60% of the male and female animals either died during study or were sacrificed in a moribund condition. A significant increase in liver to body weight ratio was observed in males and females at the 4.8 and 4.86 ng/kg/day level. A dose dependent trend of increased relative liver weight was observed. These changes were not accompanied by other signs of hepatic toxicity. It is interesting to note that the female guinea pig is more sensitive to the chronic effects of TCDD than to the acute toxic effects. This was also observed in S-D rats in the Kociba et al. (1978) chronic toxicity study.

Hamsters are relatively insensitive to the acute toxic effects of TCDD, although the response to TCDD in the liver is qualitatively consistent with other species. A dose-dependent increase in absolute liver weight was observed in hamsters 50 days after administration of a single i.p. injection of 0, 5, 25, 100, 500, 1000, 2000, and 3000 ug TCDD/kg (Table XI). Graphing the Olson et al. (1980) data using a Michaelis-Menten function resulted in a very poor fit (Graph H). These data are extremely variable which may be due to the timing of the measurement in relation to the $T_{1/2}$ of TCDD in hamsters. Peak liver concentrations of TCDD are reached at 3 days in hamsters, therefore liver weight measurements taken at 50 days may not accurately reflect tissue toxicity (Olson et al. 1980a; Gasiewicz 1979).

To assess the hepatoxic effects of TCDD on C57BL/6J mice Vos et al. (1974) administered male mice weekly doses of 0, 0.2, 1.0, 5.0 and 25.0 ug TCDD/kg. Mice were killed at 2 and 6 weeks to assess the systemic effect of TCDD. The relative liver weights generally increased with increasing

dose. A statistically significant increase was seen at 2 weeks and at 6 weeks at the 1.0 ug/kg dose level (equivalent to 0.14 ug TCDD/kg/day)(Table XI). Hepatic damage was characterized as degeneration of liver cells around the central vein and proliferation of bile duct epithelial cells (at 25 ug TCDD/kg). At the 5 ug TCDD/kg level, necrotic hepatocytes and inflammatory cells were noted in the centrilobular region. At the lowest dose 0.2 ug TCDD/kg (equivalent to 0.28 ug TCDD/kg/day) centrilobular fatty change was noted. Fatty change increased in severity with increasing dose. Microscopic observation of the liver was the most sensitive indicator of frank hepatic toxicity following TCDD exposure.

Shen et al. (1991) investigated the role of TCDD in liver injury in C57BL/6J and DBA mice administered a single dose of 0, 3, and 150 ug TCDD/kg and 30, and 60 ug TCDD/kg respectively (Table XI). Increased liver/bwt ratio and decreased body weight were the most sensitive indicators of toxicity. On day 7 the C57BL/6J mice had an equivalent increase in relative liver weight at 1/10th the dose required to elicit the same response in DBA mice, however too few dose levels were used to assess a dose response relationship. Changes in liver/bwt ratio are a good surrogate for histopathological changes in the liver of these mice however, in guinea pigs this relationship was not found. In the DBA mouse no fatty change was observed at 30 ug TCDD/kg, but mid-zonal fatty change was noted at 600 ug TCDD/kg at 7 d. Focal necrosis was noted at 30 ug TCDD/kg 7 d and at 600 ug TCDD/kg at 3 and 7 days post dosing. In the C57BL/6J mouse fatty changes were noted at 3 ug TCDD/kg at 3 and 7 days, and severe diffuse fatty changes were noted at 150 ug TCDD/kg at 3 and 7 days. Focal necrosis and focal acute and chronic inflammation was observed at 150 ug TCDD/kg at 1 and 3 days post-dosing. (Shen et al. 1991) Hence, the sensitivity for necrosis and fatty change is again approximately 10-fold between the C57BL/6J and the DBA.

The differences in TCDD sensitivity between C57BL/6J and DBA mice is believed to be mediated in part at the level of the Ah receptor gene locus. Poland and Knutson (1982) hypothesized TCDD's mechanism of toxicity as TCDD binding to an endogenous cytosolic protein (Ah receptor) in a manner steroid hormones bind to their receptors. This receptor-ligand complex interacts with DNA to regulate transcription of several proteins, including the cytochrome P-450IA1 which is assayed as the enzyme aryl hydrocarbon hydroxylase (AHH). TCDD is not mutagenic and these DNA interactions do not damage DNA. To further clarify the relationship between receptor binding and toxicity, Birnbaum et al. (1990) used C57BL/6J Ah^{d^d} congenic "resistant" mice and wild type Ah^{b^b} "sensitive" mice. A single dose of 0, 400, 800, 1600, 2400, and 3200 ug TCDD/kg TCDD was administered to Ah^{d^d} mice and 0, 50, 100, 200, 300, and 400 ug TCDD/kg TCDD to Ah^{b^b} mice. Relative liver weights were significantly increased at all doses. This is expected since doses were much higher than the dose administered by Shen et al. 1991 in which 3 ug TCDD/kg that resulted in increased relative liver weights. The severity of a variety of liver lesions were compared in the congenic and wild type mice. Hepatocellular cytomegaly and hepatocellular karyomegaly occurred at 8 times the dose in Ah^{d^d} mice compared to Ah^{b^b} wild type mice. The differences were greater for fatty change in the liver and bile duct hyperplasia. Qualitatively the toxicity seen in these two strains of mice were similar; but the doses that result in this toxicity differ by 8-24 times. (Birnbaum et al. 1990) In male C57BL/6J mice administered single oral doses of 95, 145, and 190 ug TCDD/kg, a dose-related increase liver, and liver/bwt ratio was observed. In male DBA mice administered a single dose of 1370, 1870 and 2610 ug TCDD/kg TCDD an increase in absolute and relative liver weight was observed at every dose, but not with a dose response trend (Chapman and Schiller 1985). These same authors reported a dose-dependent decrease in serum glucose, total serum cholesterol, cholesterol ester, serum triglycerides,

and an increase in liver triglycerides in both strains of mice. The dose response data is reported graphically.

Pohjanvirta studied two strains of rats, Long Evans (L/E) a species sensitive to TCDD with a LD_{50} of 10 ug TCDD/kg and the Han/Wistar (H/W) rat with a LD_{50} of >3000 ug TCDD/kg. (Pohjanvirta 1990). In assessing target organ toxicity the L/E rats received a single ip injection of 5, and 50 ug TCDD/kg. The H/W rats received an additional dose of 500 ug TCDD/kg. Relative organ weights were assessed on day 1, 4, 8, 16 and 39-40. The only difference observed in relative liver weights was the initial statistically significant increase on day 1 in the H/W rats at the two highest doses. Both species were observed to have a statistically significant increase in relative liver weight on day 3 to day 16. There were marked strain differences observed histopathologically. In L/E rats 50 ug TCDD/kg ($5 \times LD_{50}$) four days post-dosing resulted in multinucleated and swollen hepatocytes. Only minor lesions were observed in H/W rats administered up to 500 ug TCDD/kg (Pohjanvirta 1989). Distribution of TCDD to the liver did not account for any differences in liver toxicity. Both strains had peak TCDD liver concentrations on day 8 and the rate of liver tissue disappearance appeared equivalent. This is unlike the thymus distribution in which was qualitatively very different between these two strains (Pohjanvirta 1990).

A dose-dependent increase in relative liver weight was observed in male and female Sprague-Dawley rats (Kociba et al. 1976) administered 0.001, 0.01, 0.1, 1.0 ug TCDD/kg/day 5 days/week for 13 weeks (Equivalent doses: 0.0007, 0.007, 0.07, 0.7 ug TCDD/kg/day). Liver to body weight ratios (Table XI) were increased in males at the 0.1 and 1.0 ug TCDD/kg dose level. Female rats were more sensitive, having a statistically significant increase in relative liver weight additionally at the 0.01 dose level. This is illustrated using the Power Law function (Graph 1). The R^2 values for males and females (0.93, 0.94) are high for the Power Law indicating

Table XI Liver Weight Changes

Species/Strain	Dosing Regimen	Effect	Dose ug TCDD/ kg - Response				NOAEL	LOAEL	Reference	
Hartley Guinea Pigs female	TCDD administered orally 1 X week for 8 weeks	increase absolute liver weight increase liver/bwt estimated from graph	0 ug/kg 0.008 0.04 0.2 1.0	0 ug/kg/day 0.01 0.06 0.29 1.13	0 % change liver wt +0.3% +8.5% +8.5% N/A	0% change liver/bwt +4% 2% +14.3% N/A			Harris, 1973	
Hartley Guinea pigs (230-340) Males	oral continuous feed 90 days	increase liver/bwt reported as % control (total dose reported)	0 ug/kg 0.011 0.055 0.141	0 ug/kg/day 0.12 ug/kg/day 0.61 ug/kg/day 1.9 ug/kg/day	0 -9.7% (decrease) +18.1% +24.0%		0.055 ug/kg	0.44 ug/kg	DeCaprio, 1986	
Hartley Guinea pigs (230-340) females	oral continuous feed 90 days	increase liver/bwt reported as % control (total dose reported)	0 ug/kg 0.011 0.061 0.137	0 ug/kg/day 0.12 ug/kg/day 0.68 ug/kg/day 1.9 ug/kg/day	0 +4.4% -0.6% (decrease) +28%		0.061	0.44	DeCaprio, 1986	
Rhesus Monkeys - 70 kg	single oral gavage	increase liver as percent bwt 1-6 wks	0 70 350		0 +5.8% +7.0%			70 ug/kg Lowest dose tested	McConnell, 1978	
Female syrian golden hamster 51-60g	single i.p. injection	change liver weight 50 days	0 ug/kg 5 25 100 500 1000 2000 5000		0 -8.3% +49% +8.6% +22.1% +42% +27.1% 19%		100 ug/kg	500 ug/kg	Olson, Holscher, and Neal, 1980	
C57BL/6J male mice	weekly doses p.o.	increase liver/bwt ratio reported at 2 weeks and 6 weeks	ug/wk (2) (6) 0 (0) (0) 0.2 (4) (12) 1.0 (2) (6) 5.0 (10) (30) 25 (50) (150)		0 1.3% 11.2% 15.5% 17.5%	0 6.8% 17.6% 26.4% 42.6%	0.4-2 weeks 1-2.6 wks	2.0-2 wk 6.0-6 wk	Vos, 1974	
C57BL/6J male mice 20-28 g	single i.p. in olive oil	increase liver/bwt ratio as % control 1, 3, 7 days post dosing	0 dose 3 150		0 day 1 5% 2%	0 day 3 +3.6% +11%*	0 day 7 +22% +41%*	3-3 days N/A-7 days	150-3d 3-7d	Shen, 1991
DBA male mice 20-28g	single i.p. in olive oil	increase liver/bwt ratio as % control 1, 3, 7 days post dosing	0 ug/kg 30 ug/kg 600 ug/kg		0 day 1 5.8% +9.8%*	0 day 3 0 0	0 day 7 +11.5% +27%*	30 ug/kg 1-3 days	30 ug/kg 7d	Shen, 1991
C57BL/6J male mice DBA male mice	single p.o.	increase liver/bwt ratio 30 days post dosing	0 ug/kg C57BL/6J 95 1370 145 1870 190 2610	0 DBA	0 C57BL/6J 13.9% 32% 41.7%	0 DBA 53% 36.8% 46.5%	N/A	95	Chapman and Schiller, 1985	
		increase liver/bwt ratio reported as ug/kg/day and (total dose)	0 (0) ug/kg 0.001 (0.065) 0.01 (0.65) 0.1 (6.5) 1.0 (65)		0 (measured dose) N/A 0.0037 ug/g liver 0.0346 0.2840	0 3.2% 4.5% 22% 35%	0.065 ug/kg	0.65 ug/kg	Kociba et al., 1976	
Sprague Dawley rats males	p.o. 5 wk 13 weeks	increase Liver/bwt ratio reported as ug/kg/day and (total dose)	0 (0) ug/kg 0.001 (0.065) 0.01 (0.65) 0.1 (6.5) 1.0 (65)		0 (measured dose) N/A 0.0026 ug/g liver 0.0360 ug/g liver 0.3210 ug/g liver	0 3.2% 4.5%* 22%* 35%*	0.065 ug/kg	0.65 ug/kg	Kociba et al., 1976	

a good fit to the data. The "g" values for these functions indicate a sublinear dose response. Attempts to fit the Michaelis-Menten function resulted in a very poor fit suggesting that a sigmoid dose-response may not characterize this phenomenon. The female rats were also more sensitive than male rats as measured by histopathology, clinical chemistry and relative liver weight. Microscopic examination revealed some hyperplasia in the bile ducts and altered hepatocytes at the 1.0 ug TCDD/kg dose level of both sexes, characterized as multinucleated and irregularly shaped cells, lipid accumulation, and some necrosis. Slight changes in hepatocytes "variations in size and shape" were noted in the 0.1 ug TCDD/kg level. No abnormalities were noted microscopically in the 0.001 or 0.01 dose levels. Clinical chemistry also revealed a statistically significant increase in total bilirubin, direct bilirubin, and alkaline phosphatase at the two highest doses in females (0.1 and 1.0 ug TCDD/kg) and in the highest dose in males. Increase relative liver weight was the most sensitive indicator of liver toxicity in this study. This study was terminated at 13 weeks at which time a parallel study reported that steady state concentrations of TCDD are found in the fat and the liver (Rose 1976).

G. Biochemical Endpoints

The mechanism of TCDD toxicity involves the formation of a receptor-ligand complex which results in an increase in the transcription of P450 liver enzymes. The increase in enzyme production may account for the increase in liver weight, and may also be responsible for other toxic endpoints.

Cytochrome P450 is a family of enzymes which catalyze the metabolism of a wide range

of endogenous lipophilic compounds (steroids, fatty acids, cholesterol derivatives) as well as a number of xenobiotics. There is a large body of literature which point to major differences between different animal species, and humans when comparing specific cytochrome P450 forms and substrate specificity. These differences are also important when comparing sexual differences and age related effects in animals. The differences in species, sex and age sensitivity can be attributed to which cytochrome P450s are constitutively expressed and the inducibility of all isozymes. If the mechanism of toxic effects are mediated by the cytochrome P450 then the differences observed between animals can be explained. The different responses are probably due to a number of factors, because of the complexity of the biological response. As outlined in the Whitlock chapter (this document), the effects caused by dioxin interaction with the receptor are a multi step process which, with alterations at any of the steps, will alter the response. In graphing the response of activity in a single species there is very good agreement for the levels of Ah receptor in the cytosol and nucleus, the mRNA for P450IA1 and the induction of enzymatic activity using EROD as the substrate in a single tissue. Tissue sensitivity may differ because of the presence of tissue specific structural orthologs of P450. (Wrighton, 1990; Nebert et al. 1989). There can be differences between species and sex as to which forms are constitutively expressed and their inducibility. There may also be species specific forms, which make a direct extrapolation to man difficult (Guengerich 1989). The presence or absence of a specific isozyme in a tissue or cells within a tissue will also effect the toxicity. As stated above and discussed in this document (Whitlock chapter), the multi step process of induction of the TCDD responsive enzymes is mediated through a cytosolic receptor. The differences in the receptor and the regulation of the genes and post-translational events between species and sex

can result in distinctly different toxic responses. The importance of the genetics in the effects caused by TCDD is best demonstrated in the Ah responsive and nonresponsive mice. In the homozygous mouse the animals are very sensitive to many TCDD mediated responses, while the heterozygous mouse strain is less sensitive.

The P450I family is present in rodents and humans and the family contains these two members Cytochrome P450IA1 (Cyp1A1) and P450IA2 (Cyp1A2). Both of these forms are important in the response observed following TCDD treatment. The Cyp1A1 is an ortholog of the rat P450c (also known as P448). In mammalian species this can be measured as aryl hydrocarbon hydroxylase (AHH) and is expressed at very low levels in the liver and extrahepatically. In animal studies this is the major form that is induced following polycyclic aromatic hydrocarbon treatment. Wrighton (1990) has reported that in man P450IA1 is rarely expressed in human liver and is predominantly an extrahepatic form, and as such is inducible by cigarette smoke and polycyclic aromatic hydrocarbons (PAHs).

The second member of this family is Cyp1A2, which is present the untreated animals constitutively at levels greater than Cyp1A1, is inducible by polycyclic aromatic hydrocarbons (PAHs) (Sesardic et al 1988; Quattrochi and Tukey 1989). The human Cyp1A2 (HLD) is expressed in all human livers examined. This is important because this isoform is believed to be a specific (non receptor) binding protein in hepatic microsomes, which may play an important role in the human response to TCDD (Wrighton 1989). Unlike the Cyp1A1 which in the human liver is not inducible, the Cyp1A2 is inducible by constituents of cigarette smoke and other sources of polycyclic aromatic hydrocarbons. In animal studies the Cyp1A2 is inducible following TCDD exposure.

Hook et al. (1975) characterized the induction of microsomal mixed-function oxidases (MFO) and cytochromes following TCDD treatment. There was a decrease in N-demethylation enzyme activity in the liver of female C-D rats not seen in male rats; however, there was no difference in the induction of P450^o or benzo(a)pyrene hydroxylase. In addition, Hook in an enzyme assay using tissues from male rats administered 25 ug TCDD/kg found there was liver and kidney induction of biphenyl 4-hydroxylase, biphenyl 2-hydroxylase, benzo(a)pyrene hydroxylase, UDP-glucuronyl transferase and benzphetamine N-demethylase. In the lungs, intestine, brain and testes of these animals there was no induction of biphenyl 4-hydroxylase, and biphenyl 2-hydroxylase, but there was a significant induction of all other enzymes. In guinea pigs dosed with 0.175 ug TCDD/kg biphenyl-4-hydroxylase was reported in liver, lung, and kidney tissues 4 days after administration. In rabbits administered 25 ug TCDD induction of 4-hydroxylase was observed in lung tissue and not in kidney and liver. Induction of benzo(a)pyrene hydroxylase was observed in kidney and liver, but not in the lung. These differences are difficult to interpret because only one dose was administered, but suggested definite species, sex and tissue differences in response to TCDD. The clinical manifestations of these enzyme changes are not well understood, but is a focus of current research.

To understand the tissue differences in enzyme induction, EROD: ethoxyresorufin dealkylase activity a measure of Cyp1A1 activity was quantified in the homogenized lung and liver of male Swiss mice after ip injection of 5 or 50 nmol/kg TCDD. Results indicate that the induction of P450 1A1 in the liver and lung follow a very similar pattern. Both liver and lung have an immediate and significant increase in EROD 15 fold and 25 fold respectively over the very low constitutive levels (Beebe et al. 1990). Comparing the EROD activity in the lung administered 50 nmol/kg versus the liver administered 5 nmol/kg reveals that the liver has 5 times the activity (40 nmoles resorufin/min/g liver vs. 8 nmoles/g lung). These differences may

be accounted for by differences in tissue distribution, as Gasiewicz et al. (1983) reported an approximately 7 to 10 fold difference in TCDD distribution to the liver vs lung in the three species of mice in which measurements have been made.

Kitchin and Woods (1979) evaluated a dose response relationship between TCDD and the elevation of the liver enzymes as total p-450 and benzo(a)pyrene hydroxylase in microsomes. Additionally, they administered 2 ug TCDD/kg to rats and examined the increase enzyme activities associated with a number of cytochrome p-448 type substrates (biphenyl 2-hydroxylase, biphenyl 4-hydroxylase, 4,4'-dimethyl-biphenyl hydroxylase, benzo(a)pyrene hydroxylase, EROD, and benzphetamine N-demethylase). All but the last enzyme was elevated significantly by an exposure of 2 ug TCDD/kg. In a third experiment, Kitchin and Woods (1979) administered a single po dose of 2 ug TCDD/kg to female S-D rats and reported EROD, cytochrome P-450, and AHH levels 1, 3, and 6 months post-dosing. Results indicate that enzyme remain elevated even after 6 months although the levels of AHH and EROD are substantially reduced after 6 months. Kitchin and Woods (1979) do some interesting calculations based on TCDD radiotracer experiments. TCDD levels in the liver, thymus and adipose 3 days post-dosing of 2 ug TCDD/kg resulted in tissue concentrations of 1.08, 0.37, and 1.84% of oral dose per gram of tissue (wet weight). They determined that as few as 65 molecules of TCDD in a hepatocyte can significantly increase the enzyme activity of Benzo(a)pyrene hydroxylase. (Table XII). Both total P450 and BAP hydroxylase fit the Power Law functional form with high correlation, but have different kinetic orders ($g = 0.58, 0.42$) which may indicate different factors regulate each (Graphs J and K).

Abraham et al. 1988 described the response of hepatic ethoxyresorufin-O-deethylase

(EROD) and total P450 induction in female Wistar rats (See Table XII). Rats were administered doses ranging from 1 to 3000 ng TCDD/kg via subcutaneous injection. Subcutaneous absorption was reported to be slow, but almost complete. TCDD induced a significant increase P450 activity at 0.1 ug TCDD/kg and in EROD at 0.3 ug TCDD/kg. The EROD response continued to increase even at doses 1000 times this. No maximum induction was reached in this study. The Michaelis-Menten and Hill plots graphically depict this phenomenon (Graph L and M). TCDD liver concentrations continued to increase at concentrations up to 3 ug TCDD/kg. This was attributed to steady state not being reached at day 7, but maximum induction being reached at higher doses should not be discounted based on this data.

Experiments by Abraham et al. (1980) and Kitchin and Woods (1979) (Table XII) provided sufficient doses to further analyze the four functional forms described in the introduction. Kitchin and Woods (1979) measured benzo(a)pyrene hydroxylase (aryl hydrocarbon hydroxylase - AHH) induction and total P450 concentration in the liver of female Sprague-Dawley rats 3 days after TCDD administration. Abraham et al. (1980) used Wistar rats and measured EROD and total hepatic P450 at 7 days post dosing.

For the Kitchin and Wood (1979) data the linear function of dose-response was not an appropriate fit (Graph J1-A) for the total P450 ($R^2 = .67$). The Michaelis-Menten fit of raw data (J1-B) depicts an "inertial response" in the dose range from 0.0006 to 0.06 ug TCDD/kg dose range and appears to reach maximim at 20 ug TCDD/kg indicating that this function does describe the data. The Hill function (Graph J1-C) also fits the data points with a sigmoid curve and an exponent of 1.09. A Hill exponent of 1.0 is generally interpreted as having a linear dose

response at low doses without cooperativity. However, the validity of this interpretation comes into question because the x-y plot does not depict linearity at low doses. The Power Law fit, over the entire dose-range (Graph J2-A), resulted in a "g" = 0.76, ($R^2 = 0.98$) suggesting a sublinear dose-response. Systematic application of the Power Law resulted in a sublinear response in the low dose range (0.0006 to 0.6 ug TCDD/kg) (Graph J2-B)) with a "g" = 0.035; $R^2 = 0.51$ which increased at high dose range to 0.11, $R^2 = 0.97$. This shift of "g" values from 0.035 to 0.11 or greater defines the "inertial response". Graphs L depicts the Abraham et al. (1980) P450 raw data. The Hill function applied the Abraham et al.(1980) P450 raw data has an exponent of 0.55 (sublinear indicating negative cooperativity in the low dose range, while Kitchen and Woods (1979) had an exponent of 1.09. In addition the fit to the Michaelis-Menten function does not appear to reach a maximal response (Graph J1-B) indicating this function does not describe the dataset.

It is the Power Law function that suggests a consistent response between these two datasets. The change in kinetic order between the low doses ($g = 0.036$, $R^2 = .88$ [Abraham et al., 1980]; ($g = 0.035$, $R^2 = 0.51$; [Kitchen and Woods 1979]) and high doses ($g = .14$, $R^2 = .99$ [Abraham et al. 1980]); ($g = 0.11$, $R^2 = 0.97$; [Kitchen and Woods 1979]) is demonstrated with an improved "goodness of fit".

Total P450 is a non-specific measure of response and may not be a good predictor of clinical toxicity. The induction of enzyme activity is a measure of a specific post-transcriptional event and as such may serve as a more consistent predictor of clinical outcome. Therefore, the induction of benzo(a)pyrene hydroxylase was analyzed (Kitchen and Woods 1979). The Michaelis-Menten and Hill functions both graphically depict an inertial response at low doses

and a maximum response at high doses (Graph K1). The exponent for the Hill function is 1.07 (compared to 1.09 for the P450 induction) suggesting that both responses may be mediated by similar mechanisms. Systematic analysis of subsets of doses (Graph K2-B) resulted in a comparable trend of increasing kinetic order with increased dose range. The low dose (0.0006 to 0.004 $\mu\text{g TCDD/kg}$) kinetic order (g value) was 0.21, $R^2 = 0.96$ which increased with doses in the 0.02 to 0.6 $\mu\text{g TCDD/kg}$ range ($g = \underline{0.77}$, $R^2 = 0.96$). In the highest dose range tested (0.6 to 20 $\mu\text{g TCDD/kg}$) the kinetic order decreased to 0.19, $R^2 = 0.80$.

Similar responses were observed when EROD activity was measured. Graphs M depicts the functional forms used in this analysis. Both Michaelis-Menten (Graph M1-B) and Hill functions (Graph M1-C) graphically depict a low dose "inertial" response. The Hill function has a exponent of 1 which is consistent with the total P450 response (indicating no cooperativity and suggesting linearity in low dose range). The Power Law functional fit on all data points resulted in a sublinear fit, $g = 0.52$, $R^2 = 0.95$ (Graph M2-A). Analysis of subsets of data resulted in a response consistent with the Kitchin and Woods (1979) data analysis, i.e., an increase in kinetic order from the low dose range ($g = 0.11$) to the high dose range ($g = 0.66$). For EROD induction the "Goodness of fit" for these both subsets of data was high, $R^2 = 0.99$ (Graph M2-B).

The analysis of these two datasets (Kitchin and Woods, 1979; Abraham et al., 1980) suggests differences in the low and high dose responses. The Power Law function is commonly used to describe physical phenomenon, therefore the dose response fit of this model may be best described using laws of physics. The low dose response can be characterized as an "inertial" in which activity is measurable, but at a reduced rate. Not until an "external force" acts upon the system is an altered response (manifested as change in kinetic order) measured. The Power

Law function when applied to total P450, EROD, and benzo(a)pyrene hydroxylase describes this phenomena.

In female mice (Narasimhan et al.(1993) see Table XIII) measured the individual steps of the multistep process of enzyme induction. Graphic representation of the total nuclear Ah receptor binding, mRNA for Cyp1A1, mRNA for Cyp1A2, and enzyme induction (EROD) plotted against dose demonstrated parallel slopes when fitted to a Power Law function. R^2 values indicate good fit to these functions (Graph N). This data supports the conclusion that the steps of this multistep process are regulated by similar mechanisms, and there is not likely to be post-translational modification for EROD induction. However, there is a difference in Cyp1A1 and Cyp1A2 mRNA levels.

The differential dose-response in induction of Cyp1A1 and Cyp1A2 has recently been investigated (Trischer et al. 1992). Female rats (initiated with DEN or saline) were administered TCDD biweekly p.o. for 30 weeks. Dose equivalents of 3.5, 10.7, 35.7, and 125 ng/kg/day resulted in a dose-dependent increase in hepatic Cyp1A1 and Cyp1A2 (measured by RAI). The rate of induction (different slopes) of the two enzymes differs only slightly and probably not significantly (Graph O). The differences in the Power Law function between these two enzymes is not as great as the difference detected by DeVito et al. (1992) (see below) for hepatic Cyp1A1 and Cyp1A2, possibly because of the different techniques of enzyme measurement used in this study; therefore definitive conclusions can not be made. Additionally, maximum induction of Cyp1A1 is reached at a liver concentration of 10 ppb, whereas Cyp1A2 activity continues to increase after 30 weeks at hepatic concentrations of 30 ppb. Immunolocalization of enzyme induction in this experiment indicated that hepatocytes are not

induced uniformity (Trischer et al. 1992). The mechanisms responsible for this differential induction may help in the understanding of intraspecies variability of response and prove useful in risk estimation.

The induction of skin, hepatic and lung EROD and hepatic 1A2 (acetanilide hydroxylase activity) was measured in female B6C3F1 mice administered 0, 1.5, 4.5, 15, and 150 ng/kg/day for 13 weeks (total doses 0, 0.098, 0.29, 0.98, 9.8)(DeVito et al. 1992 see Table XIV). Graphic representation of dose-response data indicates parallel slopes for all EROD induction, but a different slope for 1A2 induction (Graphs P1-P4). The fit to the Power Law functional form for EROD in the three tissues measured had a high correlation coefficient. There was also a striking similarity between the Power Law for the EROD measured in the three tissues versus the power law for hepatic Cyp1A2. The difference in induction between hepatic and lung EROD ranges from 5 fold to 18 fold which may be accounted for by differences in tissue distribution, although other factors can not be ruled until tissue dosimetry assays are run parallel to the enzyme induction assays.

Enzyme induction after a subchronic (13 week) exposure in B6C3F1 female mice was evaluated using the DeVito et al.(1992) data. The linear regression model (Graph P1) for hepatic, skin and lung EROD induction had R^2 values of 0.95, 0.91, and 0.94 respectively; however, the R^2 for hepatic 1A2 induction was 0.54. The Michaelis-Menten functional forms (Graph P2) graphically depict the "inertial" phase of the dose-response at doses less than 15 ng/kg/day (total dose = 0.975 ug TCDD/kg) for hepatic, skin and lung EROD activity. For hepatic EROD, the Hill function (Graph P3) exponent (1.1) was superlinear, whereas the exponent derived from application of the Hill plot for skin and lung EROD activity was

sublinear (exponent = 0.83, 0.84). The "g" value in the Power Law function for hepatic, skin and lung EROD induction (Graph P4) depicts a sublinear kinetic order ($g = 0.68, 0.75, \text{ and } 0.79$; $R^2 = 0.92, 0.94, 0.97$, respectively). These values are remarkably consistent, and interestingly are similar to the "g" values determined for EROD induction by Abraham et al., 1988 at a similar range of doses(at doses 0.3 to 3.0 $\mu\text{g TCDD/kg}$ "g" = 0.66, $R^2 = 0.99$. Systematic analysis of subsets of data does not reveal the same change in kinetic order seen in the Abraham et al. (1988) or the Kitchin and Woods (1979) data sets; however, the total administered doses in the DeVito et al. (1992) are not in the range of doses in which the change in kinetic order was observed. There is a notable increase in the "g" value for hepatic EROD induction (Table XV), but the R^2 values are low, and a consistent trend was not observed. Although no change in kinetic order was observed in the lung and skin EROD induction, there are no acute data comparable to the Abraham et al. (1988) or Kitchin and Woods (1979) data for lung and skin induction. Therefore, a more complete understanding of the activity in extra-hepatic tissue is not possible with the available data.

The induction of P450IA2 was also measured (DeVito et al. 1992) using the functional forms previously described. The initial X-Y plot and linear plots of data revealed a much higher degree of variability in the data values. This observation is consistent with what has been found in humans populations, that P450IA2 constitutive values vary 3000% (Grant et al. 1983). This precludes making assumptions about the Hill or Michaelis-Menten plots.

TABLE XII DOSE-RESPONSE FEMALE RATS
Liver Enzyme Activity, Total P-450, Benz(a)pyrene, and EROD

Species	Dosing Regimen	Observed Effect	Dose ug TCDD/kg		Response	NOAEL	LOAEL	Reference
Female Sprague-Dawley rats	single oral dose	increase P-450 (3 days) (Omura and Sato, 1964)	0 0.0006 0.002 0.004 0.02 0.06 0.2 0.6 2.0 5.0 20.0		0.88 0% 0.91 3.4% 0.93 5.7% 1.0 13.6% 0.87 -1.1% 1.06 20.5% 1.16 31.8% 1.13 28.4%* 1.33 51.1%* 1.52 72.4%* 1.68 90.9%*	0.2	0.6	Kitchin, 1979
Female Sprague-Dawley rats	single oral dose	increase Benzo(a)pyrene hydroxylase (nmol polar products/mg protein/hr) (DePierre, 1975) at 3 days	0 0.0006 0.002 0.004 0.02 0.06 0.2 0.6 2.0 5.0 20.0		4.9 0% 4.9 0% 6.7 36.7%* 7.2 46.9%* 8.3 69.4%* 14.0 185.7%* 59.0 1104%* 96.0 1859%* 155.0 3063%* 182.0 3614%* 189.0 3757%*	0.0006	0.002	Kitchin, 1979
Wistar Rats Female	single s.c. injection	increase P-450 concentration (p-moles X mg protein ⁻¹) measured as reduced CO difference spectra 7-days post dosing	0 ug/kg 0.001 0.003 0.010 0.030 0.100 0.300 1.00 3.00	0 (measured tissue dose) 0.0031 0.0102 0.0406 0.162 0.699 3.38 10.7 27.9	169 0% 182 7.1% 173 2.4% 184 8.9% 183 8.3% 199 17.8%* 230 36.1%* 269 59.2%* 319 88.8%*		0.100 ug/kg	Abraham, 1988
Wistar rats Female	single s.c. injection	increase EROD Activity pmoles resorufin formed X mg protein ⁻¹ X min ⁻¹ (resorufin measured spectrofluorometrically in liver homogenates 7 days post-dosing)	0 ug/kg 0.001 0.003 0.010 0.030 0.100 0.300 1.00 3.00	0 (measured tissue dose) 0.0031 0.0102 0.0406 0.162 0.699 3.38 10.7 27.9	14.3 0% 16.9 18.2% 19.0 32.9%* 21.9 53.1%* 40.7 184.6%* 89.5 525.9%* 204.4 1329.4%* 495 3361.5%* 759.8 5213.3%*		0.003 ug/kg	Abraham, 1988

TABLE XIII DOSE - RESPONSE B6C3F1 Female Mice
Liver EROD, Cyp1A1 mRNA, and Cyp1A2 mRNA and Total Nuclear Ah Receptor Binding

Species	Dosing Regimen	Effect	Administered Dose	Liver Dose $\mu\text{g/gm}$	Response		NOAEL	LOAEL	Reference
B6C3F1 female mice	single i.p. injection	24 hrs. post dosing increase EROD pmol/mg protein/min	0 $\mu\text{g/kg}$ 0.005 0.010 0.025 0.05 0.10 0.50 1.00 5.00	0 0.11 0.10 0.09 0.26 0.74 2.36 11.5 53.16	0 +51% -8.0% -11% +9.0%* +109.6%* +252%* +538.5%* +1857%*	156 pmol/mg prot/min 235 168 139 170 327* 549* 996* 3053*	0.050 $\mu\text{g/kg}$	0.10 $\mu\text{g/kg}$	Narasimhan et al., 1993
B6C3F1 female mice	single i.p. injection	24 hrs post dosing increase relative mRNA units Cyp1A1	0 $\mu\text{g/kg}$ 0.005 0.010 0.025 0.05 0.10 0.50 1.00 5.00	0 0.11 0.10 0.09 0.26 0.74 2.36 11.5 53.16	0 +0.14% +0.14% +171% +100% +571% +900% +1257% +2671%	0.07 0.06 0.06 0.19 0.14 0.47 0.70 0.95 1.94	0.10	0.50	Narasimhan et al., 1993
B6C3F1 female mice	single ip injection	24 hr. post dosing relative mRNA units Cyp1A2	0 $\mu\text{g/kg}$ 0.005 0.010 0.025 0.05 0.10 0.50 1.00 5.00	0 0.11 0.10 0.09 0.26 0.74 2.36 11.5 53.16	0 -0.14 -0.28 -0.28 -0.28 -157 +57 +243 +343	0.07 0.06 0.05 0.05 0.05 0.03 0.11 0.24 0.31	0.5	1.0	Narasimhan et al., 1993, 1992
B6C3F1 female mice	single ip injection	24 hrs post dosing Total Nuclear Ah Receptor Binding	0 $\mu\text{g/kg}$ 0.005 0.010 0.025 0.05 0.10 0.50 1.00 5.00	0 0.11 0.10 0.09 0.26 0.74 2.36 11.5 53.16		N/A 4.3 7.2 6.2 8.2 6.5 10.6 45.4 111.3			Narasimhan et al., 1993

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TABLE XIV DOSE - RESPONSE B6C3F1 Female Mice
Liver EROD, Skin EROD, Lung EROD, Hepatic 1a2 Activity

Species	Dosing Regimen	Effect	Administered Dose	Response		NOAEL	LOAEL	Reference
B6C3F1 female mice	repeat dose 5 X per week 13 weeks (65 doses) oral	increase hepatic EROD nm/mg protein/min	0 ug/kg/day (0 ug/kg total) 0.0015 (0.0075 ug/kg) 0.0045 (0.292 ug/kg) 0.015 (0.975 ug/kg) 0.045 (2.925 ug/kg) 0.150 (9.75 ug/kg)	100% 220% 260% 610% 1780% 3965%	126.25 mean EROD 271.4 323.4 763.6 2245.25 4996.0			DeVito et al. 1992
B6C3F1 female mice	repeat dose 5 X per week 13 weeks (65 doses) oral	increase skin EROD nm/mg/protein/min	0 ug/kg/day (0 ug/kg total) 0.0015 (0.0075 ug/kg) 0.0045 (0.292 ug/kg) 0.015 (0.975 ug/kg) 0.045 (2.925 ug/kg) 0.150 (9.75 ug/kg)	100% 170% 280% 1040% 1760% 5280%	0.89 mean EROD act. 1.54 2.5 9.29 15.65 47.0			DeVito et al., 1992
B6C3F1 female mice	repeat dose 5 X per week 13 weeks (65 doses) oral	increase lung EROD nm/mg/protein/min	0 ug/kg/day (0 ug/kg total) 0.0015 (0.0075 ug/kg) 0.0045 (0.292 ug/kg) 0.015 (0.975 ug/kg) 0.045 (2.925 ug/kg) 0.150 (9.75 ug/kg)	100% 217% 545% 1614% 2833% 8786%	4.2 9.15 22.9 67.8 119 369			DeVito et al., 1992
B6C3F1 female mice	repeat dose 5 X per week 13 weeks (65 doses) oral	increase hepatic 1a2 acetanilide 4-hydroxylase	0 ug/kg/day (0 ug/kg total) 0.0015 (0.0075 ug/kg) 0.0045 (0.292 ug/kg) 0.015 (0.975 ug/kg) 0.045 (2.925 ug/kg) 0.150 (9.75 ug/kg)	100% 130% 180% 290% 360% 420%	277.0 357.2 618.4 798.4 920.6 1164.4			DeVito et al., 1992

The fit to the linear model is also poor, $R^2 = 0.54$. The Power Law for P450IA2 induction resulted in a "g" = 0.24, $R^2 = 0.73$, a sublinear functional fit. In addition, the absolute number for this exponent is lower than the "g" value for hepatic, skin or lung EROD induction. The evaluation of data subsets reveals a completely opposite pattern than that observed with EROD, a trend toward a decrease in the kinetic order with increasing dose. Because of the variability of the data and low R^2 the significance of these observations can not be determined.

Table XV Power Law Functional Fit Selected Subsets of Doses from Devito et al. 1992								
Dose ng/kg day	Hepatic EROD		Lung EROD		Skin EROD		Hepatic 1A2	
1.5 - 15	.47	.69	.87	.92	.75	.80	.34	.59
1.5 - 45	.85	.63	.78	.94	.71	.85	.27	.65
1.5 - 150	.68	.92	.79	.96	.75	.93	.24	.73
4.5 - 150	.12	.96	.77	.95	.81	.91	.11	.53
15 - 150	.82	.94	.74	.91	.74	.81	.18	.36

Human and animal P450 enzymes differ both quantitatively and qualitatively. Understanding the role of these enzymes in the multistep process of TCDD induced carcinogenesis is essential if enzymes are to be used as response surrogates. Additionally, the role of these enzymes in immunotoxicity, male reproductive toxicity, teratogenicity, or other non-cancer endpoints is not understood and is an area where additional research is needed.

The role of Cyp1A1 in the toxicity of TCDD is not yet fully understood. Cyp1A1 is constitutively expressed both hepatically and extrahepatically in rodents. In man Cyp1A1 is present and inducible extrahepatically, and current investigations in its role in lung cancer

indicate that genetic variations of the CYP1A1 gene may increase the relative risk of lung cancer in humans.

Induction of Cyp1A1 by TCDD in the liver has been used to evaluate cancer risk. Evidence that rodents and man may induce this enzyme differently may indicate that the estimation of human health risks based on rodent Cyp1A1 induction is not appropriate. Cyp1A2 is present constitutively in human livers at widely variable levels, but is not found extrahepatically in humans and in rodents (Wrighton and Stevens 1992). In rodents Cyp1A2 is found constitutively at higher levels than rodent Cyp1A1 (Trischer et al. 1992; Kedderis et al. 1991). The induction of Cyp1A2 may alter the rate of metabolism of endogenous materials thereby altering their function. If enzyme induction is used as a surrogate for TCDD toxicity then extrahepatic induction of Cyp1A1 and hepatic induction of Cyp1A2 are areas of investigation relevant to human health effects.

Sunahara et al. (1989) reported a dose-dependent decrease in hepatic EGF receptor after administration of 1.0-25.0 ug TCDD/kg po in Sprague-Dawley female rats (Table XVI). EGF is not produced in the liver, but extrahepatically, therefore the levels of EGF in adrenalectomized S-D rats was also investigated. A decrease in the EGF receptor binding was found from 1.0 to 5.0 ug TCDD/kg. Graph Q depicts similar hepatic EGF receptor binding in intact and adrenalectomized S-D rats administered a dose range of 1 to 5 ug TCDD/kg. However, at doses below 1.0 ug TCDD/kg in adrenalectomized animals there was a marked increase in EGF receptor binding levels. Unfortunately, doses between 0.001 and 0.5 ug TCDD/kg were not tested in intact rats.

An 80-90% decrease in high and low affinity hepatic EGF binding was observed in

C57BL/6J mice 7 days after treatment with 30 ug TCDD/kg. There was no decrease in EGF receptor mRNA as a consequence of TCDD treatment indicating that the effect may occur post-transcriptionally. The transcriptional alteration of an EGF receptor ligand has been a hypothesized effect of TCDD (Lin et al. 1991). The significance of these decreases to carcinogenesis was discussed in this document (Lucier Chapter). EGF may be involved in the carcinogenic mechanism, but there are not clear cut dose-response relationships in the path from decreased hepatic EGF to cancer.

Epidermal growth factor is believed to be a regulator of cell proliferation (Clarke et al., 1991). A dose-dependent increase in EGFr detected immunohistochemically in mouse embryonic uterine epithelial cells (GD14) of C57BL/6N mice was observed (Abbott and Birnbaum 1990a). A consequence of this is hyperplasia of the uterine epithelial cells, and hydronephrosis. There was not enough data points available for this endpoint to fit it to a dose-response curve.

The level of EGF receptor in embryonic palate closure has been studied in C57BL/6n mice (Abbott and Birnbaum 1989) and in humans (Abbott and Birnbaum 1991). Although statistically significant changes in EGF receptor level were not found in the medial epithelial cells of human palate after exposure to TCDD the C57BL/6N mice the EGF receptor level was altered. In normal the normal palate, closure involves the decrease in EGF receptor and a subsequent programmed cell death, or lack of cell growth. A sufficient decrease in EGF receptor does not occur in the TCDD treated palate cultures preventing normal palate closure. It has been hypothesized that other factors (TGF- α , or TGF- β) play a role in its regulation, but sufficient data is not available to determine a dose-response relationship (Abbott et al. 1991)

TABLE XVI DOSE-RESPONSE FEMALE SPRAGUE-DAWLEY RATS
Epidermal Growth Factor Response Intact V. Adrenalectomized rats

Species	Dosing regimen	Effect	Dose	Response % Change	NOAEL	LOAEL	Reference
Female Sprague-Dawley rats	single p.o.	% change EGF receptor binding levels 10 days post-dosing	0 ug/kg 1.0 2.5 5.0 10.0 25.0	0 -14% -19% -39% -54% -64%	no significance determined	no significance determined	Sunuhara et al., 1989
Female Sprague-Dawley rats (200-250 g) adrenalectomized.	single p.o.	% change EGF receptor binding levels 10 days post dosing	0 ug/kg 0.001 0.01 0.10 0.50 1.0 2.5 5.0	-3% +27% +2% +7% -12% -48% -43%	no significance determined	no significance determined	Sunuhara et al., 1989

III. CONCLUSION

Understanding the consistencies and inconsistencies of dose response relationships is necessary and can add confidence to a risk assessment. TCDD has been extensively studied with various species and strains of animals using different protocols resulting in many consistencies in response. Thymic atrophy, wasting syndrome and liver toxicity are seen in most controlled animal experiments with TCDD. TCDD is also acutely toxic in many species tested, but the sensitivity to acute toxicity differs with species and strain and does not appear as a constant predictor of other endpoints. Female rats and guinea pigs are less sensitive to acute toxicity than males, but are more sensitive to chronic toxicity to liver. Hamsters are relatively insensitive to acute toxicity but are very sensitive to fetal toxicity (hydronephrosis). Carcinogenic responses are less well understood but TCDD is clearly a low-dose rodent carcinogen. Where humans respond to each of these endpoints is a critical question in risk assessment which can not be easily answered without a better understanding of the underlying mechanisms of toxicity.

The segregation of toxic effects with the Ah receptor is an often reported phenomenon and it is clear that most responses to TCDD in cells in culture and in animal tissues segregate with the Ah receptor. Generally atypical tissue responses within species are also Ah receptor mediated, for example, the response of BALB/cByJ mice (Ah^b/Ah^b) to TCDD in the PFC assay for immunoresponse is very sensitive, whereas the DBA mouse (Ah^d/Ah^d) is much less sensitive (Silkworth et al., 1989). The kinetics of tissue distribution appear to have some role in different sensitivities. For instance, the half-life in the thymus of the hamster is short and this species requires a comparatively high dose of TCDD to produce thymic atrophy. Pohjanvirta et al., (1989, 1990) reported the same phenomenon in Long Evans and Han Wistar rats, finding

that the half-life in the thymus was related to the animals sensitivity to thymic atrophy. These differences can be accounted for by several factors: primarily pharmacokinetic and dynamic differences. However, as with steroids, tissue and cell factors may also be involved to account for specificity.

Cyp1A1 or 1A2 induction have served as the major surrogates to TCDD activity and has led to a better understanding of tissue susceptibility and species differences. Induction of Cyp1A1 in humans is difficult to quantify however, Cyp1A2 has been proposed as a reasonable biomarker when coupled with tissue levels of TCDD (or congeners). The level of Cyp1A2 in the human liver (which has highly variable constitutive levels) may account for differences in human responses. The role of Cyp1A1 and Cyp1A2 induction in cancer is described in this document (Lucier chapter). The relationships between enzyme induction and the increase in toxicity at non-cancer endpoints is not well understood, and this is an area where further research is needed.

In animal studies graphic analysis of the data revealed that the Power Law function was the best fit for dose-response relationships of Total Ah receptor binding, Cyp1A1 mRNA concentrations and EROD activity. The consistency of the power function seen with the graphic analysis of these endpoints (Graph K) indicates that there is little post-translational modification. The "goodness of fit" to the power function was high for these responses and lines were parallel, confirming what is known of the biological relationships between receptor, mRNA and protein. Graph M shows excellent correlation coefficients for the EROD activity in three tissues, revealing a good fit ($R^2 > 0.90$) to the Power Law function. The consistency of the Power Law function demonstrates that EROD activity in extrahepatic tissues is probably not

modified, and from this, indicates that in mice liver EROD activity is a good surrogate for extra hepatic tissue activity. The difference in the power law for hepatic Cyp1A2 confirms what is known about Cyp1A2: that is, it regulated differently than Cyp1A1. Finally, the analysis of selected data sets with the Power Law function has added to the understanding of the dose-response relationships for these endpoints. The enzyme endpoints are easily analyzed and may prove to be a simple surrogate for some other toxic effects. An "inertial" response in the low dose range can be characterized when applying this function to total P450, EROD and benzo(a)pyrene hydroxylase in the liver. This suggests that the response at low doses may be part of an adaptive response to environmental levels of TCDD ("external forces") and that only at higher doses does the system respond with an increase in kinetic order. The curve fitting for the Cyp1A1/Cyp1A2 regulated endpoints is contrasted to the immunotoxicity data, where the measured endpoints are reached, presumably, through a more complex and less well understood pathway.

Immunotoxicity and thymic atrophy are frequently reported toxic responses to TCDD administration. The application of the Power Law function to these endpoints suggests a very different response than that seen in enzyme induction. At low doses a high kinetic order (reaction rate) is seen contrasted with a lower kinetic order at high doses. This suggests that additional factors, not directly related to enzyme induction, are involved in these clinical endpoints. Although the immune system may be extremely sensitive to the toxic effects of TCDD there does not seem to be an assay that has developed the sophistication to make interspecies comparisons.

Some organ weight changes were sensitive clinical endpoints. Thymic atrophy and liver weight increases were observed after a single oral dose of 1 ug TCDD/kg in mice, and with a

continuous dose of 4.9 TCDD ng kg day in guinea pigs (See Table III and Table XI). Although individual data sets fit the Power Law function with a high correlation coefficient, there wasn't enough consistency between study methodology to adequately compare studies. Current research in the cellular mechanism of thymic atrophy and understanding of thymocyte maturation has developed to the point where a mechanistic model can be proposed. Additional quantitative data on thymocyte maturation across species is needed for this model to develop.

The development of a in vitro model for cleft palate has enabled direct species comparison, and has been useful in understanding these species differences. Qualitatively the measured endpoints in this model have shown consistency across species and have helped to understand the role of the EGF receptor in programmed cell death. There were not enough data points to analyze this endpoint using the Power Law function. Although, qualitative comparisons indicate that rat and human palate are less sensitive than the palates of C57BL/6N mouse. Because of the understanding of the mechanism for cleft palate formation a mechanistic model can be developed.

From a public health perspective, the use of non-cancer endpoints may be more significant in assessing human health risks because some of these effects occur at lower doses and have been reported to occur under general population exposure conditions, i.e. short to moderate exposure periods, or acute accidental exposures. A comparison of tissue-dose with effect in a variety of studies exemplifies this (Table XVII). The apparent differences between endpoints for cancer and non-cancer risks are in the extrapolation procedure which are questioned in the modelling chapter (Lucier and Gallo).

Kociba et al. (1978) performed a rodent bioassay to assess the carcinogenic risk of TCDD study supplying Sprague-Dawley rats with feed containing concentrations equivalent to

0, 0.001, 0.01, and 0.1 ug TCDD/kg/day. The administered dose associated with a statistically significant increase in hepatocellular carcinoma in female rats was 0.1 ug TCDD/kg/day. At 0.01 ug TCDD/kg/day female rats were found to have a statistically significant increase in hepatocellular nodules and alveolar hyperplasia. These doses produced liver concentrations of 24,000 ppt and 5100 ppt, respectively (Kociba 1978).

Using an initiator-promoter dosing regimen, Trischer et al., (1992) found an increase in Cyp1A1 and Cyp 1A2 in rodent liver tissue after administration of weekly doses equivalent to 3.5 ng/kg/day to 125 ng/kg/day. A dose-dependent increase in these biochemical endpoints was significant at all doses. The measured concentration of TCDD in the liver was reported as 480 ppt to 1990 ppt.

In contrast to these two studies, liver concentrations of TCDD (tissue dose) that statistically increases non-cancer endpoints are as low as 0.084 ppt. Narasimhan et al., (1993) administered a single i.p. dose of 0 to 2.5 ug TCDD/kg to B6C3F1 female mice, and found a statistically significant decrease in splenic plaque forming cells (PFCs/spleen) at the 0.05 ug TCDD/kg dose level. The liver concentration that resulted in this decrease was 0.084 ppt.(calculated from Narasimhan et al. 1993)

Ma et al. 1991 administered female C57BL/6J mice single i.p. injections of TCDD and measured the decrease in hepatic protein tyrosine phosphorylation. The EC₅₀ for this decreased response was 0.05 ug TCDD/kg which was approximately equivalent to a tissue dose of 0.084 ppt. (the tissue distribution data used to calculate this was from Narasimhan et al., 1993). This data also reports an effect that is not clearly nuclear-Ah receptor mediated, the decrease in protein tyrosine phosphorylation.

Table XVII Summary LOEL Non-Cancer Endpoints vs. Cancer Endpoints See text for description of study protocols		
Endpoint	LOAEL - Tissue dose - liver ppt	Reference
increase hepatic hyperplastic nodules	5100 ppt	2 year Rodent Bioassay - Kociba et al., 1978
increase Cyp1A1/Cyp1A2	480 ppt	Initiation-Promotion Protocol - Trischer et al., 1992
increase benzo(a)pyrene hydroxylase (Ahh)	5.4 ppt	Kitchin and Woods, 1979
increase EROD	10.2 ppt	Abraham et al., 1988
reduction in spermatogenesis in male offspring	maternal tissue dose 806 ppt fetal liver dose at 0.083 ppt	Mably et al. 1992
Increase in protein tyrosine phosphorylation	0.084 ppt (estimated)	Ma et al., 1992
decrease splenic plaque forming cells	0.084 ppt	Narasimhan et al., 1993

The increase hepatic enzyme activity is a well documented phenomenon which may ultimately be responsible for alterations in clinical endpoints. Kitchin and Woods, 1979 found that enzyme activity (benzo(a)pyrene hydroxylase) was significantly increased in Sprague-Dawley rats after the single oral administration of 0.002 ug TCDD/kg (LOAEL). Based on [1,6-³H] TCDD administration the tissue concentration in the liver that resulted in this increased enzyme activity was 5.4 ppt. Kitchin and Woods (1979) estimated that this level is equivalent to 65 molecules of TCDD per liver cell. Abraham et al. (1988) reported a statistically significant increase in ethoxyresorufin O-deethylase activity (EROD) after a single s.c. administration of 0.003 ug TCDD/kg resulting in a measured ¹⁴C-TCDD liver concentration of 10.2 ppt. These two studies are remarkably consistent, in reporting liver concentrations which are less than 1/200 the concentrations in the carcinogenicity bioassays.

Other studies have also found very low TCDD concentrations that result in adverse effects. Mabry et al. (1992) reported that a single maternal dose of 0.064 ug TCDD/kg resulted in a significant and permanent reduction in spermatogenesis in their male offspring. If, as VanBerg et al. (1987) reported, that 0.13% of TCDD reaches the fetus, then the fetal dose that resulted in these effects would be 0.083 ppt.

Clearly hepatocellular carcinoma is not the most sensitive effect of TCDD administration in animals. Induction of cancer appears to occur after continuous exposure to doses that result in high levels of enzyme induction. The most significant public health concern from TCDD exposure may be immunotoxicity, reproductive toxicity and other effects that are seen in human and animal after low dose acute exposures. Empirical analysis of these effects will help to determine the biological significance of these observations and result in a more relevant risk assessment.

The evaluation of dose-response relationships has demonstrated many consistencies and inconsistencies in the patterns of toxicity between tissues, strains, and species. The development of new models has been proposed for cleft palate development and thymic atrophy because they are well understood and sufficient data has been collected to analyze additional data needs and start mechanistic model development. Future research focused on cleft palate, thymocyte maturation and immunotoxic endpoints, and male reproductive toxicity should result in progress toward this end.

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Appendix 1

The Use of Power Laws, Michaelis-Menten and Hill's Function in Dose Response: The Mathematical Rationale

The use of a particular empirical function in phenomenological dose-response analysis is guided by conceptual considerations concerning infinitesimal (differential) incremental changes in response at a given dose level. In the following x and y denote dose and response in appropriate units, $y = f(x)$ the dose response function and dx , dy are the differentials of the dose and the response. As a simple example, one may consider the case of linear response: the underlying assumption is that the infinitesimal change in response is always proportional to, and with a constant proportionality ratio, a , to the infinitesimal change in dose at all dose levels. So, $dy = a dx$, which is integrated to give $y = ax + b$, b being the "response at zero dose".

A nonlinear dose-response function is derived if one assumes that at any given levee of dose and infinitesimal change in response is proportional to an infinitesimal change in dose but with a proportionality ratio that depends on the levels of dose and response. The specific assumption that leads to a Power Law is that the incremental change in response will be proportional to the incremental change in dose as well as to the total response at that point, but inversely proportional to the total level of dose: $dy = g dx \frac{y}{x}$, where g is a proportionality constant. This relationship gives, $y = ax^g$, where a is an integration constant. The differential relationship above essentially states that the local rate of change of response with increasing dose is proportional to the total response normalized over the total dose. This concept, i.e. the rate of change of the "dependent" variable with respect to the "independent"

variable being proportional to their ratio, underlies much of the S-system approach to nonlinear phenomenon and employs a type of scaling that is common in many physiological and other biological systems. More complicated nonlinear dose-response functions are derived if one assumes a similar but generalized relationship between incremental response and dose locally, i.e. at a given point of the dose-response curve, as follows:

$$dx = g(x) dx \frac{y}{x}$$

where the proportionality constant g is now replaced by a more general "local modifier function" $g(x)$. By assigning an appropriate form to $g(x)$ one can appropriately modify the relationship between dose and response so as to reflect dominance of different biological phenomenon over different ranges of the dose-response curve. For example, one can choose a functional form for $g(x)$ such that for small doses the proportionality factor is close to unity and therefore the relationship $dy = g dx \frac{y}{x}$ becomes linear or assumes another constant value, g , in which case the power law behavior (with power g) dominates at low doses. At the same time one may define $g(x)$ so that e.g., at high doses the incremental change in response becomes vanishingly small. A functional form that combines both these requirements is:

In that case

$$g(x) = \frac{gb}{b+x^g}$$

$$\frac{1}{y} \frac{dy}{dx} = \frac{1}{x} \frac{gb}{b+x^g}$$

and, using the property

$$\int \frac{dx}{x(b+x^g)} = \frac{1}{bg} \ln \left| \frac{x^g}{b+x^g} \right|$$

and one gets

biological systems. More complicated nonlinear dose-response functions are derived if one assumes a similar but generalized relationship between incremental response and dose locally, i.e. at a given point of the dose-response curve, as follows:

$$dy = g(x) dx \frac{y}{x}$$

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$$g(x) = \frac{gb}{b+x^g}$$

In that case

$$\frac{1}{y} \frac{dy}{dx} = \frac{1}{x} \frac{gb}{b+x^g}$$

and, using the property

$$\int \frac{dx}{x(b+x^g)} = \frac{1}{bg} \ln \left| \frac{x^g}{b+x^g} \right|$$

and one gets

$$y = \frac{ax^g}{b+x^g}$$

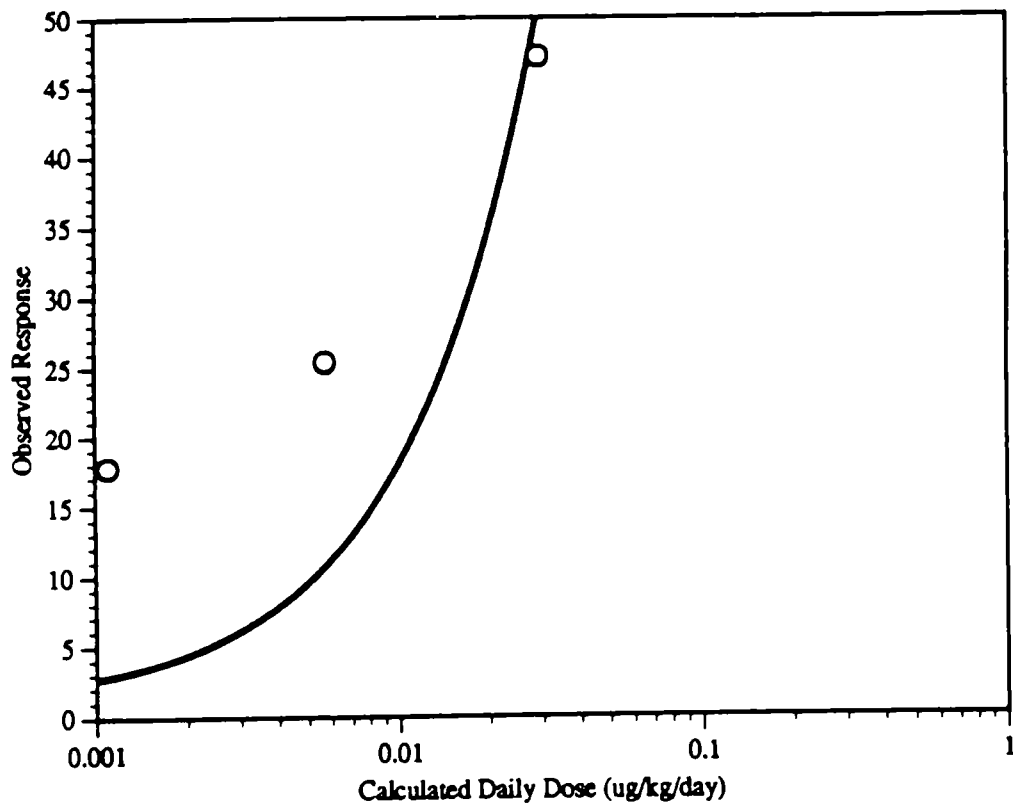
where a is an integration constant. The above expression is the well known Hill function (which for $g=1$) becomes the Michaelis-Menten equation.

GRAPH A1
Dose-Response
(Graph in Log-Linear Coordinates)
for

Female Guinea Pigs
(Harris et al., 1973)

Dose: repeat oral dose 1 X weekly for 8 weeks

Response: % decrease thymic weight



Functional Form of Sigmoid Dose-Response
(Best Fit Estimate for "1 + Michaelis-Menten" type response)
 $f(x) = 1 + 4.745175E+6 \cdot x / (2.771639E+3 + x)$, $\chi^2 = 447.7731$

GRAPH A2

Dose-Response

(Graph in Log-Linear Coordinates)

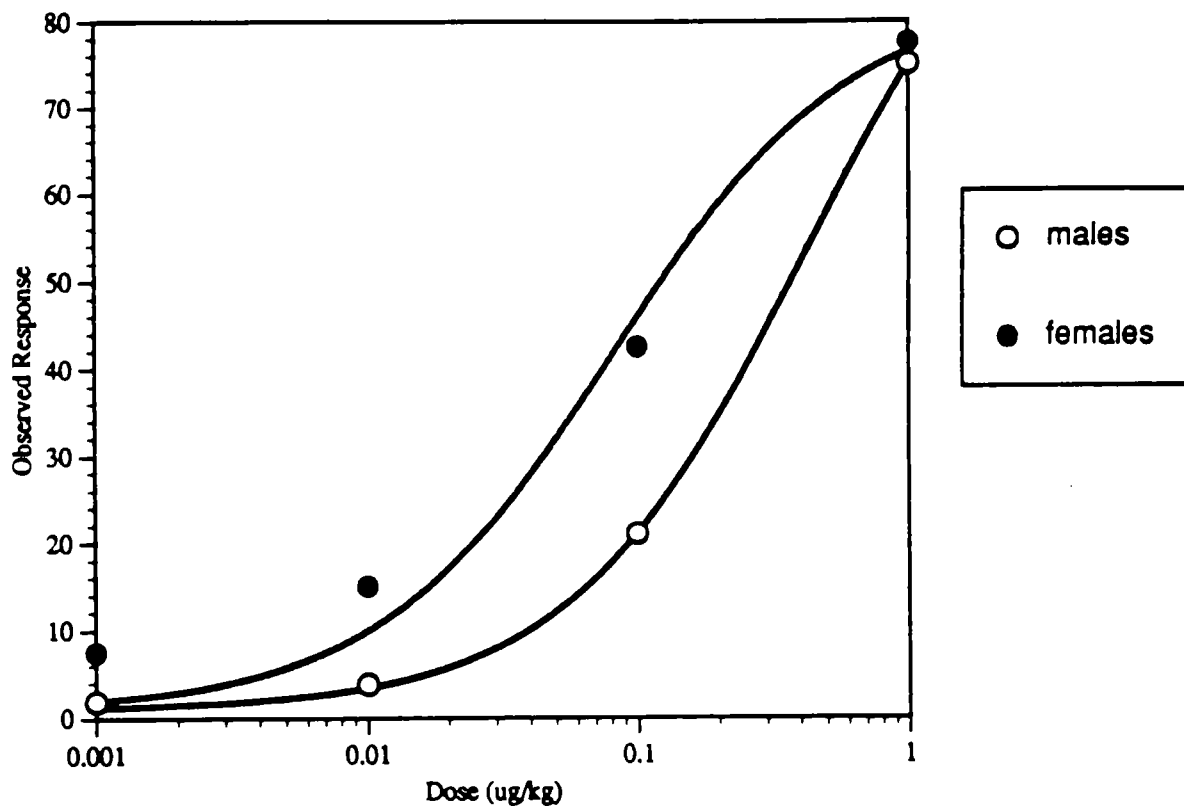
for

Male and Female Sprague-Dawley Rats

(Kociba et al, 1976)

Dose: repeat dose 5 X per week for 13 weeks

Response: % Decrease thymus weight



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "1 + Michaelis-Menten" type response)

males, $f(x) = 1 + 1.054825 + 2 \cdot x / (4.255322E-1 + x)$, $\chi^2 = .5741008$

females, $f(x) = 1 + 8.165068E+1 \cdot x / (8.180588E-2 + x)$, $\chi^2 = 69.15886$

GRAPH B1

Dose-Response

(Graph in Log-Linear Coordinates)

for

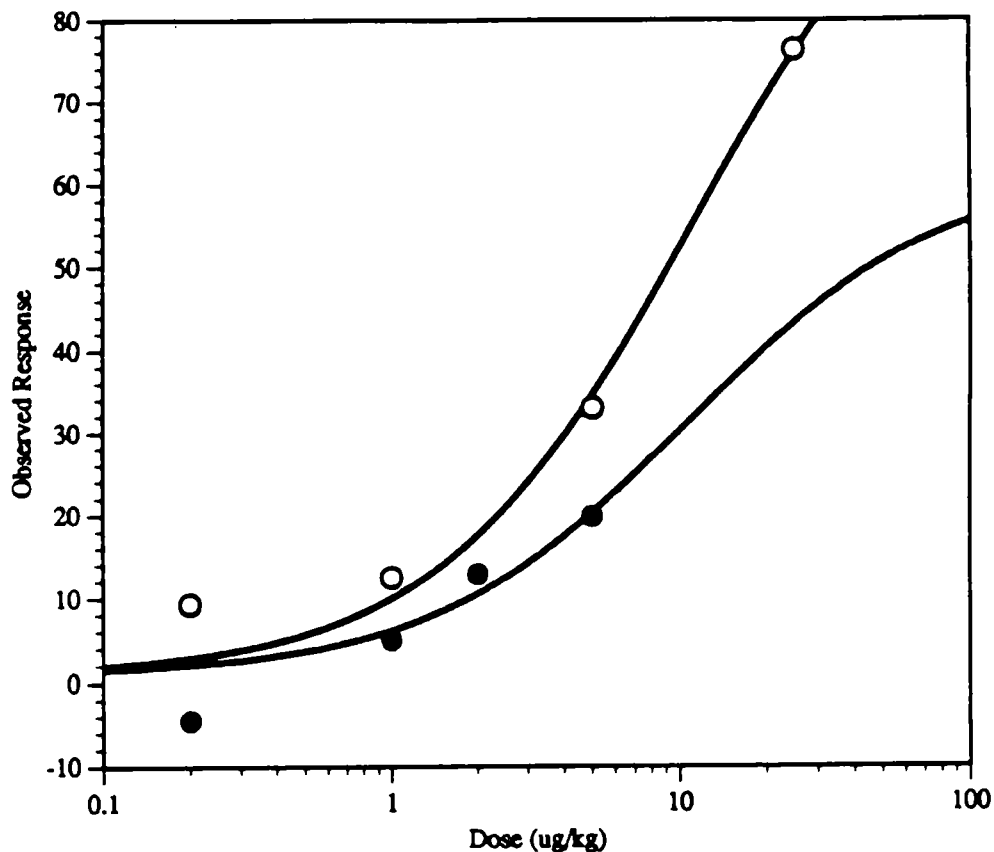
Male C57BL/6 Sch St. mice, (Vos, 1974)

Female C57BL/6 mice (Kerkvliet and Brauner, 1990)

Dose: single oral

Response: % decrease in thymic weight 2 weeks (Vos) and 7 days

(Kerkvliet and Brauner) post dosing



○ Vos
● Kerkvliet and Brauner

Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "1 + Michaelis-Menten" type response)

Vos, $f(x) = 1 + 6.046204E+1 \cdot x / (1.061148E+1 + x)$, $\chi^2 = 50.5892$

Kerkvliet and Brauner, $f(x) = 1 + 1.081043E+2 \cdot x / (1.098941E+1 + x)$, $\chi^2 = 49.71537$

GRAPH B2

Dose-Response

(Graph in Log-Linear Coordinates)

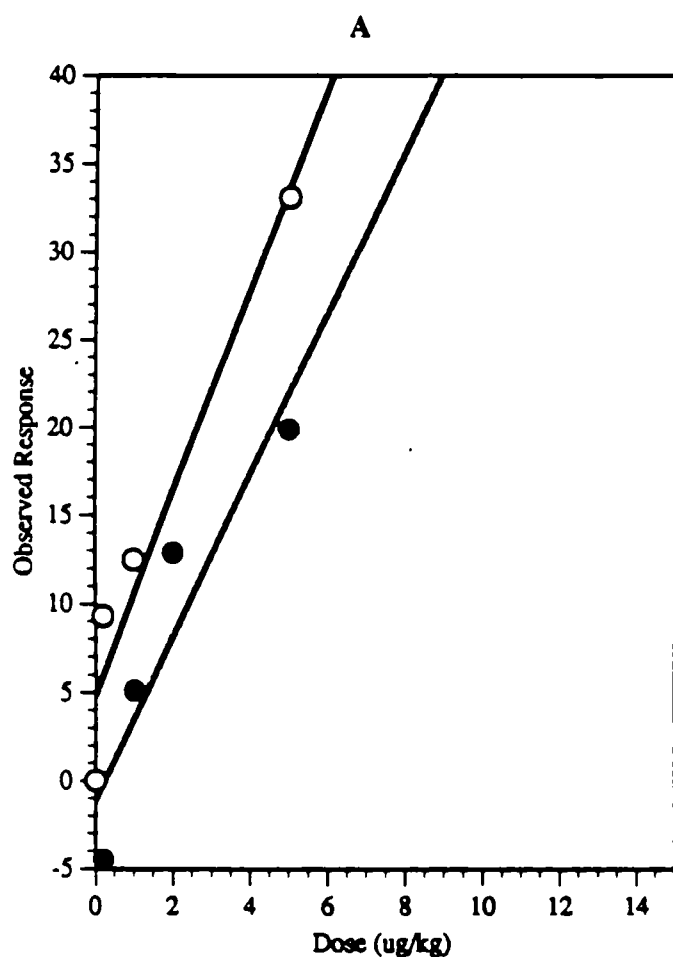
for

Male C57BL/6 Sch St. mice, (Vos, 1974)

Female C57BL/6 mice (Kerkvliet and Brauner, 1990)

Dose: single oral

Response: % decrease in thymic weight 2 weeks (Vos) and 7 days
(Kerkvliet and Brauner) post dosing

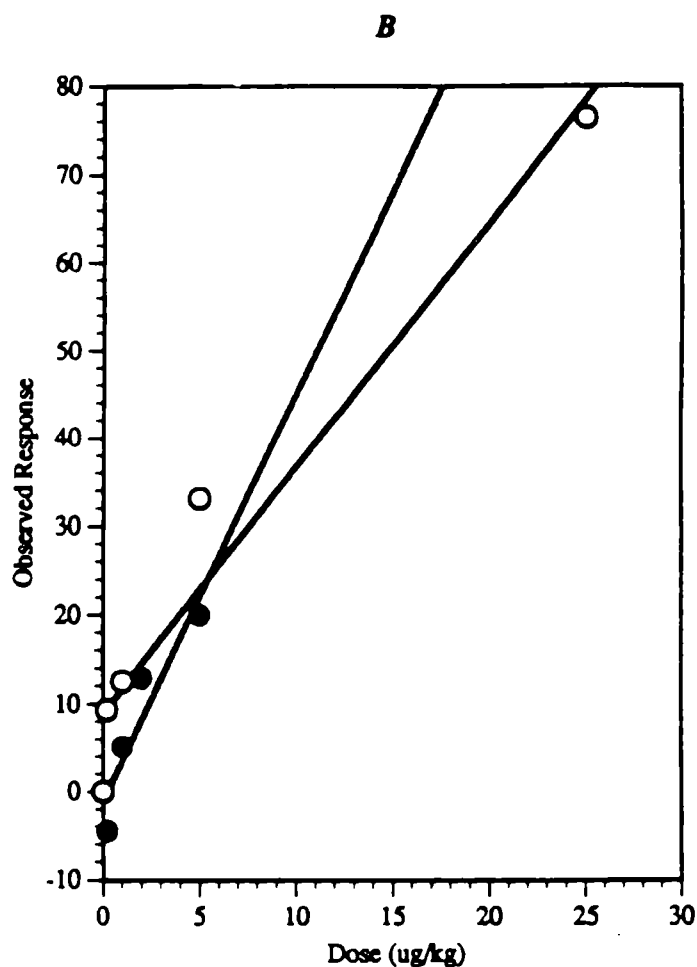


Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for Linear type response)

$$\text{Vos, } f(x) = 5.767803E+0 \cdot x + 4.784906E+0, R^2 = .93$$

$$\text{Kerkvliet, } f(x) = 4.613001E+0 \cdot x + 1.106652E+0, R^2 = .85$$



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Linear type response)

$$\text{Vos, } f(x) = 2.784140E+0 \cdot x + 8.886964E+0, R^2 = .95$$

$$\text{Kerkvliet, } F(x) = 4.613001E+0 \cdot x + 1.106652E+0, R^2 = .85$$

○ Vos

● Kerkvliet and Brauner

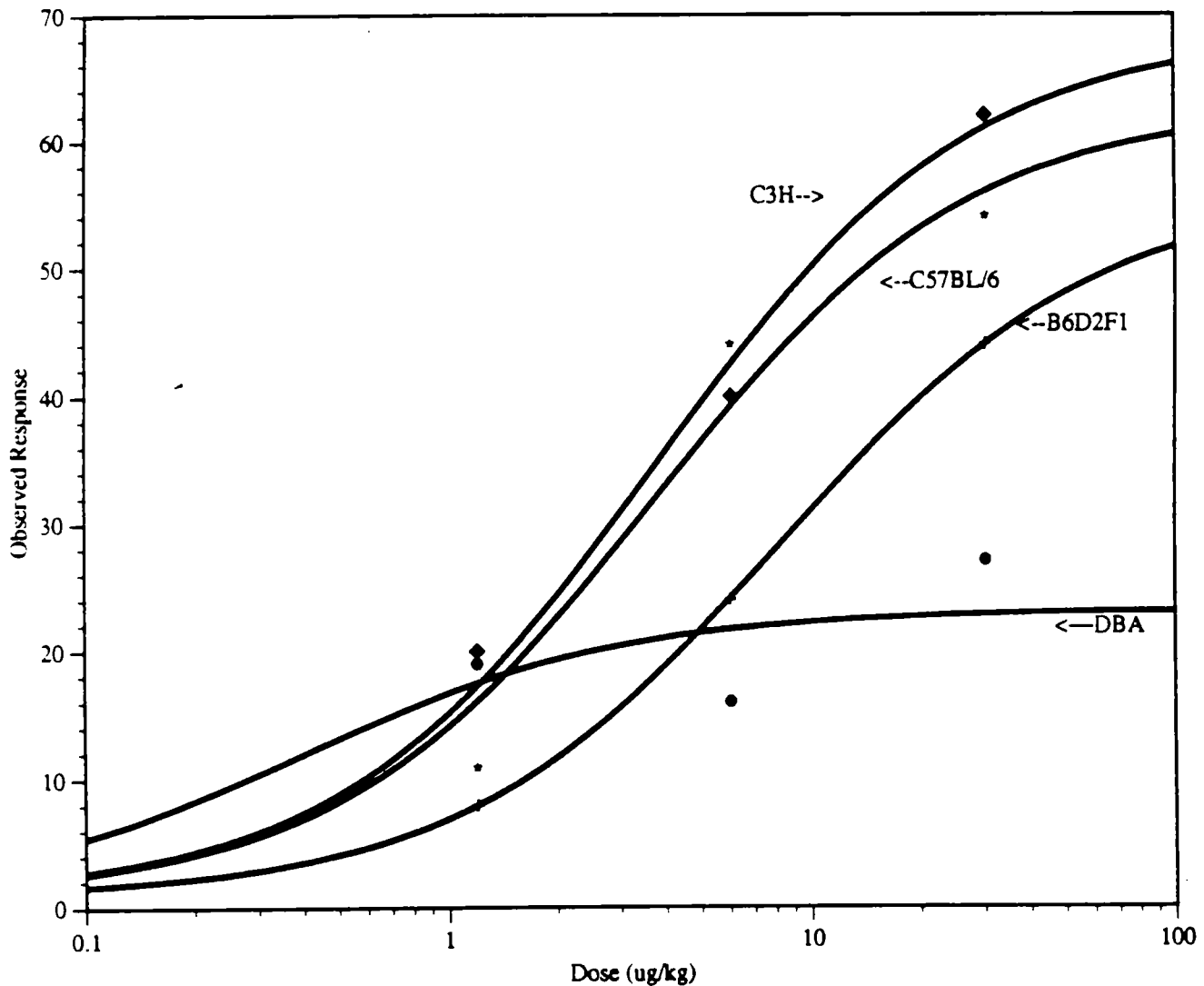
Graph B3
Dose-Response
 (Graph in Log-Linear Coordinates)

for
 Male mice with differing suseptibility to he Ah induction
 (Vecchi et al., 1983)

Dose: single ip

Response: % decrease thymus weigh 12 days post-dosing

Michaelis Menten Function



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "1+ Michaelis-Menten" type response)

$$\text{B6D2F1 } f(x) = y = 1 + 5.477307E+1 * x / (8.229451E+0 + x), \text{chi}^2 = 0.33$$

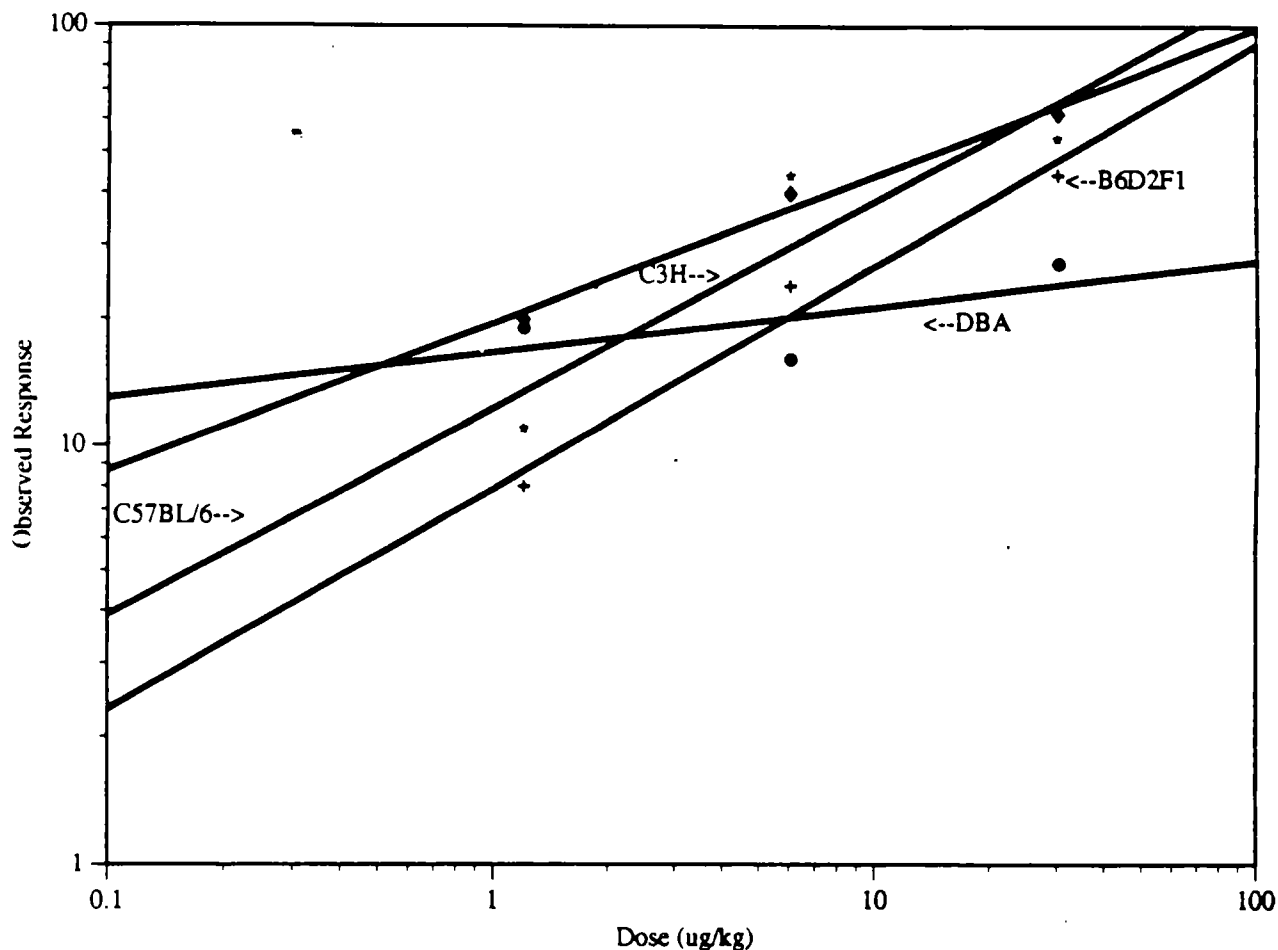
$$\text{C57BL/6J } f(x) = y = 1 + 6.171059E+1 * x / (3.697454E+0 + x), \text{chi}^2 = 48.9$$

$$\text{C3H } f(x) = y = 1 + 6.754428E+1 * x / (3.767704E+0 + x), \text{chi}^2 = 14.4$$

$$\text{DBA } f(x) = y = 2 + 2.20347E+1 * x / (3.000336E-1 + x), \text{chi}^2 = 52.3$$

GRAPH B4
Comparative Dose-Response
 (Graph in Log-Log Coordinates)

for
 Male mice with differing suseptibility to the Ah induction
 (Vecchi et al., 1983)
 Dose: single ip
 Response: decrease thymus weight



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

B6D2: $f(x) = 7.884996E+0 \cdot (x^{5.296098E-1})$, $R^2 = 9.729347E-1$

C57BL/6J: $f(x) = 1.223977E+1 \cdot (x^{4.942995E-1})$, $R^2 = 8.447337E-1$

C3H: $f(x) = 1.957259E+1 \cdot (x^{3.514898E-1})$, $R^2 = 9.833631E-1$

DBA: $f(x) = 1.658810E+1 \cdot (x^{1.091679E-1})$, $R^2 = 4.339738E-1$

GRAPH B5

Dose-Response

(Graph in Log-Linear Coordinates)

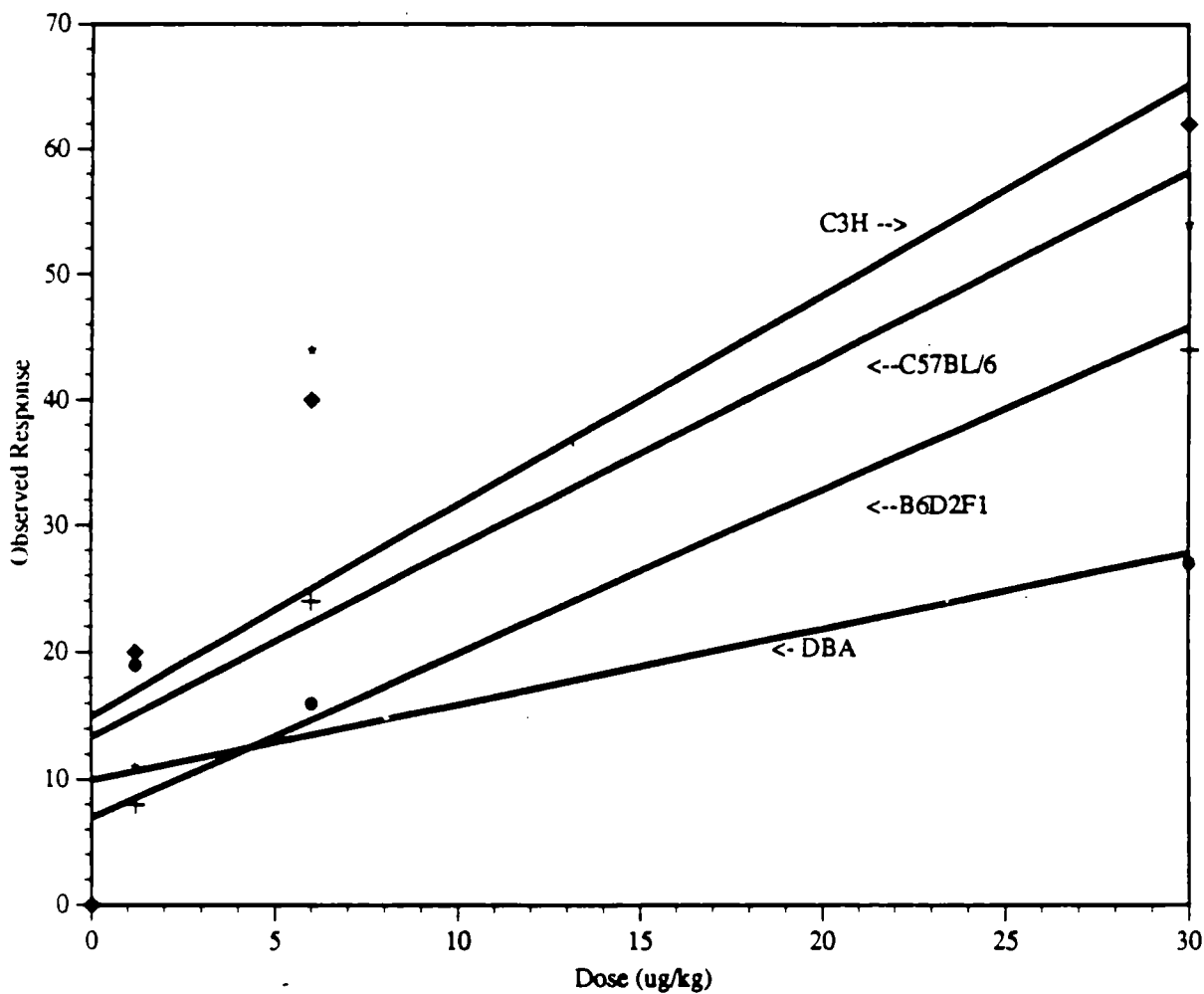
for

Male mice with differing suseptibility to the Ah induction

(Vecchi et al., 1983)

Dose: single ip

Response: % decrease thymus weight 12 days post-dosing



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for Linear function)

$$\text{C57BL/6, } f(x) = 1.145833\text{E}+0 \cdot x + 2.212500\text{E}+1, R^2 = 6.173469\text{E}-1$$

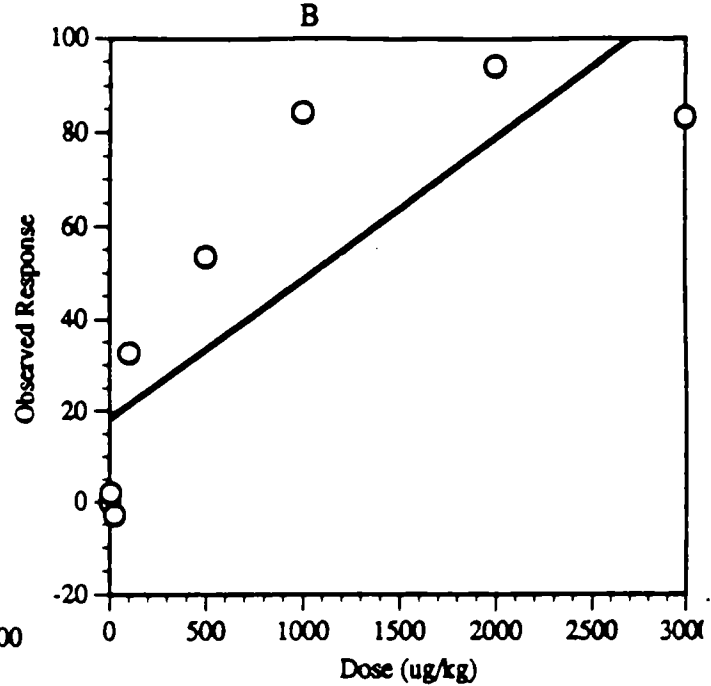
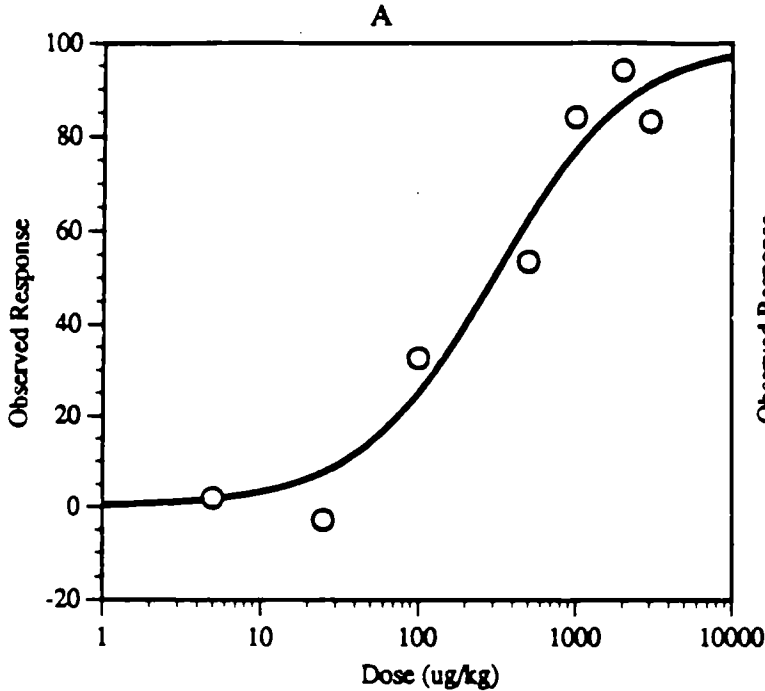
$$\text{C3H, } f(x) = 1.283602\text{E}+0 \cdot x + 2.475000\text{E}+1, R^2 = 8.888266\text{E}-1$$

$$\text{DBA, } f(x) = 3.360215\text{E}-1 \cdot x + 1.650000\text{E}+1, R^2 = 8.313934\text{E}-1$$

$$\text{B6D2F1, } f(x) = 1.115591\text{E}+0 \cdot x + 1.150000\text{E}+1, R^2 = 9.107615\text{E}-1$$

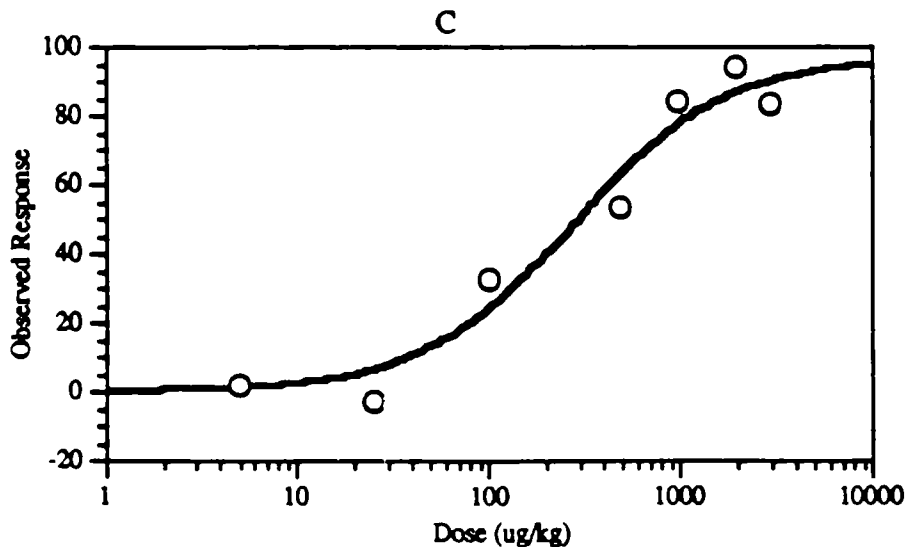
GRAPH B6
Dose-Response
 for
 male Syrian Hamsters
 Olsen et al, 1980
 Dose: single oral

Response: % response decrease thymic weight 50 days post dosing



Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for "Michaelis-Menten" type response)
 $f(x) = 0.101 + 1.001197E+2 * x / (3.023870E+2 + x)$
 $\chi^2 = 415.4893$

Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for Linear type response)
 $f(x) = 3.019634E-2 * x + 1.829978E+1$
 $R^2 = 6.781075E-1$

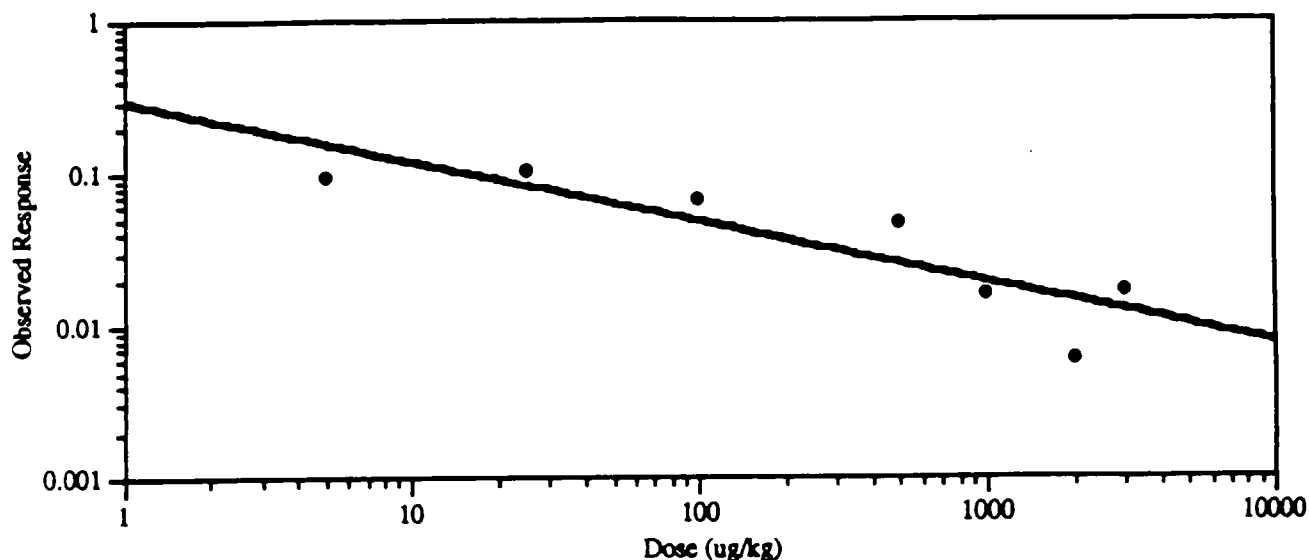


Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for "Hill plot")
 $f(x) = (9.709755E+1 * x^{1.087476E+0}) / (4.559997E+2 + x^{1.087476E+0})$
 $\chi^2 = 407.9375$

Graph B7
Dose-Response
(Graph in Log-Linear Coordinates)
Male Syrian Hamsters
(Olsen, 1980)

Dose: single oral

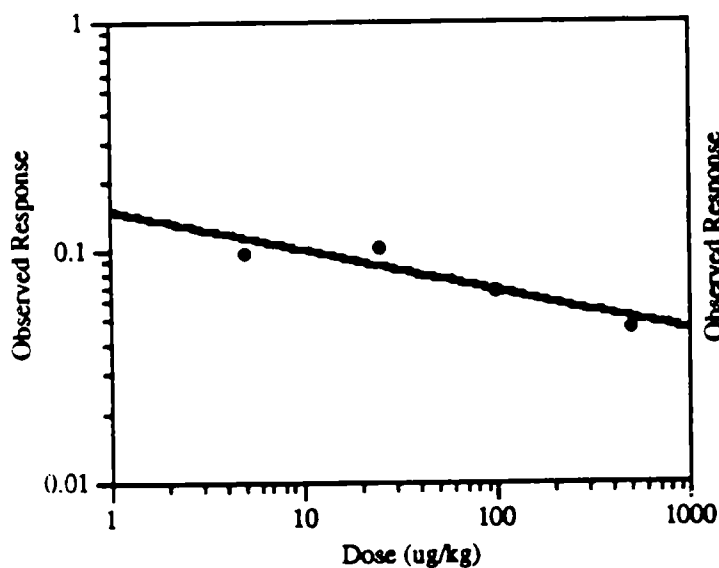
Response: response thymus weight change after 50 days-(A)- raw
data



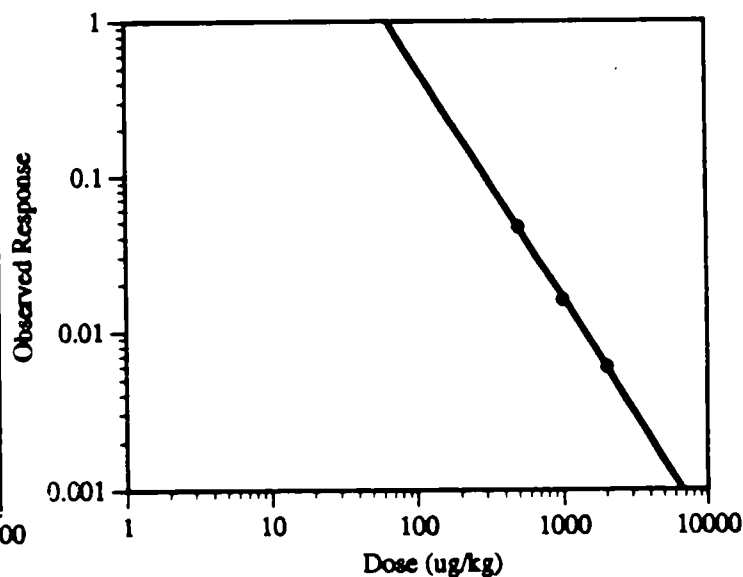
(Best Fit Estimate for Power Law)
 $f(x) = 2.931550E-1 \cdot (x^{-3.945743E-1})$
 $R^2 = 7.593673E-1$

(B)

(C)



(Best Fit Estimate for Power Law Function)
(doses = 5 - 500 ug/kg)
 $f(x) = 1.494819E-1 \cdot (x^{-1.737934E-1})$
 $R^2 = 8.487105E-1$



(Best Fit Estimate for "Power Law Function")
(doses 500-2000 ug/kg)
 $f(x) = 4.705032E+2 \cdot (x^{-1.484813E+0})$
 $R^2 = 9.992644E-1$

Graph C1

Dose-Response

(Graph in Log-Linear Coordinates)

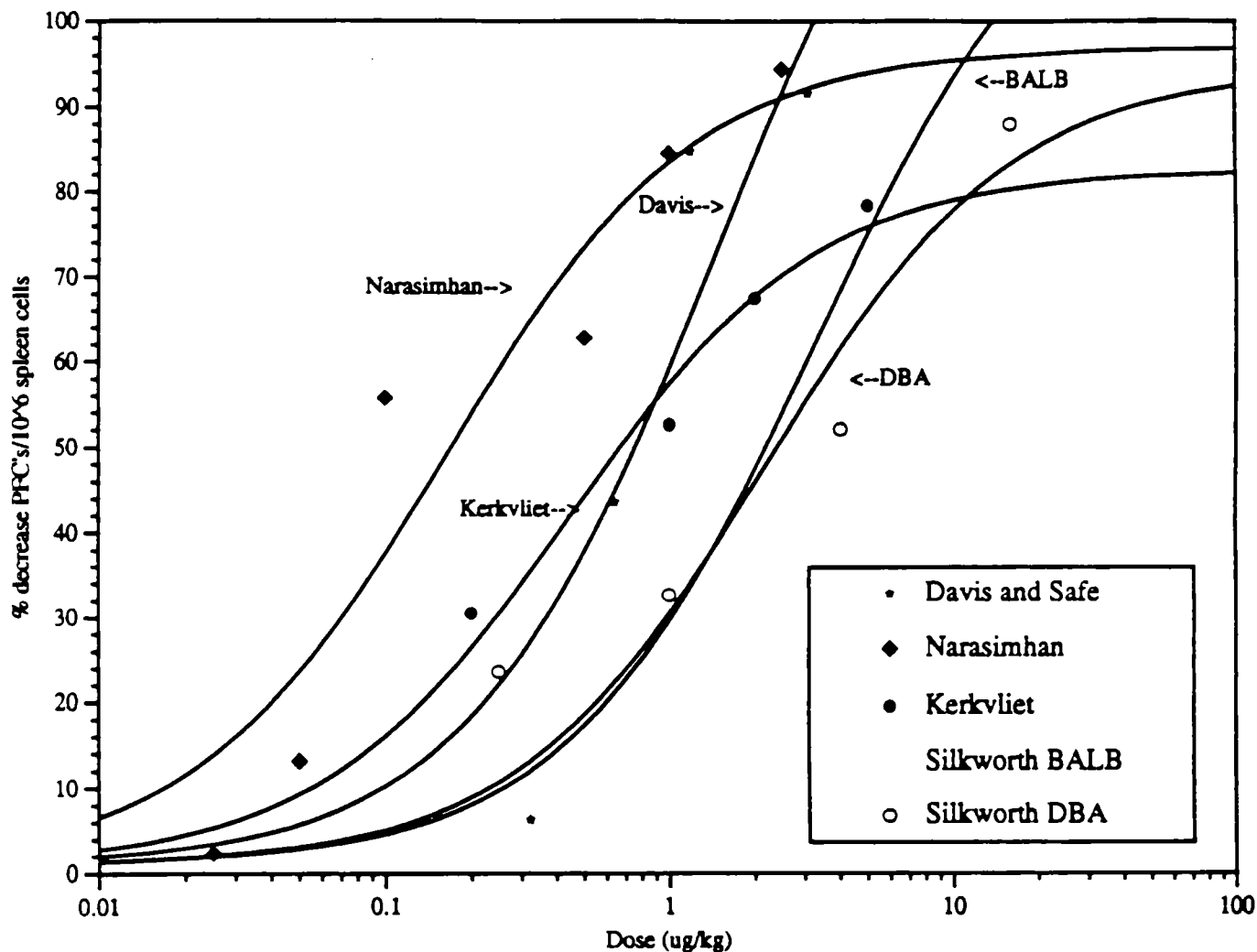
Female C57BL/6 mice (Kerkvliet and Brauner, 1990)

Male C57BL/6J mice (Davis and Safe, 1988)

B6C3F1 female mice (Narasimhan et al., 1993)

BALB and DBA male mice (Silkworth et al., 1989)

Dose: Single oral or ip administration



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "1 + Michaelis-Menten" type response)

Davis, $f(x) = 1 + 1.432263E+2 \cdot x / (1.456438E+0 + x)$, $\chi^2 = 849$

Narasimhan et al, $f(x) = 1 + (9.592833E+1 \cdot x) / (1.627160E-1 + x)$, $\chi^2 = 693$

Kerkvliet, $f(x) = 1 + (8.143631E+1 \cdot x) / (4.432174E-1 + x)$, $\chi^2 = 46.2$

Silkworth BALB, $f(x) = 1 + (1.225246E+2 \cdot x) / (3.286863E+0 + x)$, $\chi^2 = 284$

Silkworth DBA, $f(x) = 1 + (9.344592E+1 \cdot x) / (2.176351E+0 + x)$, $\chi^2 = 280$

Graph C2

Comparative Dose-Response

(Graph in Log-Log Coordinates)

Female C57BL/6 mice (Kerkvliet and Brauner, 1990)

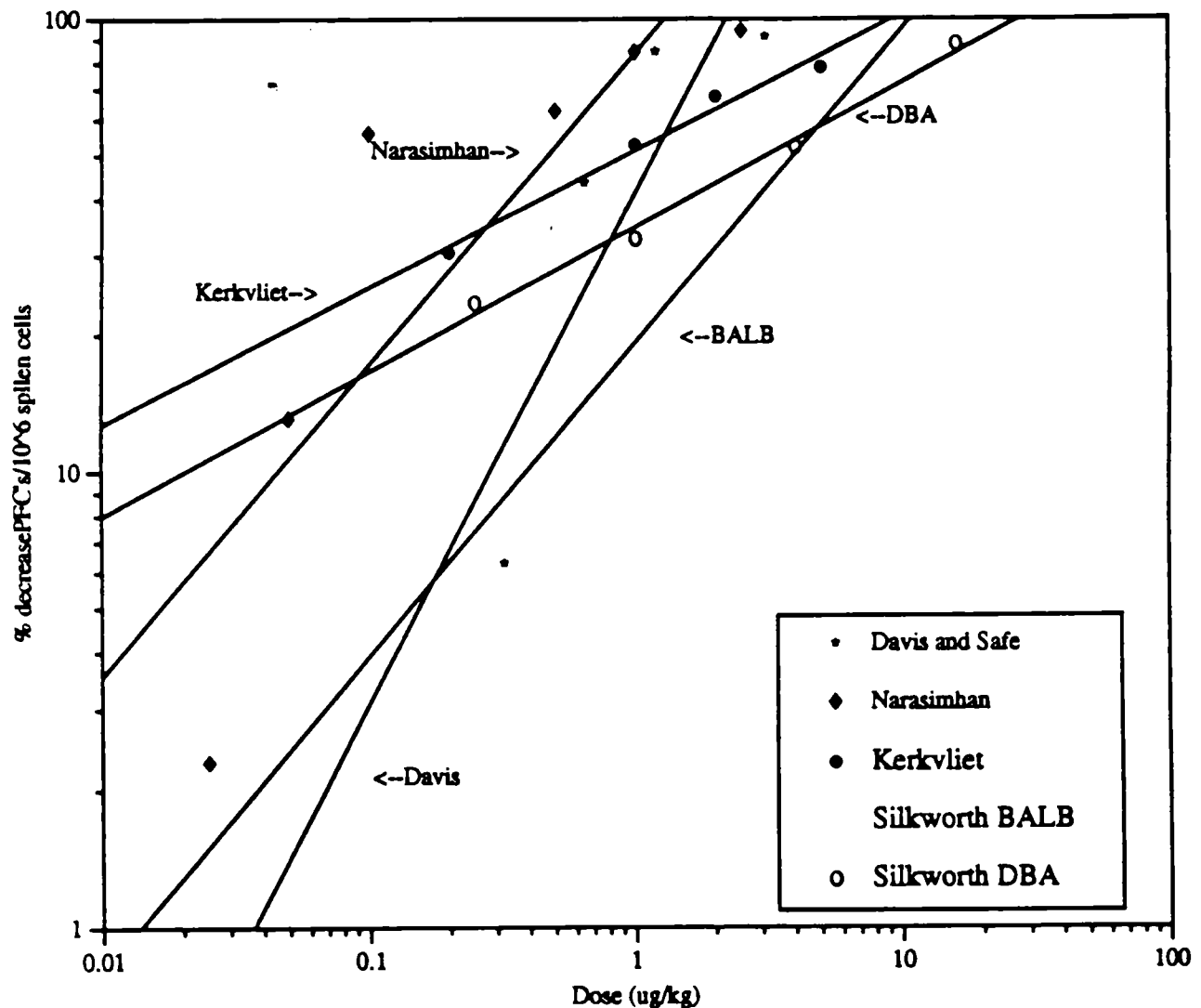
Male C57BL/6J mice (Davis and Safe, 1988)

B6C3F1 female mice (Narasimhan et al., 1993)

BALB and DBA male mice (Silkworth et al., 1989)

Dose: Single oral or ip administration

Response: % decrease PFC/10⁶ spleen cells



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

$$\text{Davis, } f(x) = 4.124948E+1 \cdot x^{1.125711E+0}, r^2 = .7501$$

$$\text{Narasimhan et al. , } f(x) = 8.380591E+1(x^{6.873447E-1}), r^2 = .7317$$

$$\text{Kerkvliet, } f(x) = 5.114275E+1 \cdot x(x^{3.027756E-1}), r^2 = .9807$$

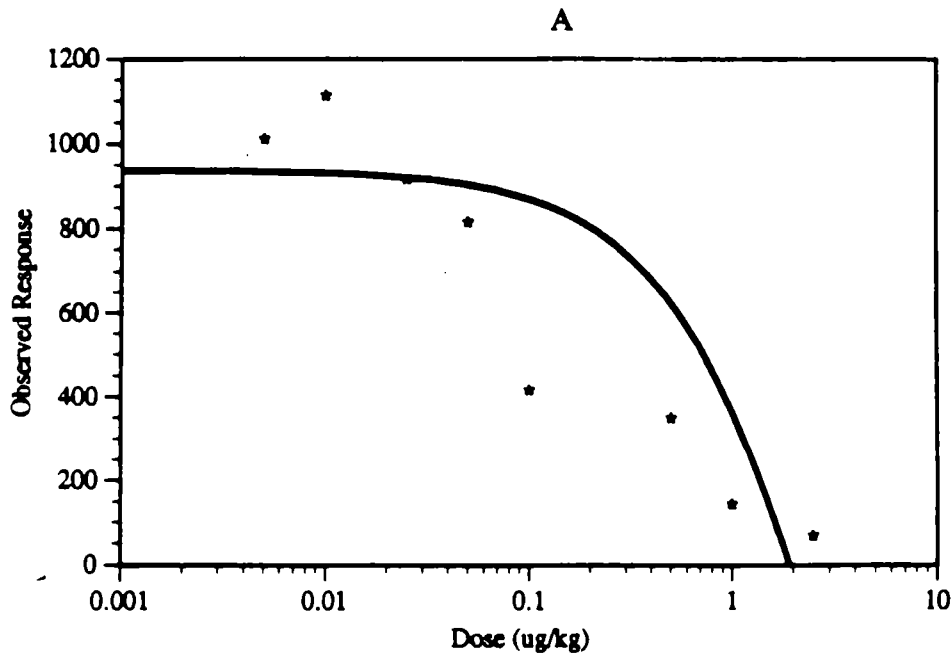
$$\text{Silkworth BALB, } f(x) = 1.929342E+1 \cdot (x^{6.915990E-1}), r^2 = .9371$$

$$\text{Silkworth DBA, } f(x) = 3.468637E+1 \cdot (x^{3.193009E-1}), r^2 = .9899$$

Chart C3
Dose-Response
(Graph in Log-Linear Coordinates)
for

Female B6C3F1 mice
(Narasimhan et al., 1993)

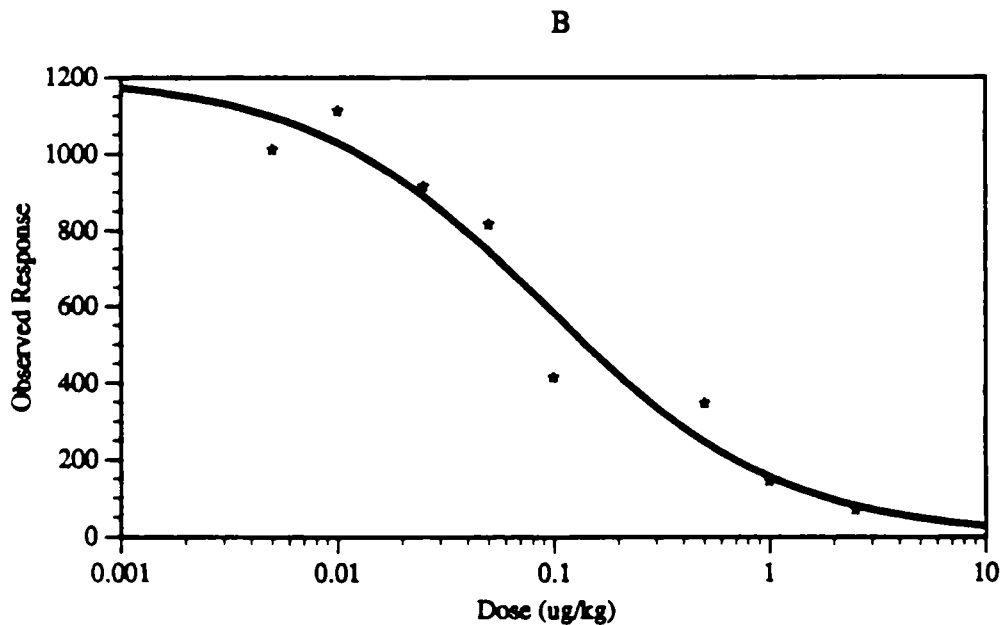
Response: number of PFC's/ 10^6 spleen cells



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Michaelis-Menten" + background parameter type response)

$$f(x) = 939 + -3.005346E+3 * x, \text{ chi}^2 = 43,447$$

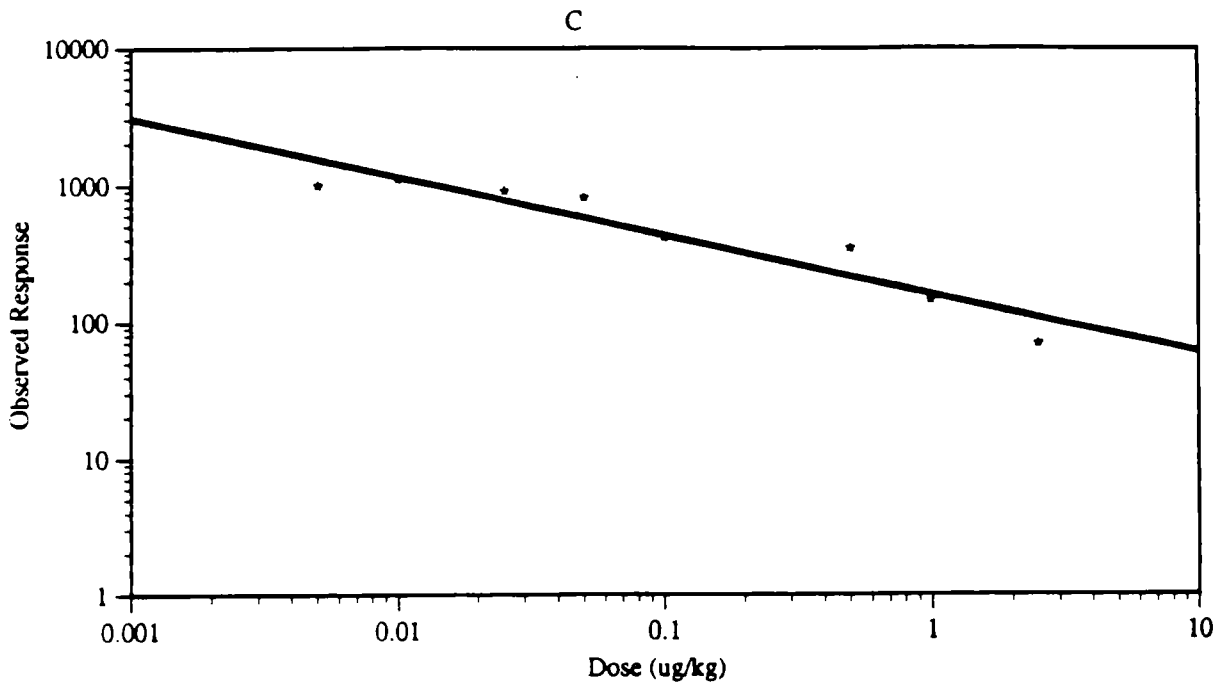


Functional Form of Sigmoid Dose-Response

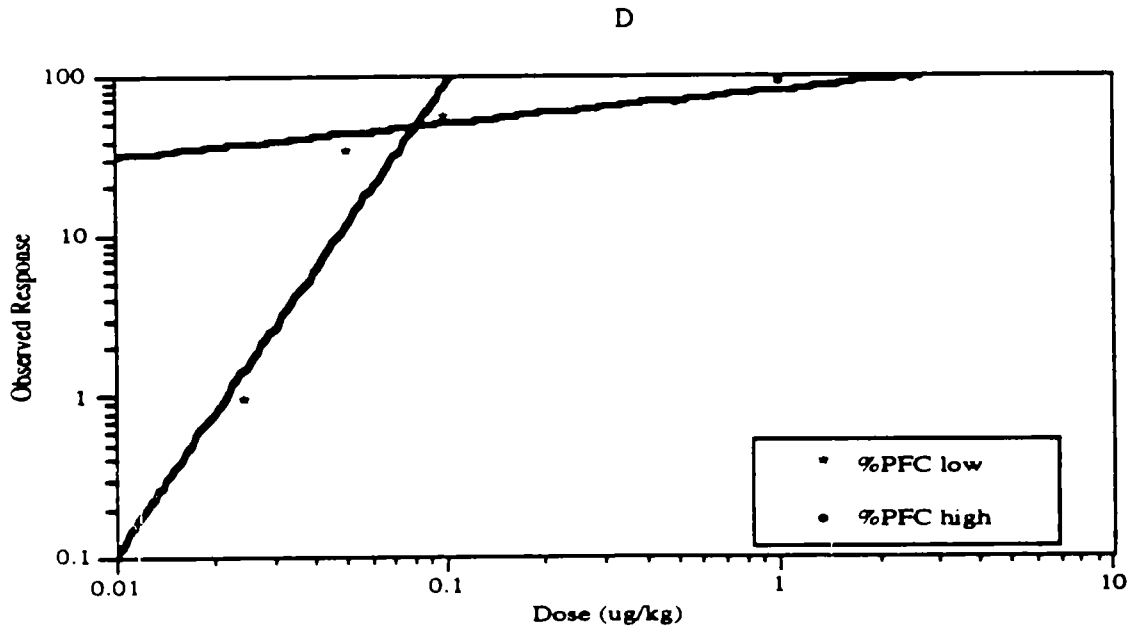
(Best Fit Estimate for "Hill function type response)

$$f(x) = 1.204213E+3 * x^{-7.995208E-1/6.767036E+0} + x^{-7.995208E-1}, \text{ chi}^2 = 5834$$

Chart C3
Dose-Response
 (Graph in Log-Linear Coordinates)
 Female B6C3F1 mice
 (Safe, 1992)
 Response: % decrease PFC



Functional Form of Dose-Response
 (Best Fit Estimate for Power Law)
 $f(x) = 1.602198E+2 * x^{-4.269049E-1}$, $r^2 = .8930$

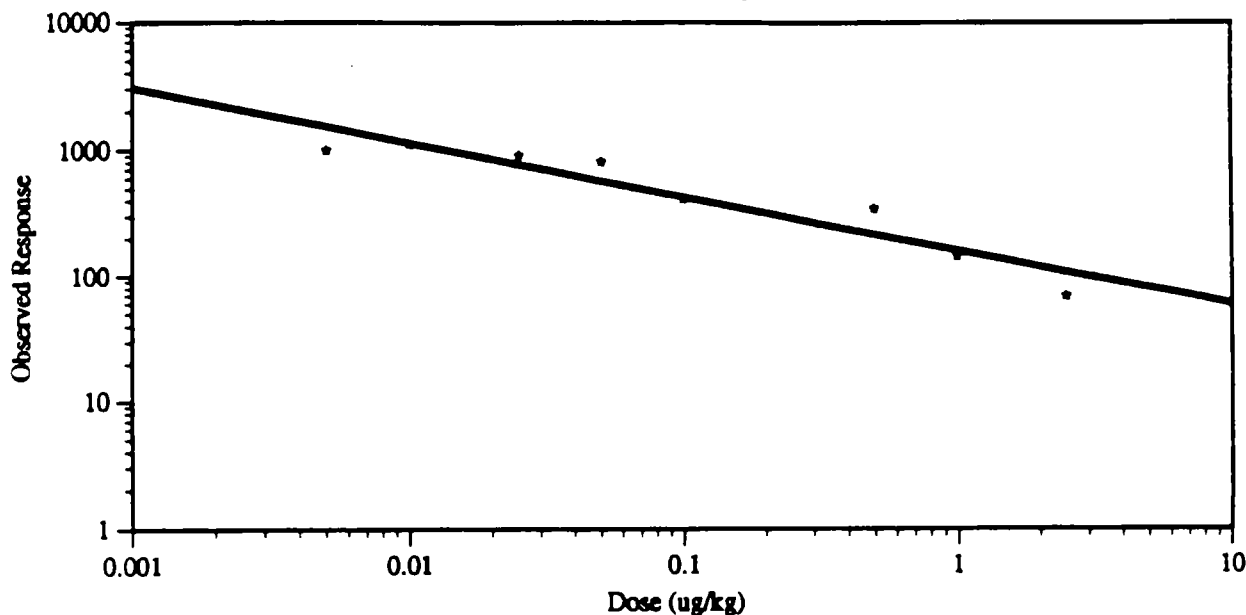


Functional Forms of Dose-Response Curves
 (Best Fit Estimates for Power Laws)
 PFC low dose $f(x) = 7.395768E+4 * (x^{2.933124E+0})$, $R^2 = 8.481507E-1$
 PFC high dose $f(x) = 8.103178E+1 * (X^{2.074615E-1})$, $R^2 = 8.233572E-1$

GRAPH C4
Dose-Response
 (Graph in Log-Linear Coordinates)
 for
 Female B6C3F1 mice , Narasimhan et al., 1993)

Response: number of PFC's/spleen
 or % response PFCs/10⁶ spleen cells

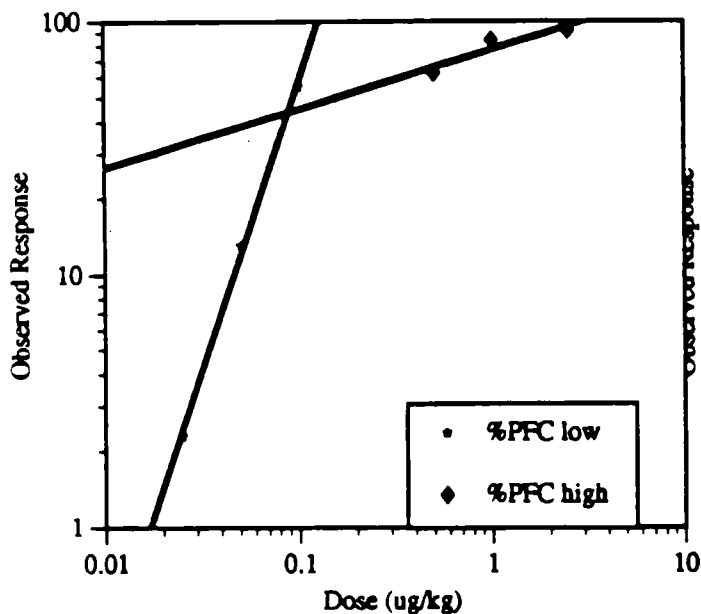
A



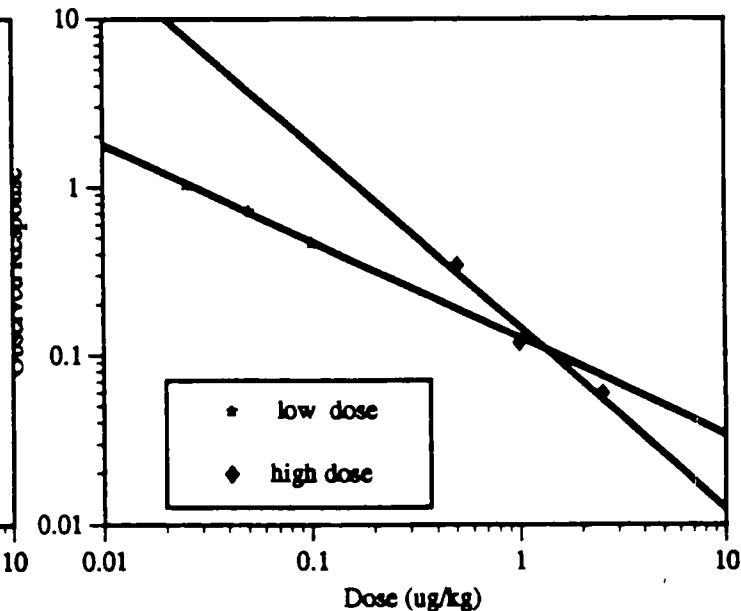
Functional Form of Dose-Response
 (Best Fit Estimate for Power Law)

$$f(x) = 1.602198E+2 * x^{-4.269049E-1}, R^2 = 8.929944E-1$$

Dose -Response - Subsets - % response-B



Dose -Response - Subsets - raw data response-C



Functional Forms of Dose-% Response Curves

PFC low $f(x) = 1.164128E+4 * x^{2.298986E+0}, R^2 = 9.972033E-1$

PFC high $f(x) = 7.748043E+1 * x^{2.341332E-1}, R^2 = 8.628811E-1$

Functional Forms of Dose- Response Curves

PFC low $f(x) = .274749E-1 * (x^{-5.729254E-1}), R^2 = 9.961E-1$

PFC high $f(x) = 1.474431E-1 * (X^{-1.078037E+0}), R^2 = 9.594E-1$

GRAPH C5

Comparative Dose-Response

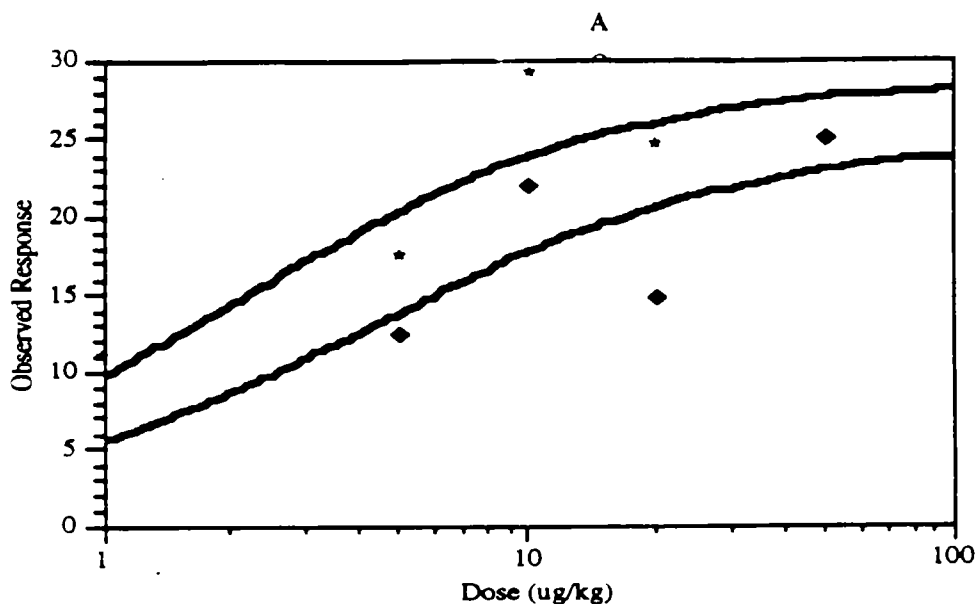
(Graph in Log-Log Coordinates)

C57BL/6J mice AhBB and Ahdd

(Kerkvleit et al. , 1990a)

Dose: single oral dose TCDD 2 days prior to an ip injection of 25 ug TNP-LPS

Response: decrease spleen cellularity

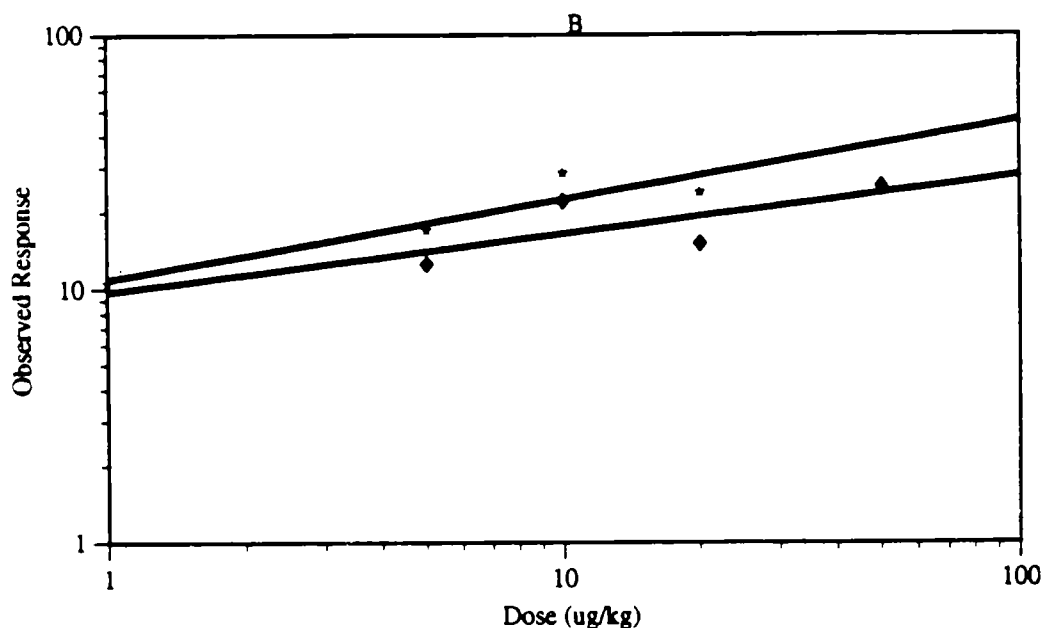


Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

Ahbb, $f(x) = 1 + 2.7767E+1 * x / (2.1853353E+0 + x)$, $\chi^2 = 38.15$

Ahdd, $f(x) = 1 + 2.385456E+1 * x / (4.32568E+0 + x)$, $\chi^2 = 56.63$

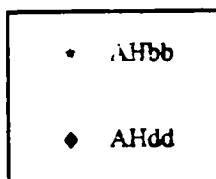


Functional Forms of Dose-Response Curves

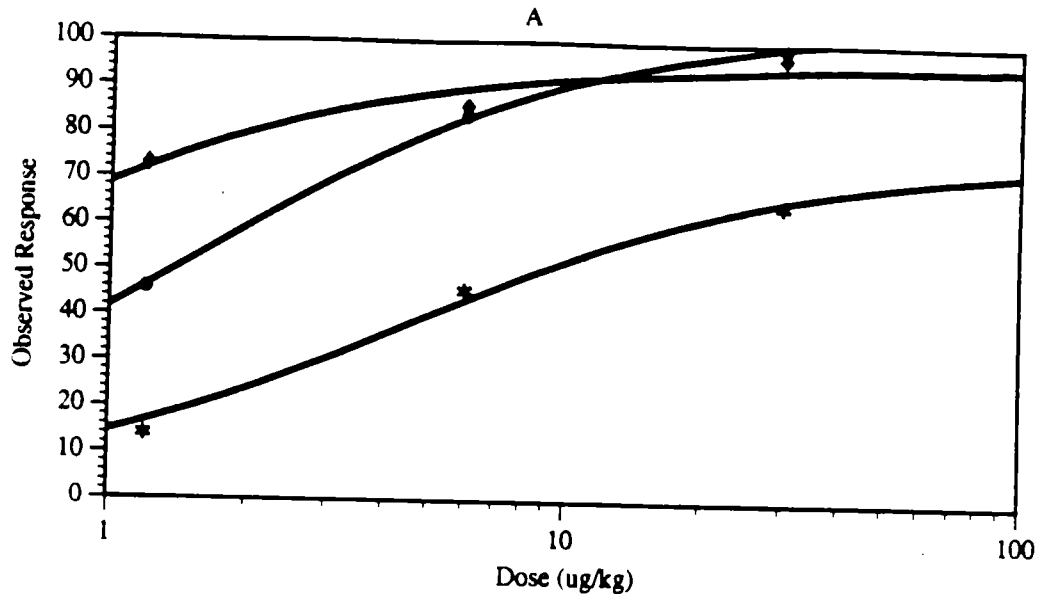
(Best Fit Estimates for Power Laws)

Ahbb: $f(x) = 1.089295E+1 * (3.140763E-1)$, $R^2 = 8.527404E-1$

Ahdd: $f(x) = 9.726441E+1 * (2.284170E-1)$, $R^2 = 4.834202E-1$



Graph D1
 Comparative Dose-Response
 (Graph in Log-Log Coordinates)
 Male Mice differing in susceptibility to Ah induction
 (Vecchi, 1983)
 Dose: single ip
 Response: % decrease PFC/splenocytes at 5 days

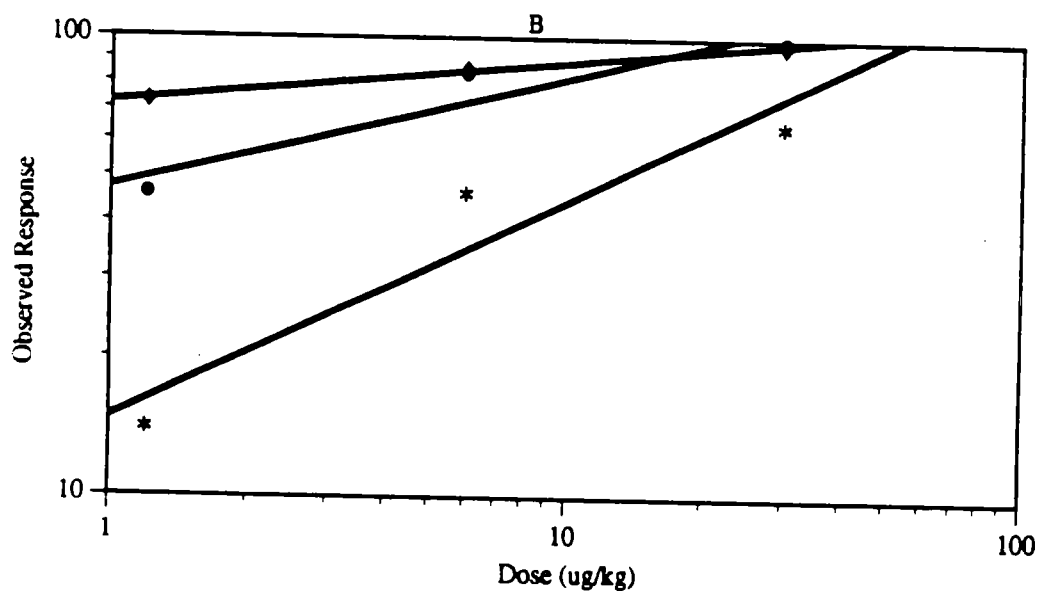


Functional Forms of Dose-Response Curves
 (Best fit Estimates for 1+ Michaelis-Menten)

C57BL/6: $f(x) = 1 + 9.448837E+1 * x / (3.957415E-1 + x)$, $\chi^2 = 21.7$

DBA: $f(x) = 1 + 7.446390E+1 * x / (4.459745E+0 + x)$, $\chi^2 = 13.7$

B6D2F1: $f(x) = 1 + 1.034210E+2 * x / (1.535931E+0 + x)$, $\chi^2 = 0.71$



Functional Forms of Dose-Response Curves
 (Best Fit Estimates for Power Laws)

C57BL/6: $f(x) = 7.235706E+1 * (x^{8.0771E-2})$, $r^2 = 9.922455E-1$

DBA2: $f(x) = 1.477200E+1 * (x^{4.769771E-1})$, $r^2 = 9.085196E-1$

B6D2F1: $f(x) = 4.738026E+1 * (x^{2.381199E-1})$, $r^2 = 9.018871E-1$

◆ C57BL/6

* DBA2

● B6D2F1

GRAPH E

Dose-Response

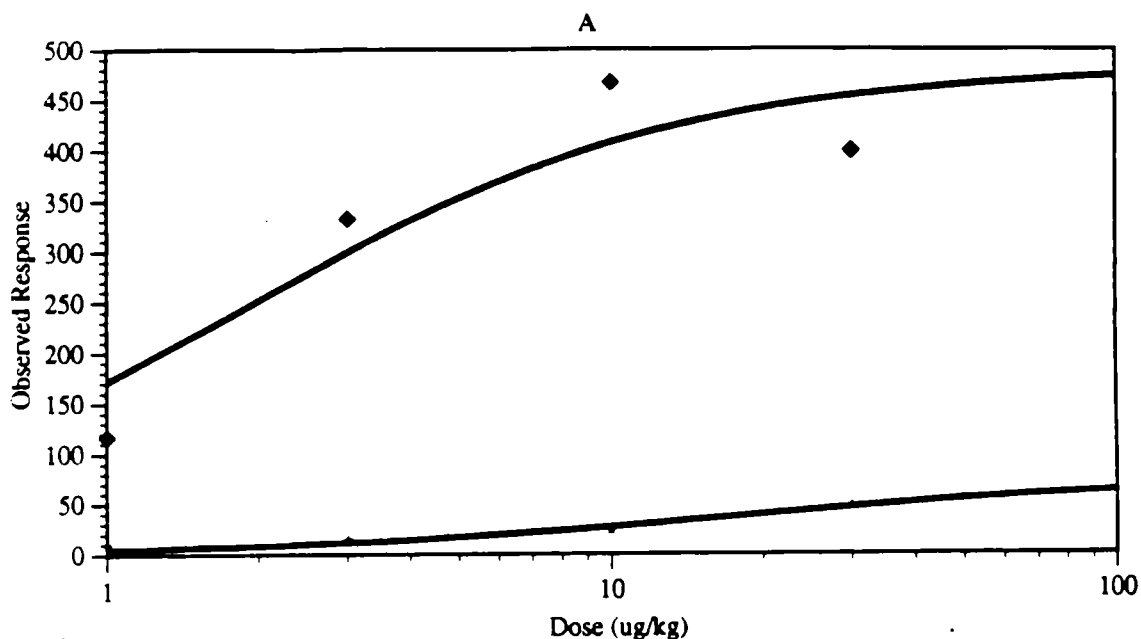
(Graph in Log-Linear Coordinates)

Female CD-1 mice

(DeVito et al. 1992))

Dose: single i.p.

Response: % decrease hepatic estrogen receptor increase hepatic AHH activity



Functional Form of Sigmoid Dose-Response

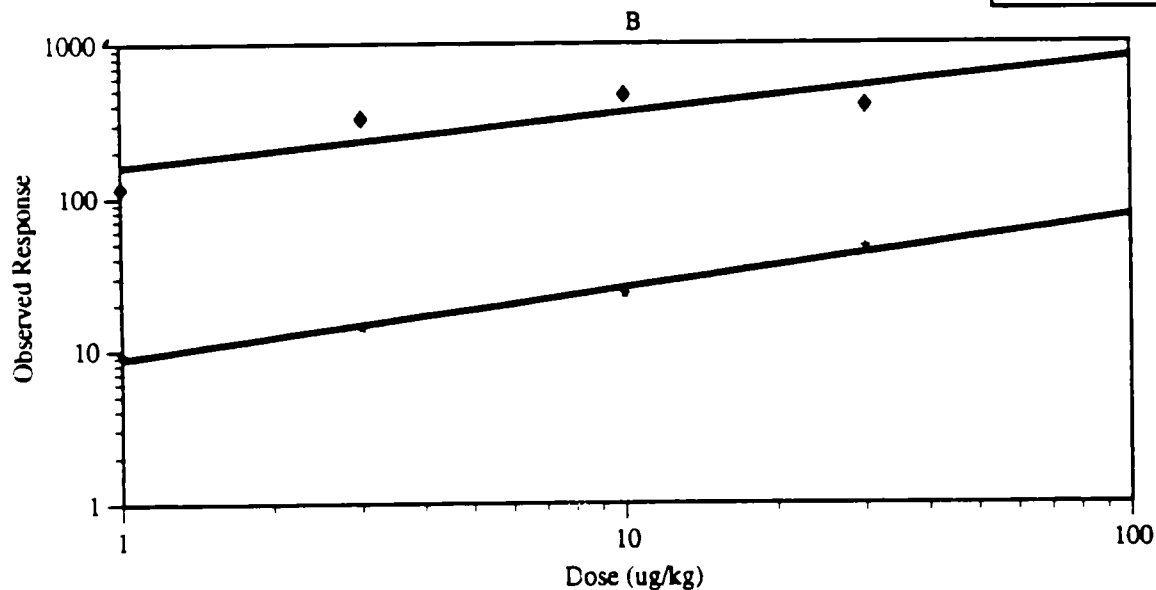
(Best Fit Estimate for "Michaelis-Menten" type response)

Hepatic AHH activity : $f(x) = 4.808068E+2 * x / (1.821770E+0 + x)$

Hepatic ER: $f(x) = 1.7132724E+1 * x / (1.761074E+1 + x)$

• Hepatic ER

◆ Hepatic AHH



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

Hepatic ER: $f(x) = 8.829252E+0 * (x^{4.667998E-1})$, $R^2 = 9.842274E-1$

Hepatic: $f(x) = 1.600130E+2 * (x^{3.533556E-1})$, $R^2 = 6.889952E-1$

GRAPH F

Dose-Response

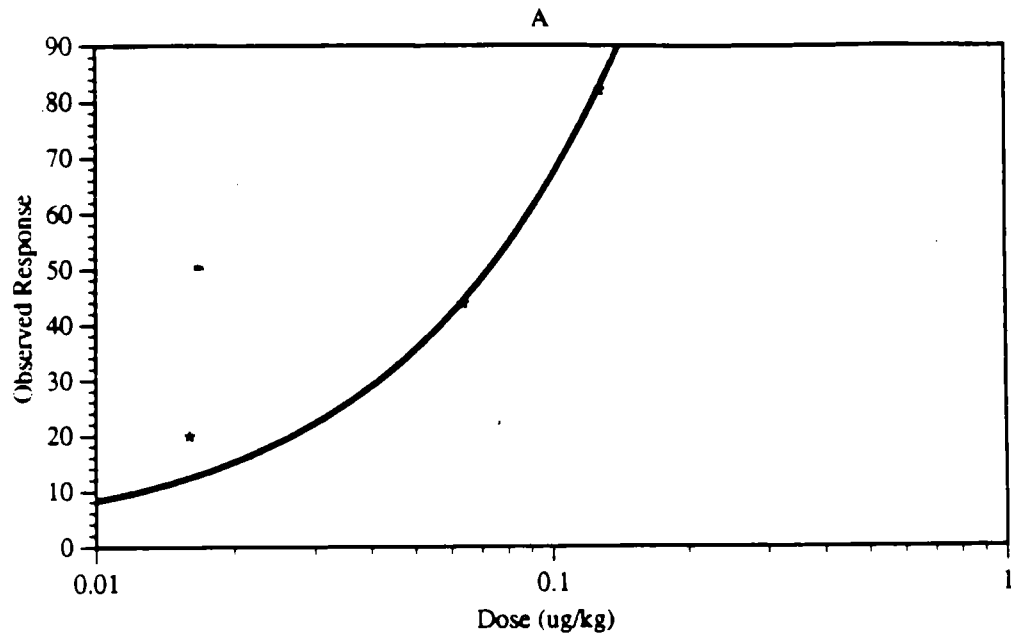
(Graph in Log-Linear Coordinates)

Female Sprague-Dawley Rats

(Astroff et. al, 1991)

Dose: single oral

Response: % decrease uterine c-fos mRNA units

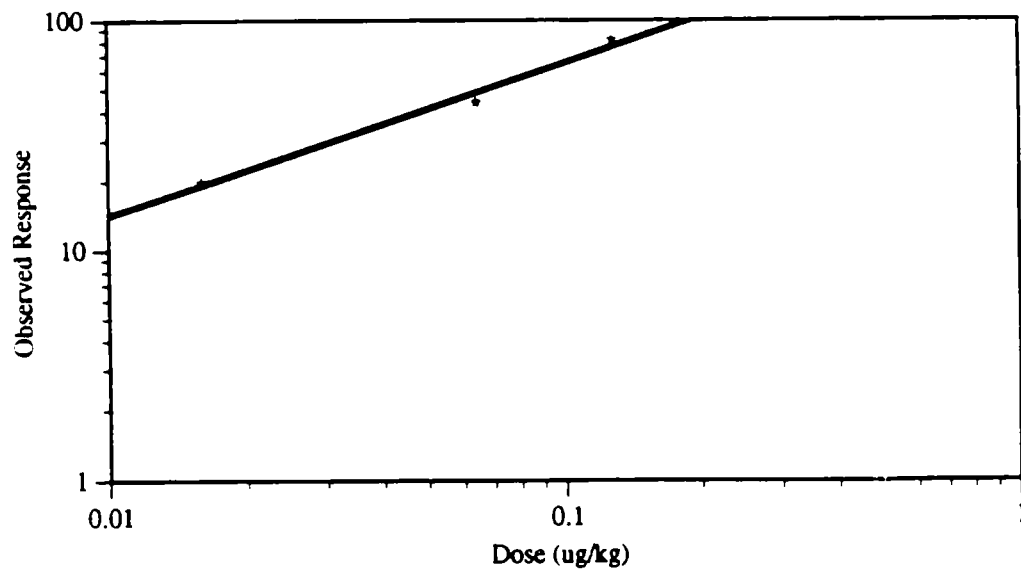


Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "1 + Michaelis-Menten" type response)

$$f(x) = 1 + 6.515702E+2 * x / (8.920061E-1 + x)$$

B

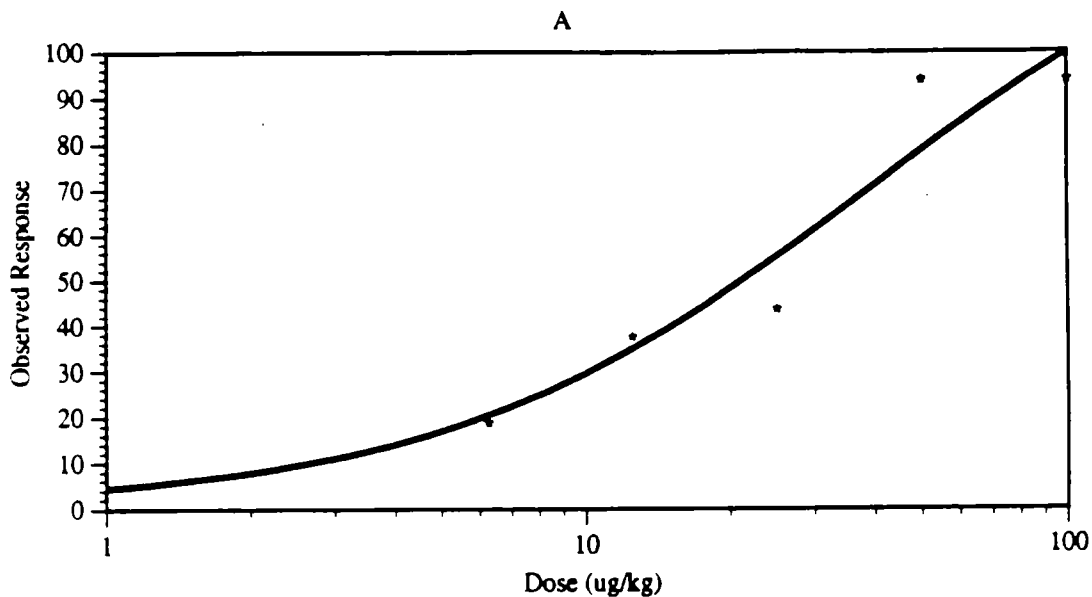


Functional Forms of Dose-Response Curves

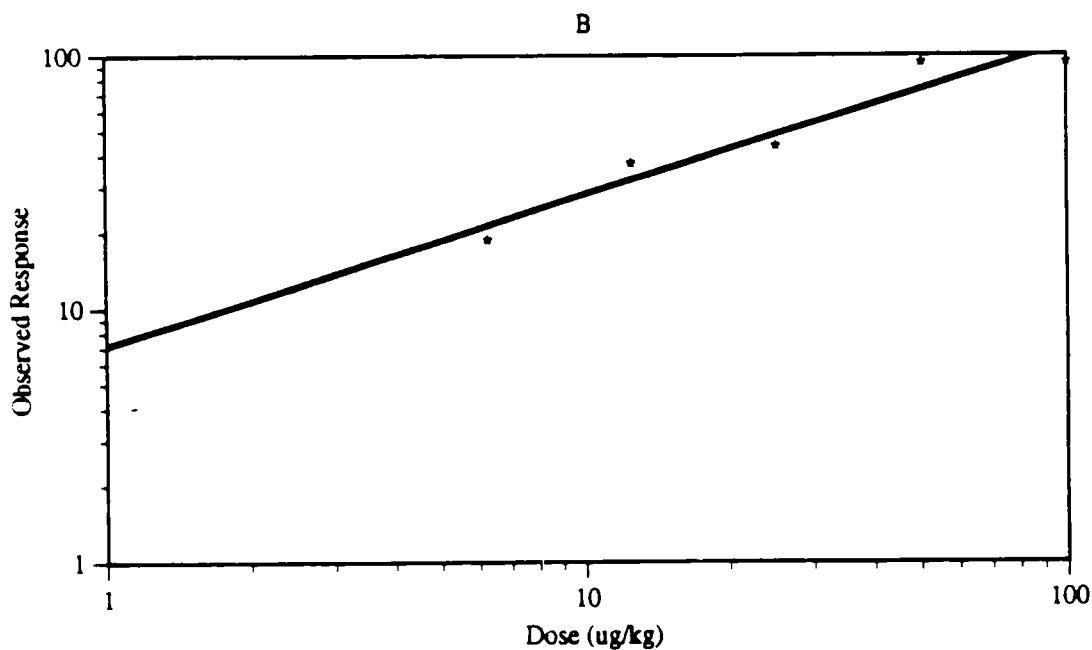
(Best Fit Estimates for Power Laws)

$$f(x) = 3.001080E+2 * (x^{6.628571E-1}), R^2 = 9.851087E-1$$

Graph G
Dose-Response
 (Graph in Log-Linear Coordinates)
 for
 Male Sprague-Dawley Rats
 (Moore et al, 1985)
 Dose: single oral
 Response: % decrease serum testosterone

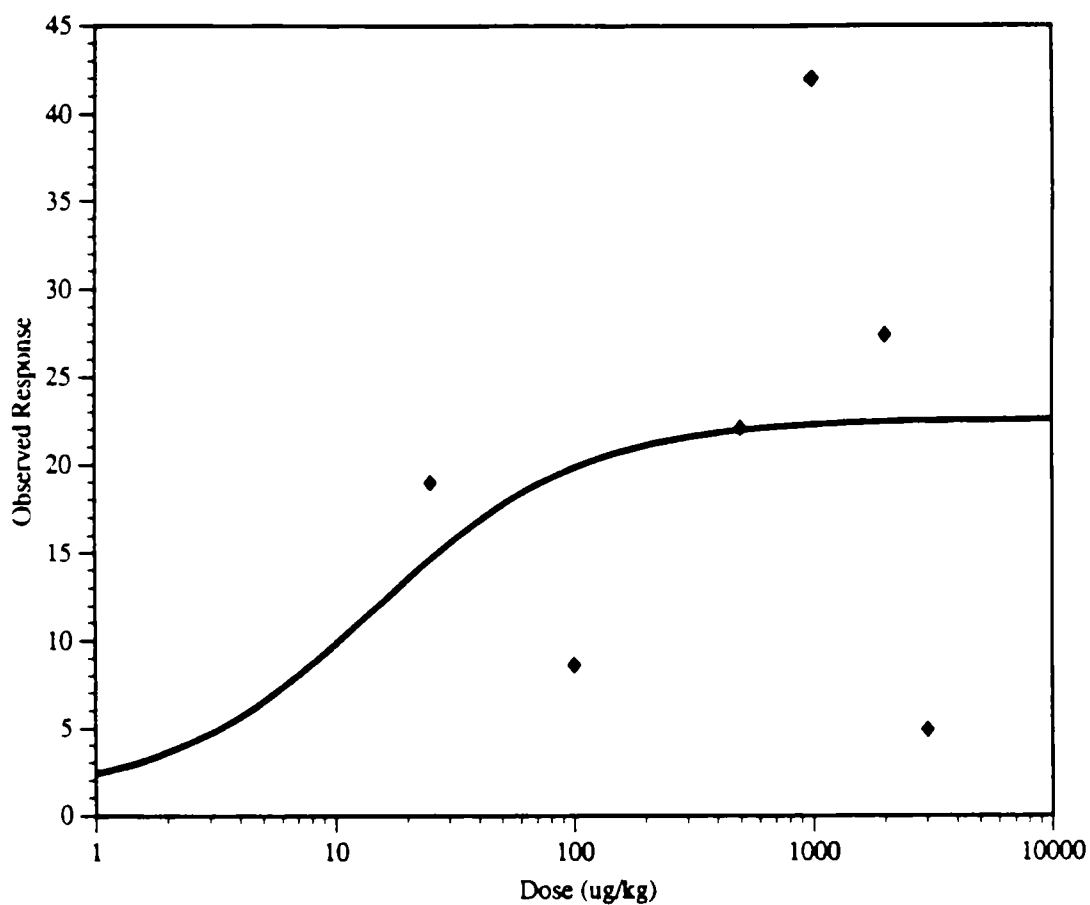


Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for Michaelis-Menten" type response)
 $f(x) = y = 1 + 1.359828E+2 * x / (3.753185E+1 + x)$



Functional Forms of Dose-Response Curves
 (Best Fit Estimates for Power Laws)
 $f(x) = 7.138131E+0 * (x^{5.988408E-1})$, $R^2 = 9.252290E-1$

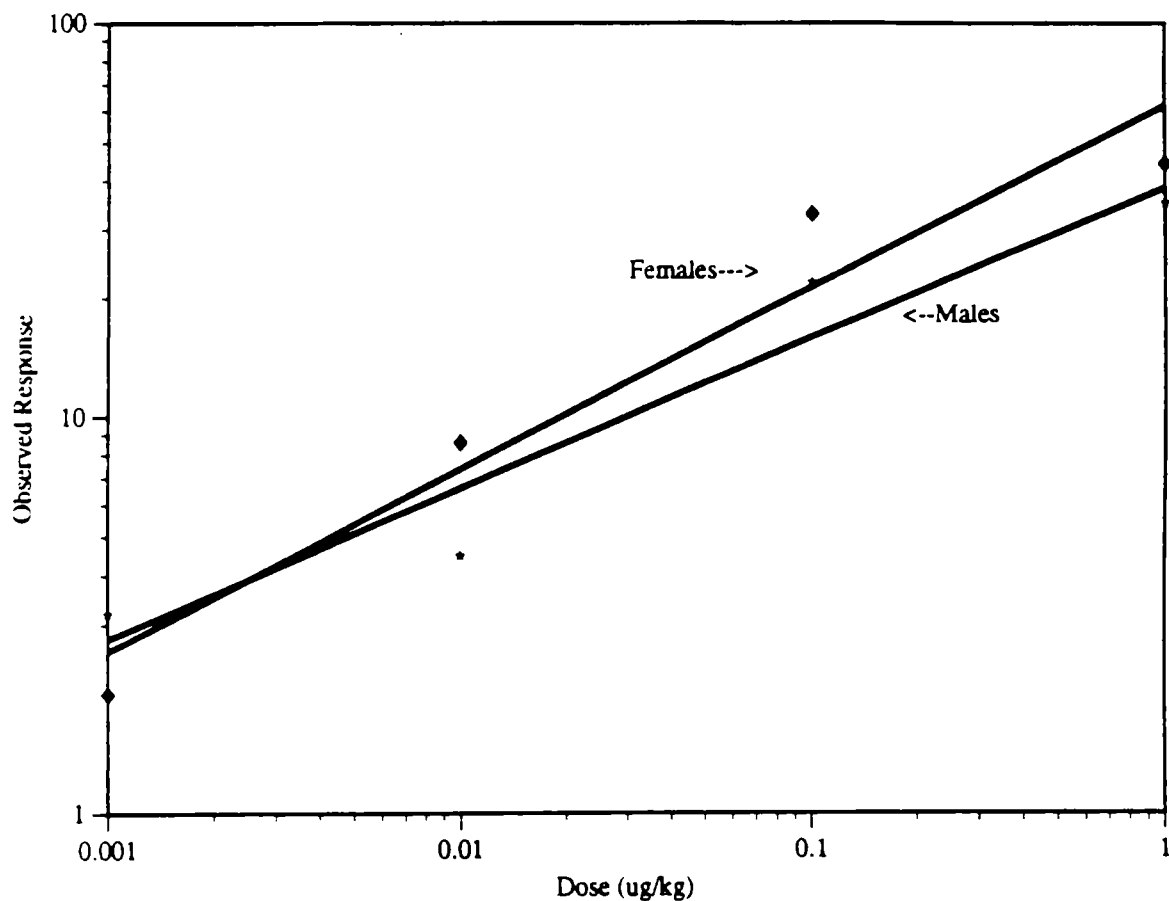
GRAPH H
Dose-Response
 (Graphin Log-Linear Coordinates)
 for
 Female Syrian Hamsters
 (Olsen et al, 1980)
 Dose: single i.p.
 Response: increase liver weight 50 days post dosing



Functional Form of Sigmoid Dose-Response
 Best Fit Estimate for "1 + Michaelis-Menten" type response)

$$f(x) = 1 + 2.156705E+1 * x / (1.447850E+1 + x)$$

GRAPH I
Comparative Dose-Response
 (Graph in Log-Log Coordinates)
 for
 Female and Male Sprague-Dawley Rats
 (Kociba, 1976)
 Dose: p.o. 5x/wk 13 weeks
 Response: increase liver/bwt ratio



Functional Forms of Dose-Response Curves
 (Best Fit Estimates for Power Laws)

Female: $f(x) = 6.181107E+1 * (x^{4.611284E-1})$, $R^2 = 9.357175E-1$
 Male: $f(x) = 3.820392E+1 * (x^{3.805964E-1})$, $R^2 = 9.310575E-1$

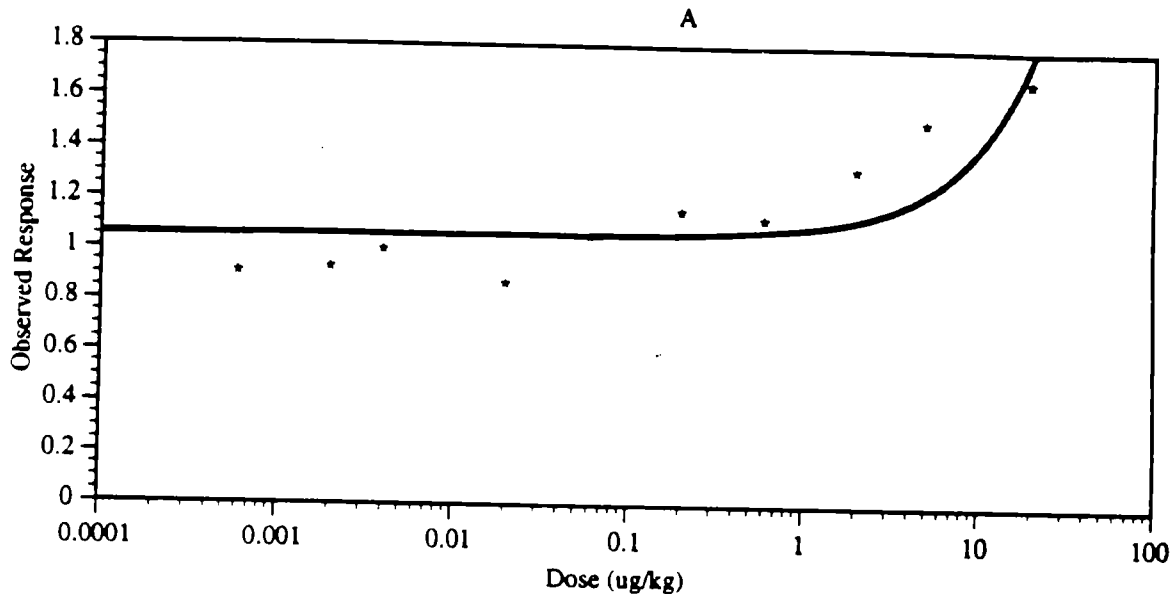
• males
 ♦ females

Graph J1
Dose-Response
(Graph in Log-Linear Coordinates)
for

Female Sprague-Dawley Rats
(Kitchin and Woods, 1979)

Dose: single oral

Response: raw data change in P-450 at 3 days

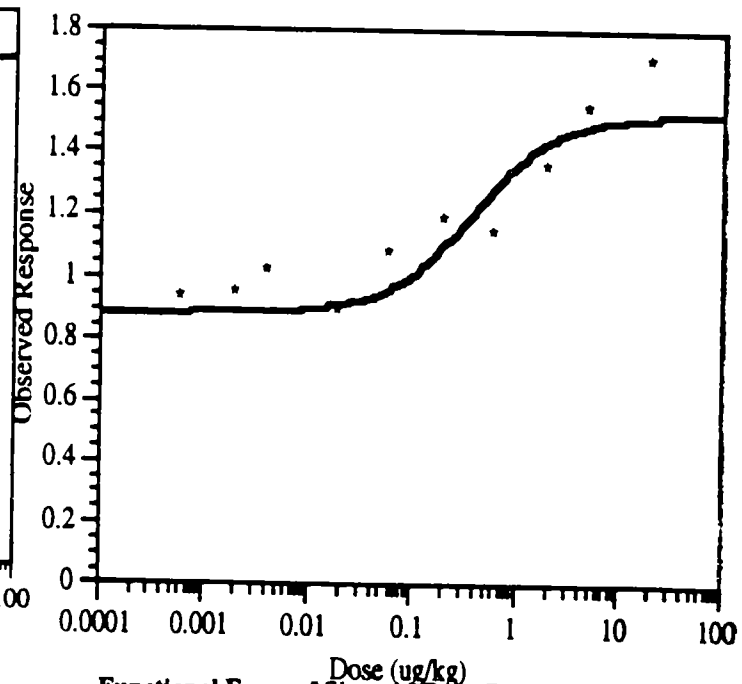
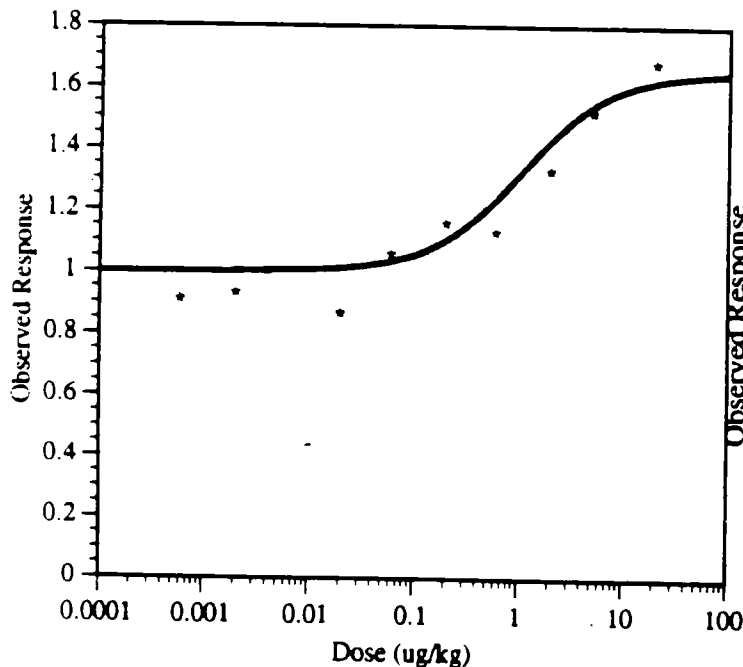


Functional form Linear Dose-Response Best Fit

$$f(x) = 3.565270E-2 * x + 1.05977E+0, R^2 = 6.72222497E-1$$

B

C



"Functional Form of Sigmoid Dose-Response

Best fit for Michaelis-Menten + background parameter response)

$$f(x) = y = 0.88 + 7.812964E-1 * x / (1.022375E+0 + x)$$

Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Hill function plot

$$f(x) = (6.466629E-1 * x^{1.08926E+0}) / (3.584389E1 + x^{1.089267E+0}) + 0.88; \text{ chi square} = 9.346E-2$$

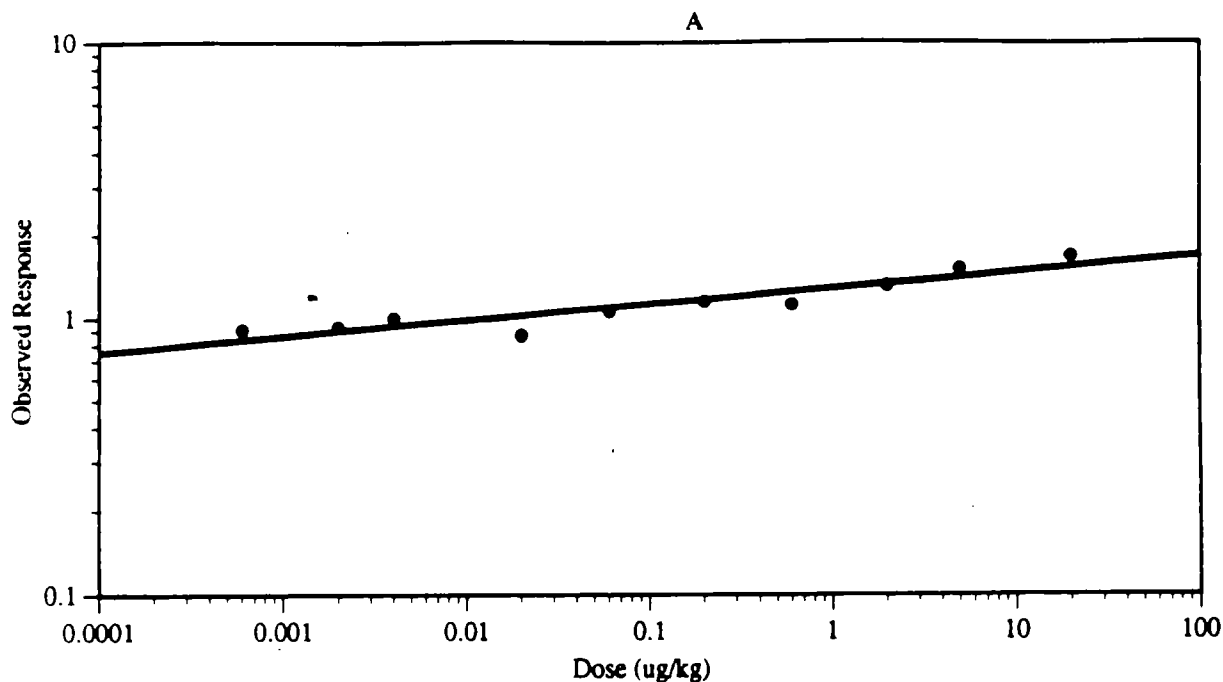
GRAPH J2

Comparative Dose-Response

(Graph in Log-Linear Coordinates) for
Female Sprague-Dawley Rats (Kitchin and Woods, 1979)

Dose: single oral

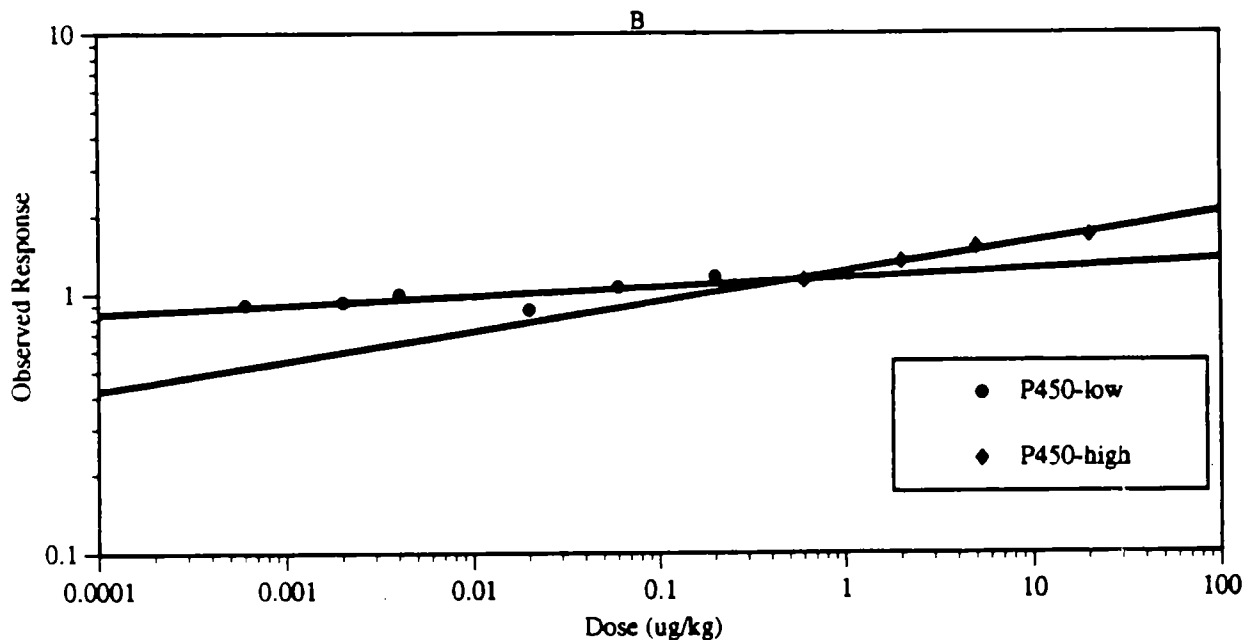
Response: raw data P450 (3 days)



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

complete data set $f(x) = 1.293664E+0 * (x^{5.855594E-2})$, $R^2 = 8.538984E-1$



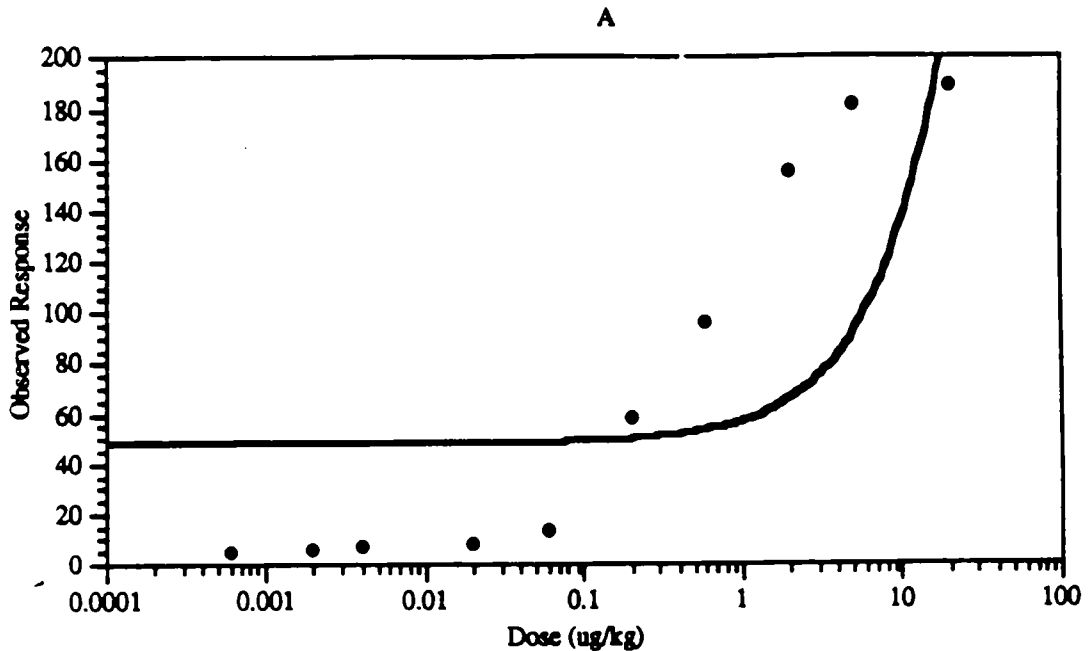
Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

(.0006 to .6 ug/kg) $f(x) = 1.152077E+0 * (x^{3.457812E-1})$, $R^2 = 6.187106E-1$

(0.6 to 20 ug/kg) $f(x) = 1.220259E+0 * (x^{1.146001E-1})$, $R^2 = 9.754329E-1$

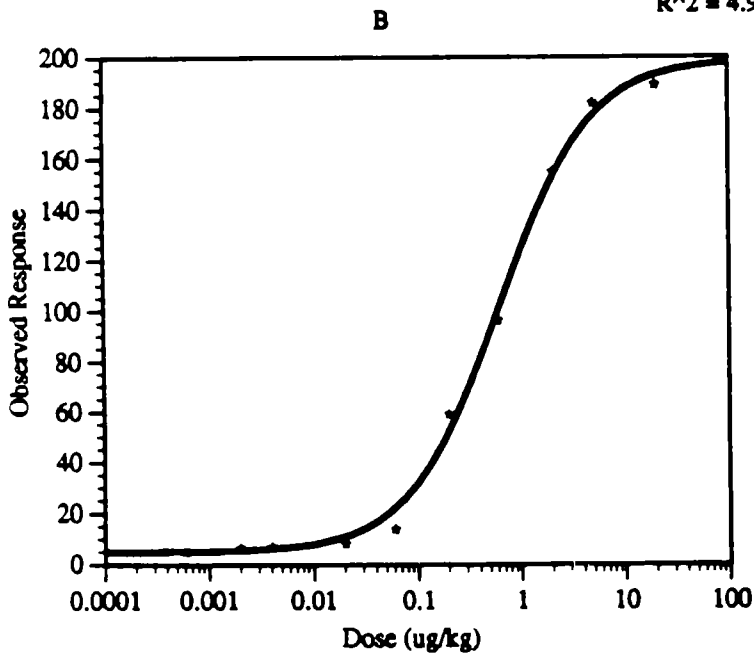
Graph K1
Dose-Response
 (Graph in Log-Linear Coordinates)
 Female Sprague-Dawley Rats
 (Kitchin, 1979)
 Dose: single oral
 Response: increase Benzo(a) pyrene hydroxylase raw data



Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for "Linear Model" type response)

$$f(x) = 8.692922E+0 \cdot x + 4.796840E+1$$

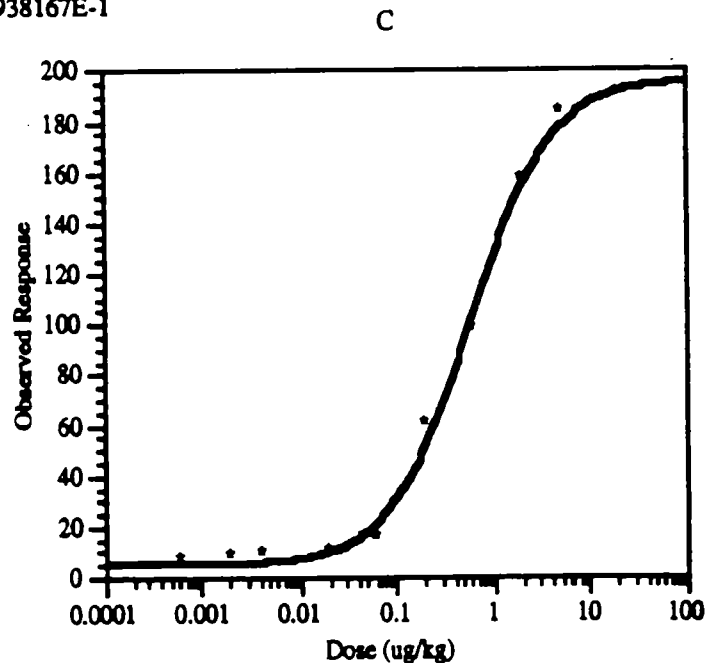
$$R^2 = 4.938167E-1$$



Functional Form of Sigmoid Dose-Response
 (Best Estimate for "Michaelis-Menten + background parameter")

$$f(x) = y = 4.9 + 1.939516E+2 \cdot x / (6.164672E-1 + x)$$

$$\chi^2 = 197.2$$



Functional Form of Sigmoid Dose-Response
 (Best for "Hill Function" + background parameter)

$$f(x) = 1.090917E+2 \cdot x^{1.066342E+0} / 5.697987E-1 + x^{1.066342E+0}$$

$$\chi^2 = 163$$

K2

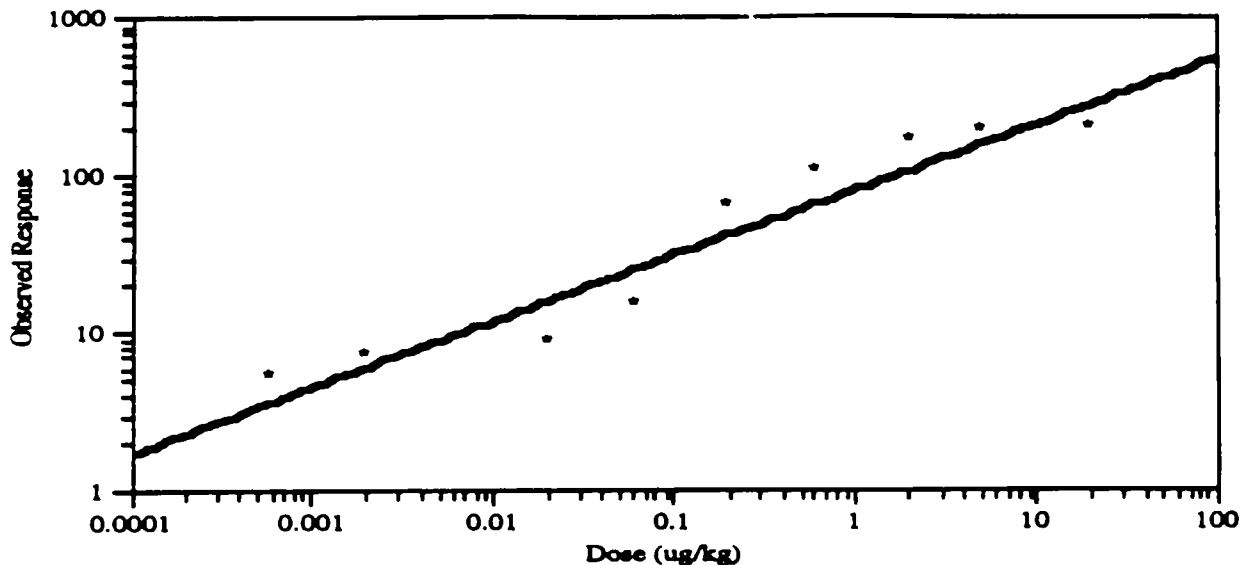
Comparative Dose-Response
(Graph in Log-Log Coordinates)

Female Sprague-Dawley Rats
(Kitchin and Woods, 1979)

Dose: single oral

Response: increase Benzo(a) pyrene hydroxylase

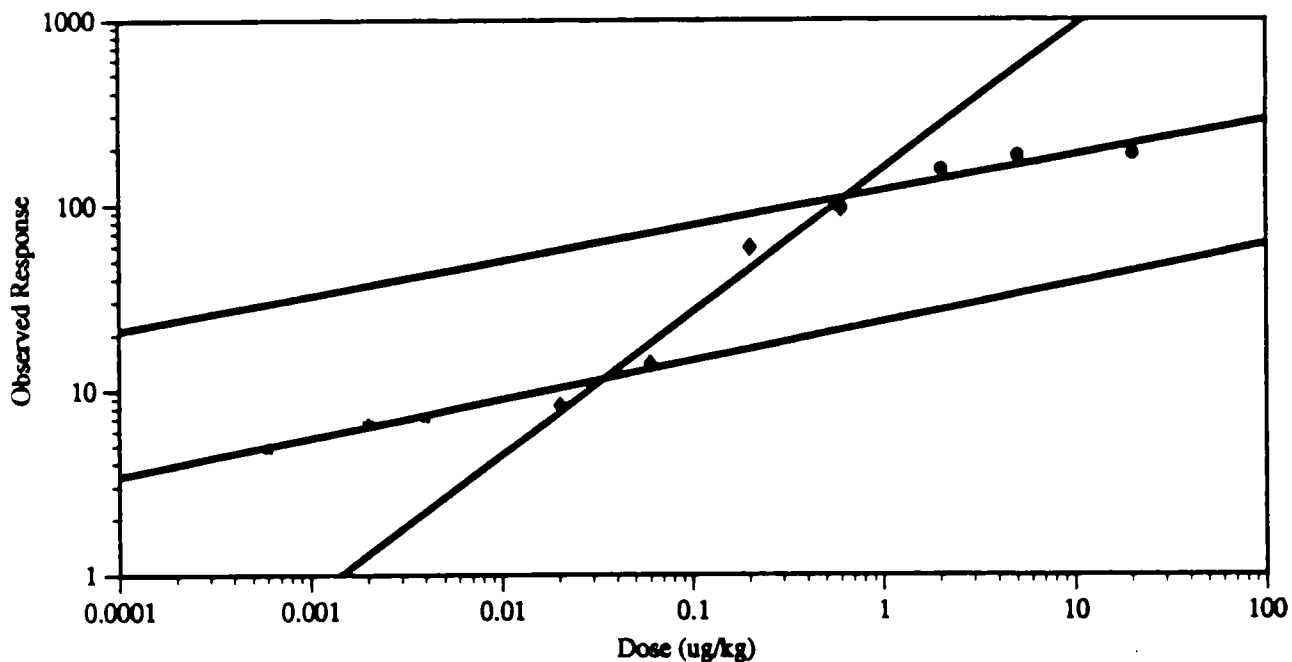
A



Functional Form of Dose-Response

(Best Fit Estimate for Power Law)

$$f(x) = 8.044850E+1 * (x^{4.195383E-1}), R^2 = 9.310005E-1$$



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

$$(x = 0.0006 \text{ to } 0.004) f(x) = 2.351223E+1 * (x^{2.091992E-1}), R^2 = 9.633734E-1$$

$$(x = 0.02 \text{ to } 0.6) f(x) = 1.572746E+2 * (x^{7.726666E-1}), R^2 = 9.638735E-1$$

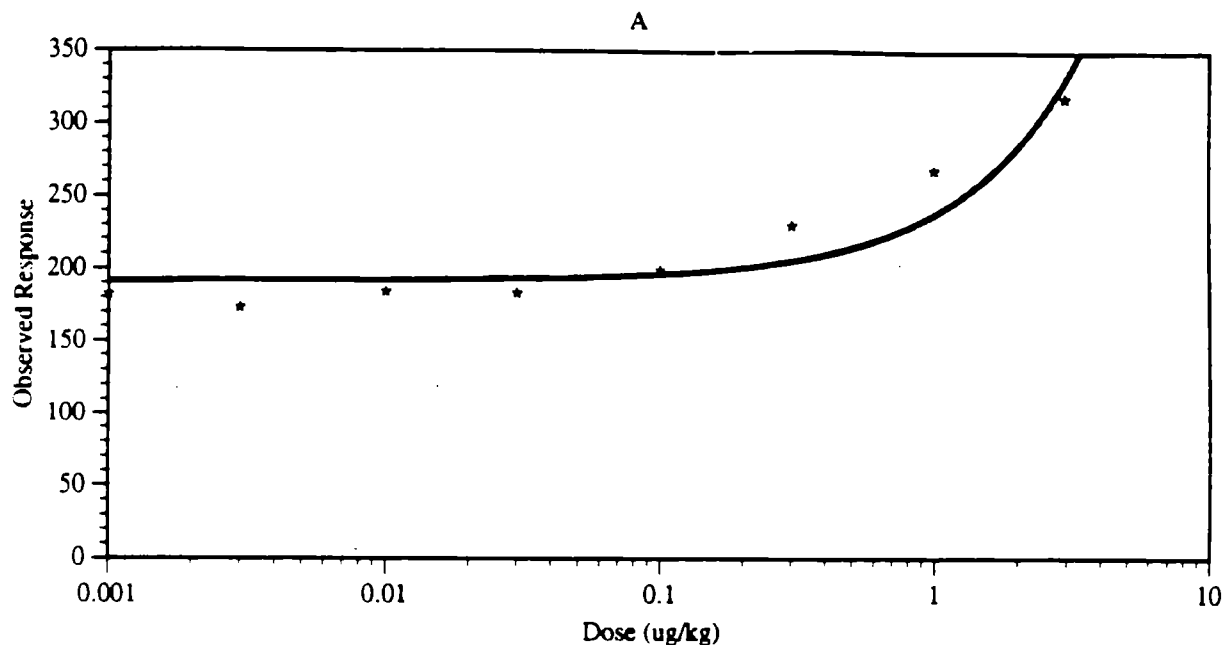
$$(x = 0.6 \text{ to } 20) f(x) = 1.2000001E+2 * (x^{1.887329E-1}), R^2 = 8.048891E-1$$

* BaP hydroxylase -Low

◆ BaP hydroxylase Mid

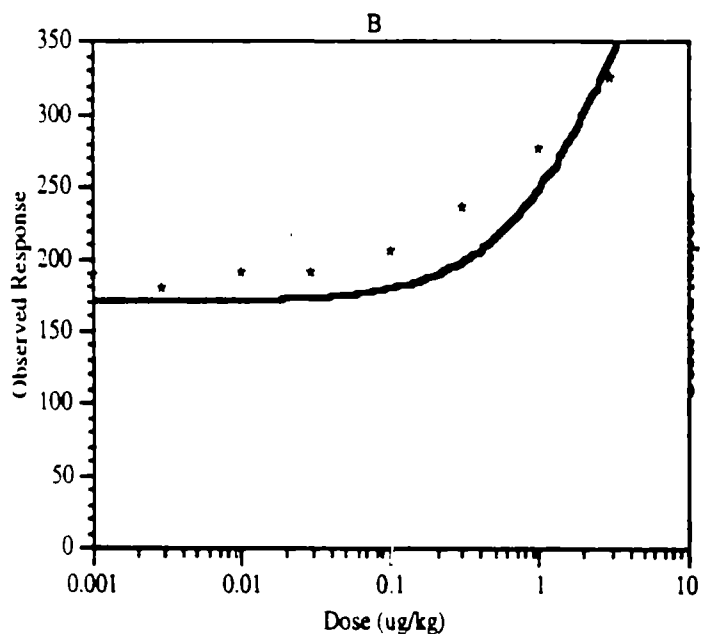
● BaP hydroxylase -High

Graph L1
Dose-Response
 (Graph in Log-Linear Coordinates)
 Female Wistar Rats
 (Abraham, 1980)
 Dose: single sc
 Response raw data P450 at 7 days



Functional Form of Dose-Response
 (Best Fit Estimate for Linear Model)

$$f(x) = 4.676353E-1 * x + 1.913979E+2, R^2 = 8.781851E-1$$

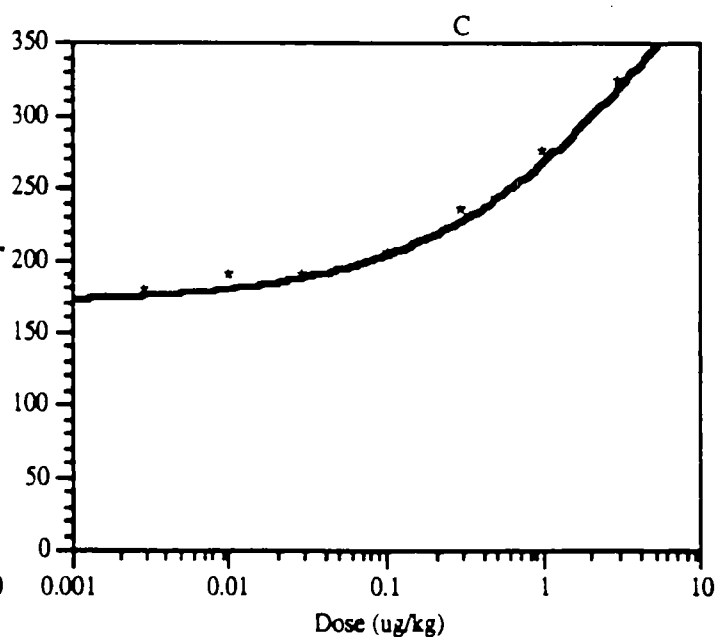


Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for " Michaelis-Menten + background parameter"

$$f(x) = y = 169 + 3.876606E+2 * x / (3.886855E+0 + x)$$

$$\text{Chi}^2 = 2769$$



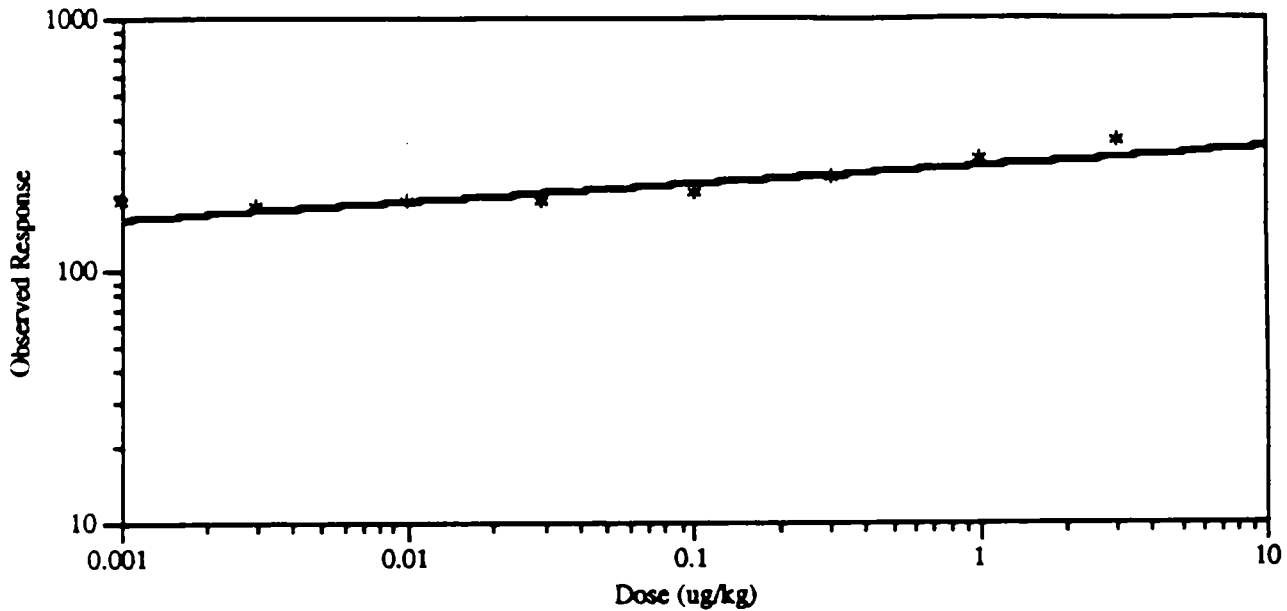
Functional Form of Dose-Response

(Best Fit Estimate for Hill function + background parameter)

$$f(x) = 3.994308E+2 * x^{5.478666E-1} / (3.021723E+0 + x^{5.478666E-1}) + 169, \text{Chi}^2 = 169$$

Graph L2
Dose-Response
(Graph in Log-LogCoordinates)
Female Wistar Rats
(Abraham et al., 1980)
Dose: single sc
Response: raw data: P450

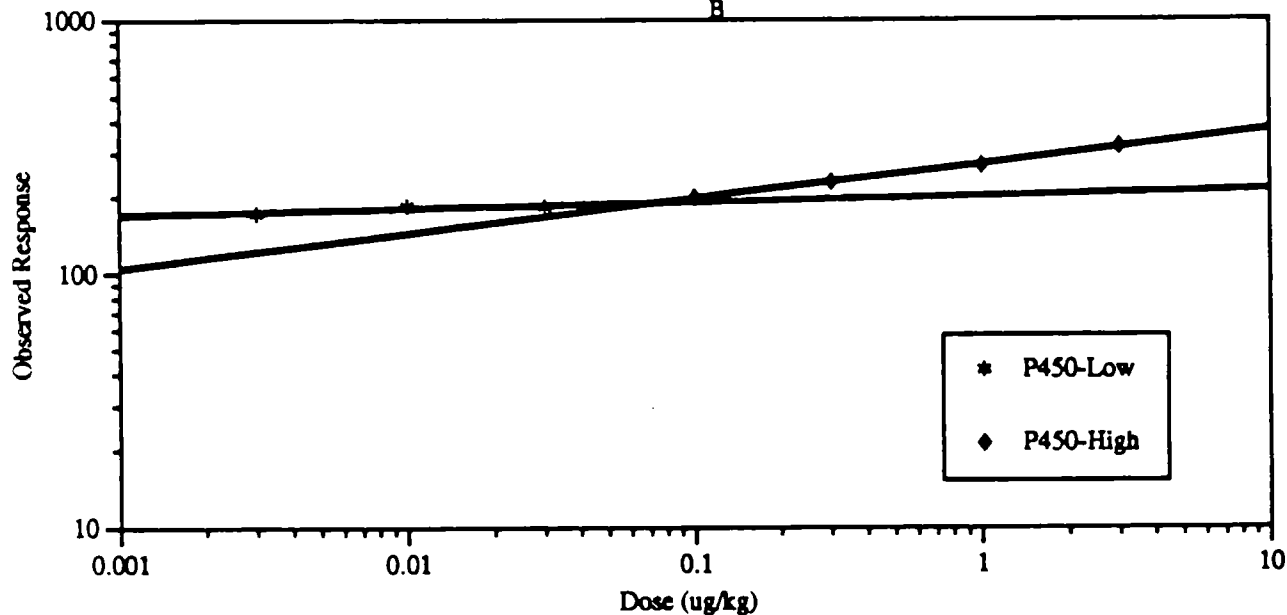
A



Functional Form of Dose-Response
(Best Fit Estimate for Power Law)

$$f(x) = 2.615298E+2 * (x^{7.141240E-2}), R^2 = 8.300624E-1$$

B

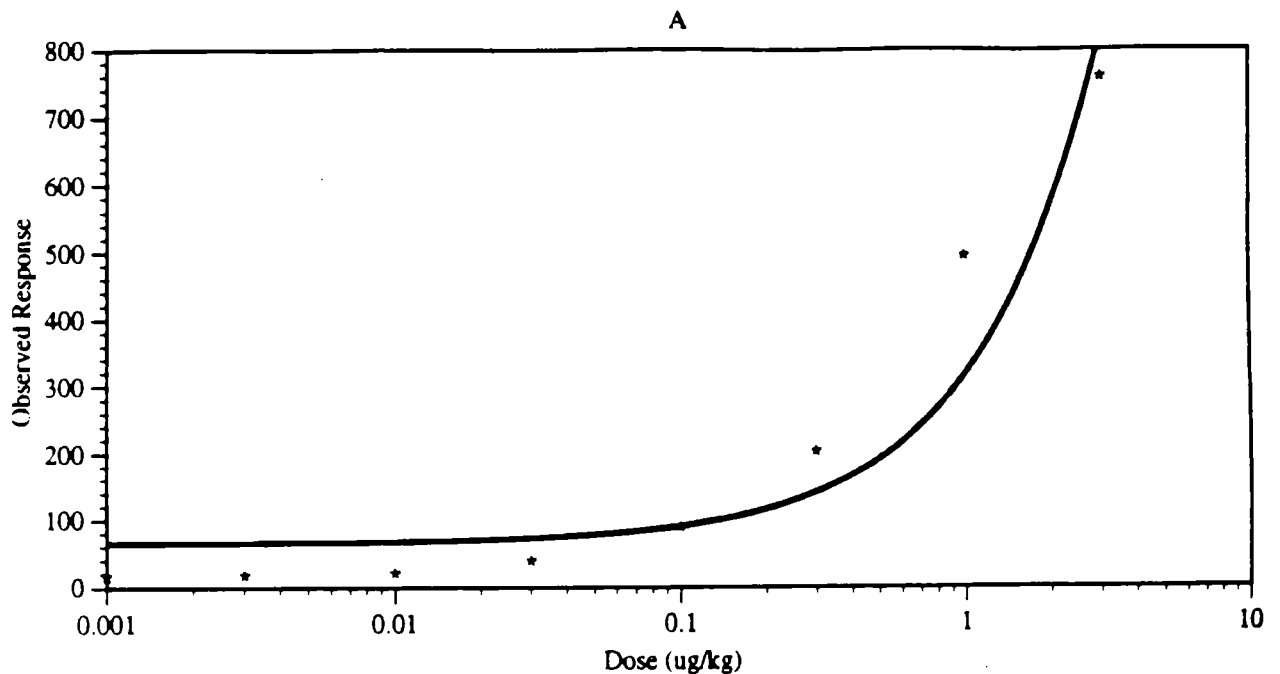


Functional Form of Dose-Response
(Best Estimate for Power Law)

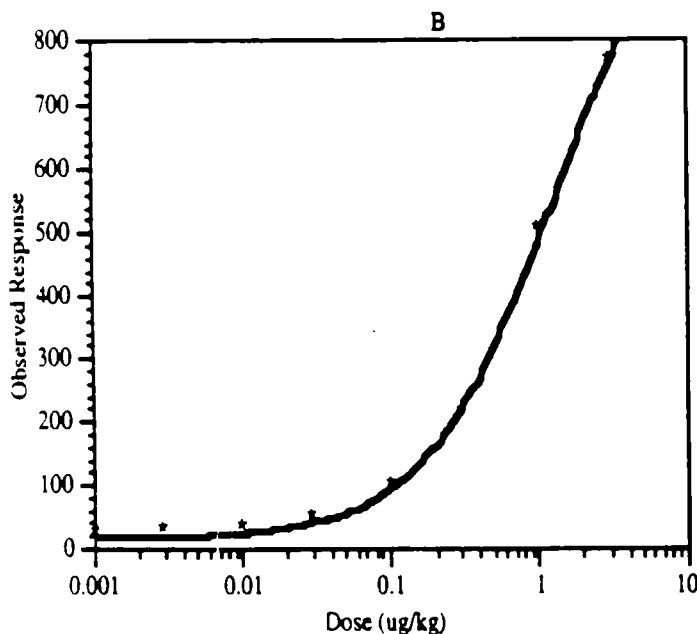
$$\text{P450-Low } f(x) = 2.134553E+2 * (x^{3.591655E-2}), R^2 = 8.762258E-1$$

$$\text{P450-High } f(x) = 2.719892E+2 * x^{1.377786E-1}, R^2 = 9.982764E-1$$

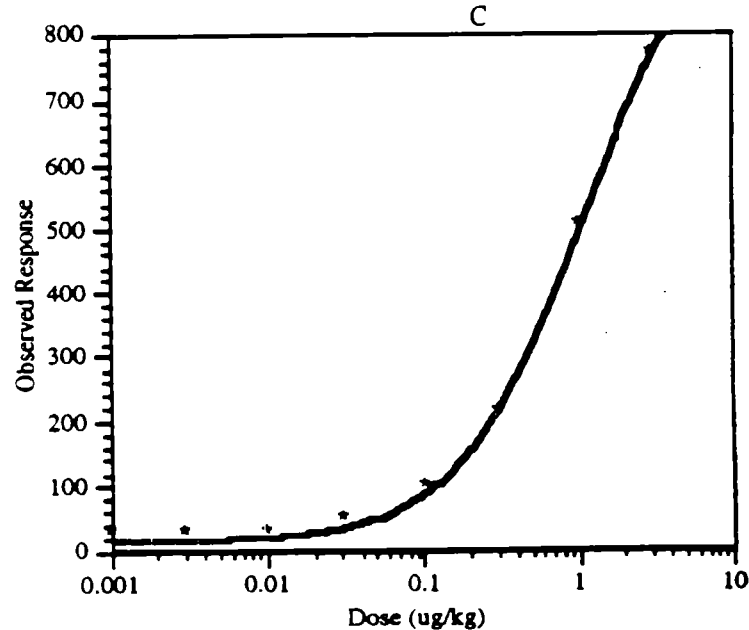
Graph M1
Dose-Response
(Graph in Log-Linear Coordinates)
Female Wistar Rats
(Abraham et al., 1980)
Dose: single sc
Response: raw data: EROD - 7 days



Functional Form of Sigmoid Dose-Response
(Best Fit Estimate for "Linear "type response)
 $f(x) = 2.527667E+2 \cdot x + 6.538808E+1$, $R^2 = 0.911$



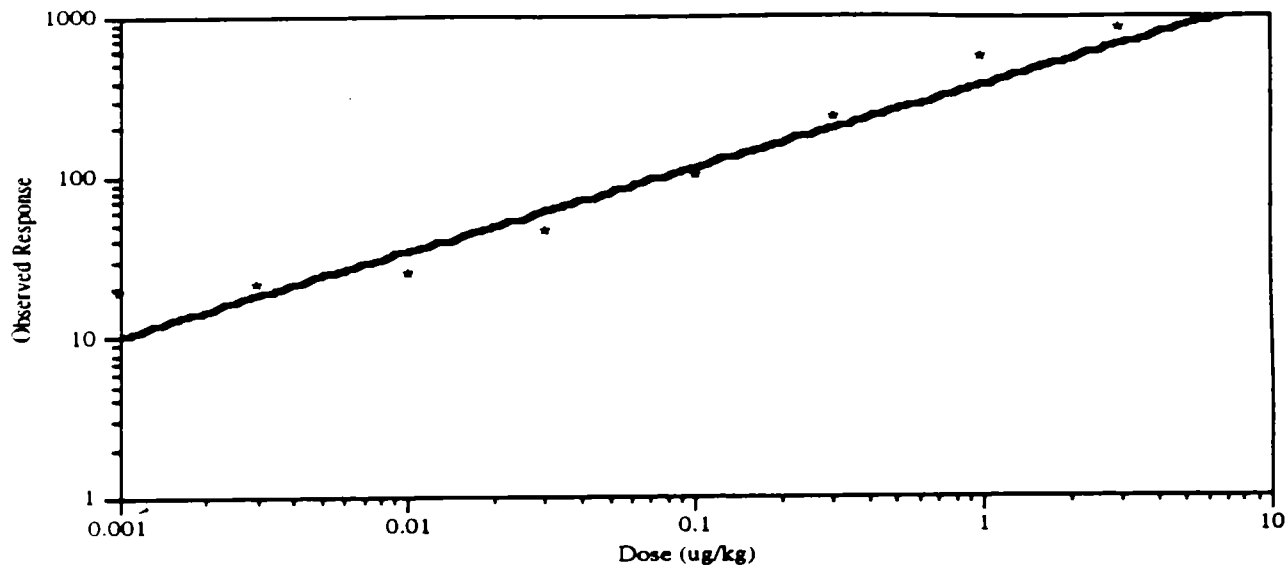
Functional Form of Sigmoid Dose-Response
(Best Fit Estimate for "Michaelis-Menten" + background parameter"
type response)
 $f(x) = 14.3 + 1.066009E+3 \cdot x / (1.273480E+0 + x)$, $\text{Chi}^2 = 343$



Functional Form of Sigmoid Dose-Response
(Best Fit Estimate for "Hill plot with background parameter")
 $f(x) = (9.877489E+2 \cdot x^{1.088899E+0} / (1.072719E+0 + x^{1.088899E+0})) + 14.3$, $\text{Chi}^2 = 176$

Graph M2
Comparative Dose-Response
Dose-Response
 (Graph in Log-Log Coordinates)
 Female Sprague-Dawley Rats
 Abraham et al., 1988
 Dose: single sc
 Response: raw data EROD (7 days)

A

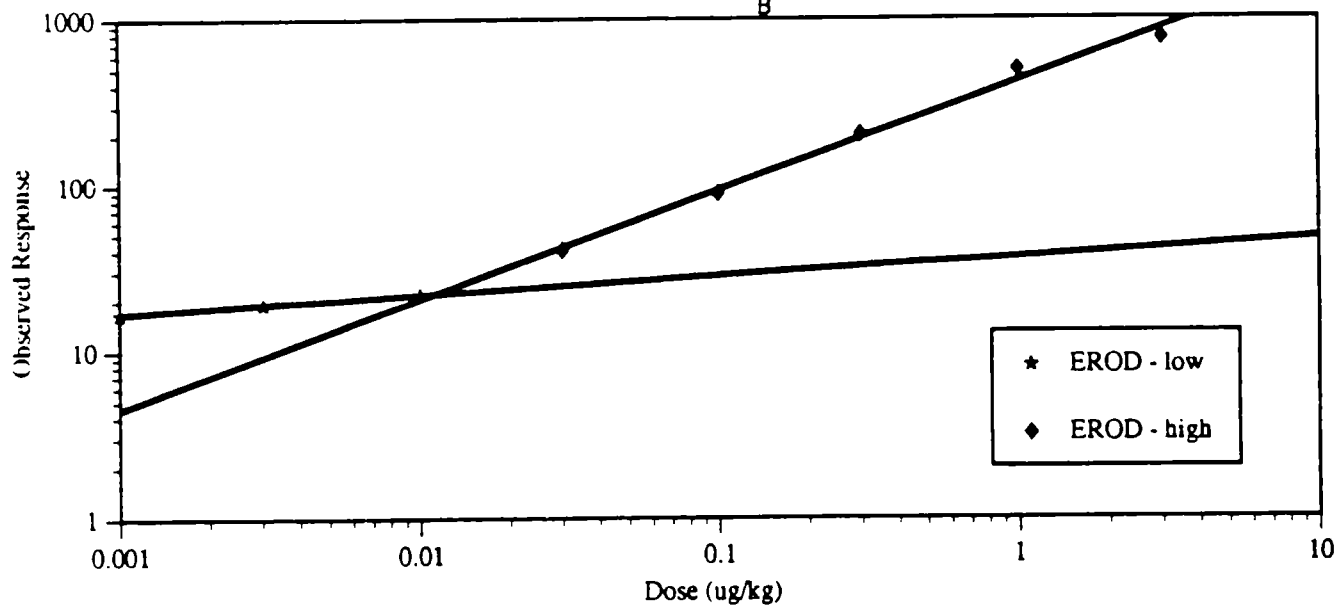


Functional Form of Dose-Response

(Best Fit Estimate for Power Law)

$$f(x) = 3.724271E+2 * (x^{5.226669E-1}), R^2 = 9.528637E-1$$

B



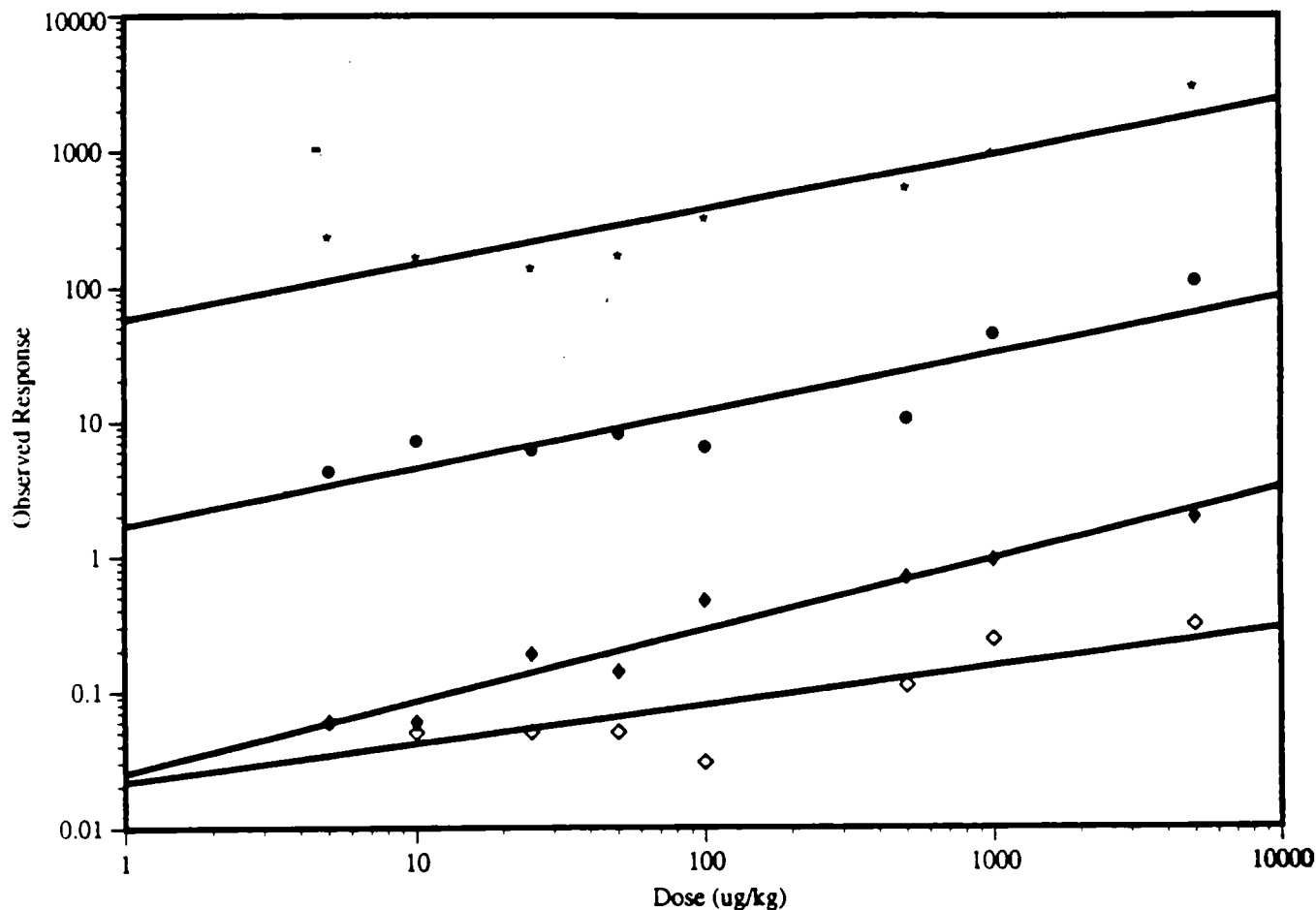
Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

$$\text{EROD - low } f(x) = 3.671388E+1 * (x^{1.126439E-1}), R^2 = 9.991464E-1$$

$$\text{EROD - high } f(x) = 4.236494E+2 * (x^{6.572701E-1}), R^2 = 9.912442E-1$$

GRAPH N.
Comparativ Dose-Response
 (Graph in Log-Log Coordinates)
 for
 Female B6C3F1 Mice
 (Safe 1993)
 Dose: single i.p. injection
 Response: 3 days post dosing increase EROD, Cyp1A1, Cyp1A2, Total nuclear Ah Receptor Binding



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

$$\text{EROD } f(x) = 5.810675E+1 * (x^{4.071389E-1}), R^2 = 8.276920E-1$$

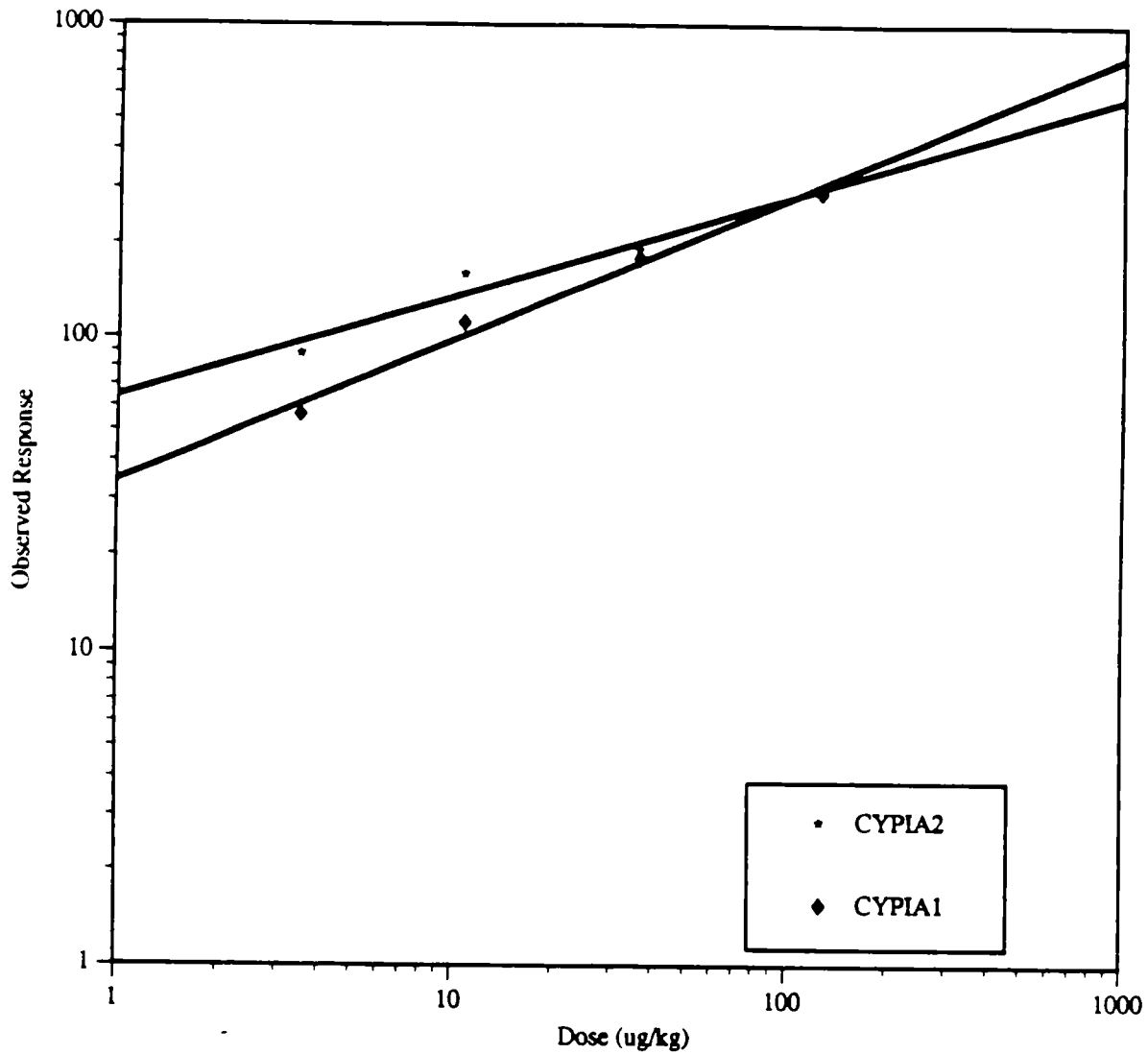
$$\text{Cyp1A1 } f(x) = 2.529055E-2 * (x^{5.273765E-1}), R^2 = 9.472254E-1$$

$$\text{Total Binding } f(x) = 1.694760E+0 * (x^{4.264534E-1}), R^2 = 8.042876E-1$$

$$\text{Cyp1A2 } f(x) = 2.169244E-2 * (x^{2.824955E-1}), R^2 = 6.557616E-1$$

- EROD pmol/mg/min
- ◆ Cyp1A1 mRNA
- total receptor binding
- ◇ Cyp1A2

GRAPH O
Comparative Dose-Response
 (Graph in Log-Log Coordinates)
 Female Sprague-Dawley Rats
 (Trischer et al., 1992)
 Dose: p.o. biweekly for 30 weeks
 Response: increase enzyme activity



Functional Forms of Dose-Response Curves
 (Best Fit Estimates for Power Laws)

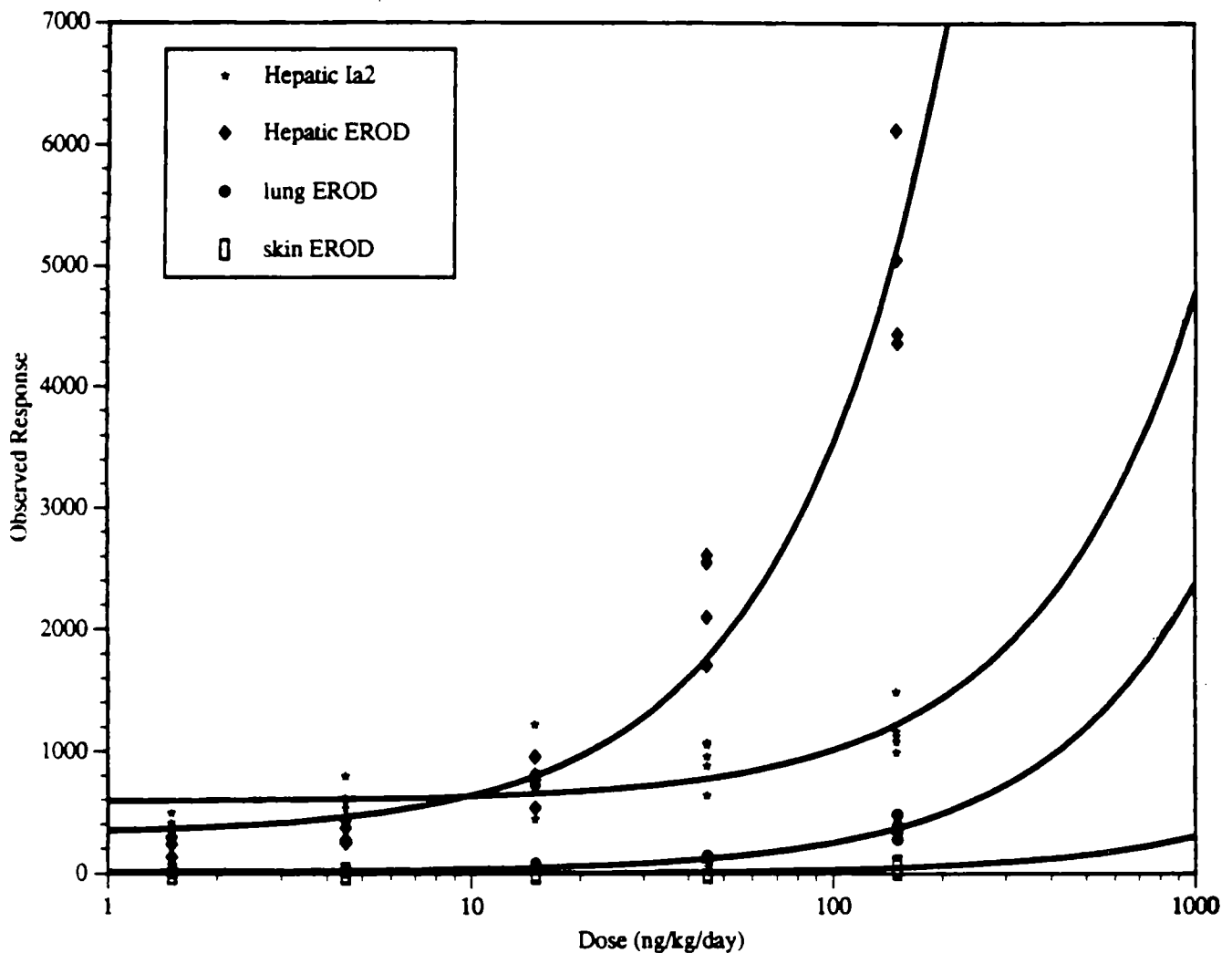
Cyp1A1 $f(x) = 6.47089E+1 * (x^{3.193647E-1})$, $R^2 = 9.546921E-1$

Cyp1A2 $f(x) = 3.453639E+1 * (x^{4.540268E-1})$, $R^2 = 9.869648E-1$

GRAPH P1
Comparative Dose-Response
 (Graph in Log-Linear Coordinates)

for
 Female B6C3F1 Mice
 (DeVito et al., 1992a)

Response: 13 week dosing 5 x per week % increase Hepatic EROD, Hepatic 1a2,
 Skin and Lung EROD



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Linear Function)

Hepatic EROD: $f(x) = 4.188324E+0 \cdot x + 5.909044E+2$, $R^2 = 5.364690E-1$

Hepatic 1a2: $f(x) = 3.2.205153E+1 \cdot x + 3.213650E+2$, $R^2 = 9.458424E-1$

skin EROD: $f(x) = 2.996198E-1 \cdot x + 2.254425E+0$, $R^2 = 9.113437E-1$

lung EROD: $f(x) = 2.360102E+0 \cdot x + 1.561678E+1$, $R^2 = 9.399031E-1$

Graph P2

Dose-Response

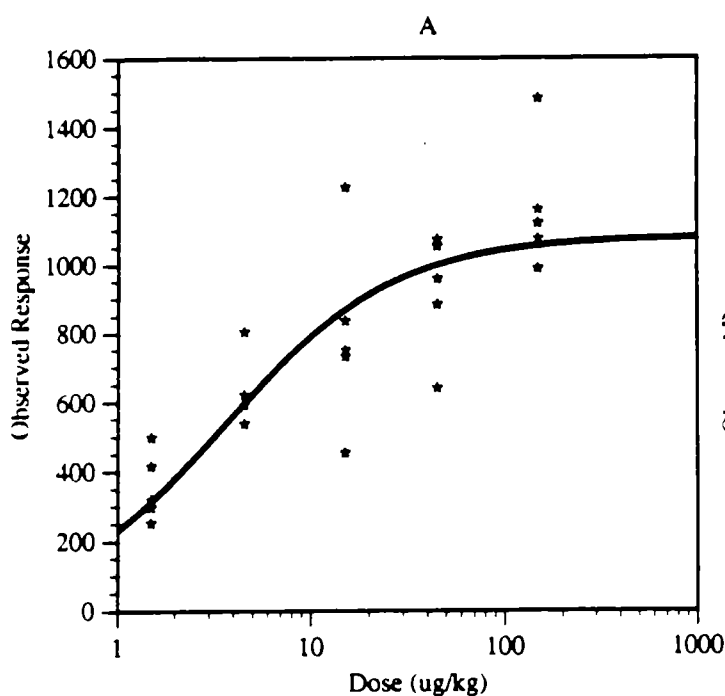
(Graph in Log-Log Coordinates)

Female B6C3F1 mice

(DeVito et al, 1992a)

Dose: 5 X weekly 13 weeks

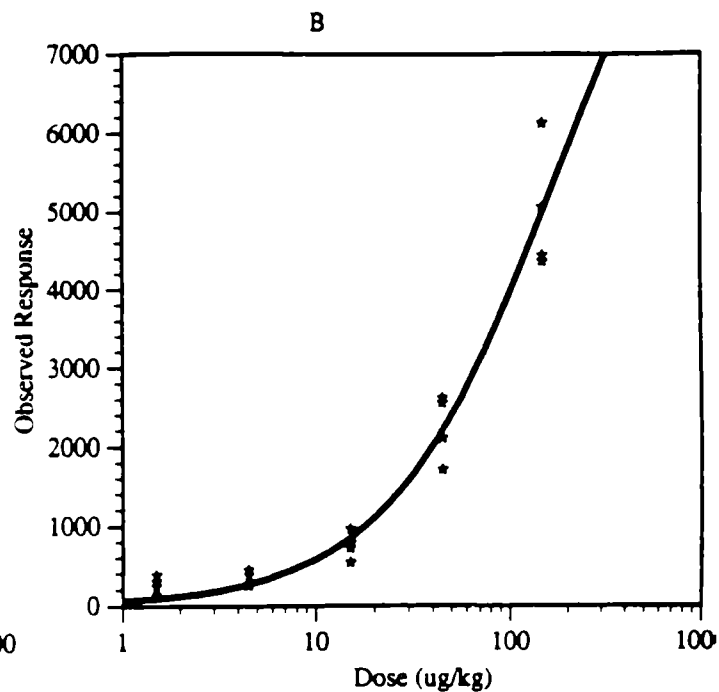
Response: % increase Cyp1A2 level, Hepatic EROD, Lung EROD and Skin EROD



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Michaelis-Menten" type response)

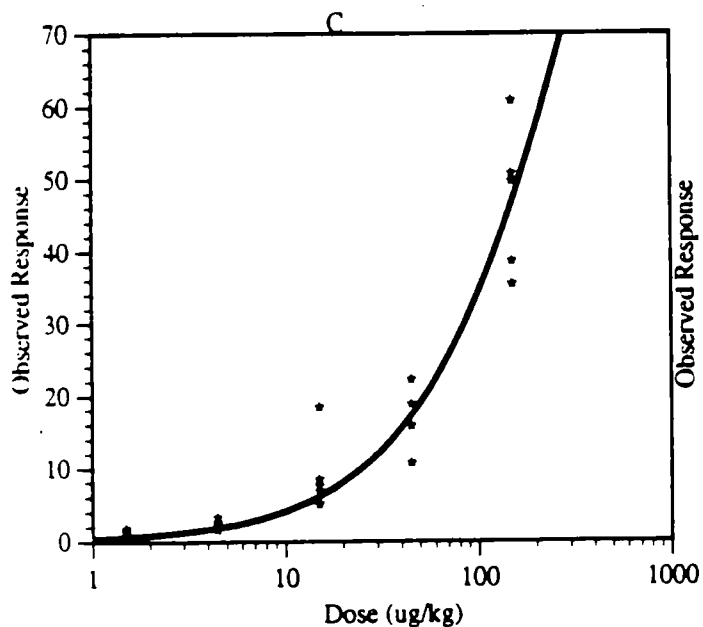
Hepatic 1A2 $f(x) = 1 + 1.080720E+3 * x / (3.669929E+0 + x)$



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Michaelis-Menten" type response)

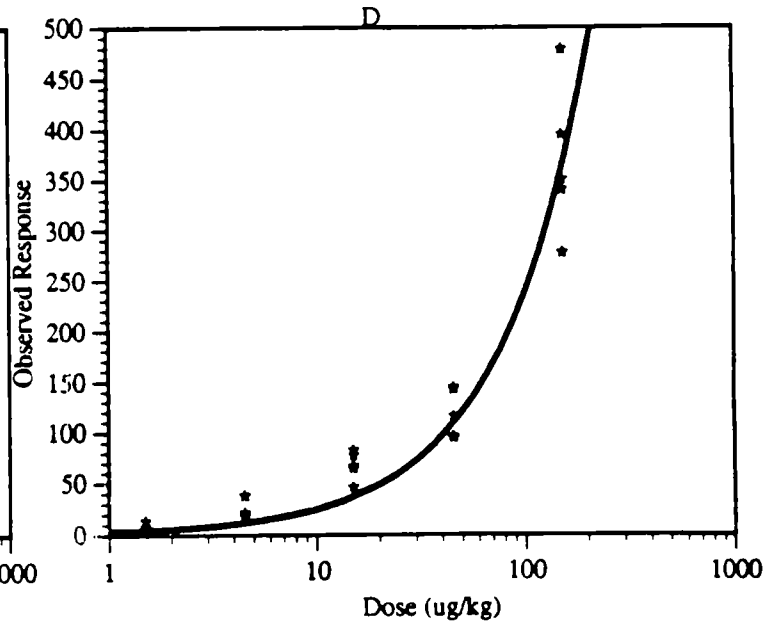
Hepatic EROD $f(x) = 1.088571E+4 * x / (1.766552E+2 + x)$



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Michaelis-Menten" type response)

Skin EROD $f(x) = 1.734444E+2 * x / (4.054377E+2 + x)$



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Michaelis-Menten" type response)

Lung EROD $f(x) = 1 + 2.521625E+9 * x / (-1.0431179E+9 + x)$

Graph P3

Dose-Response

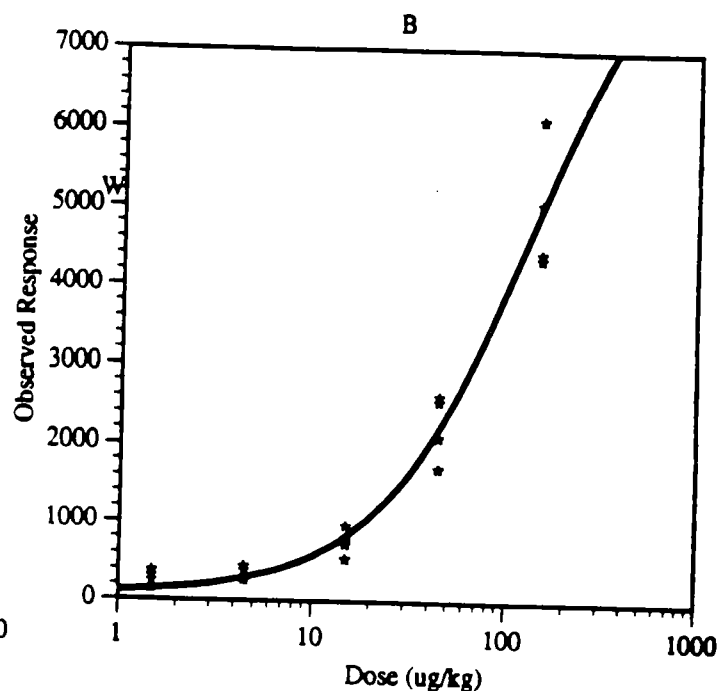
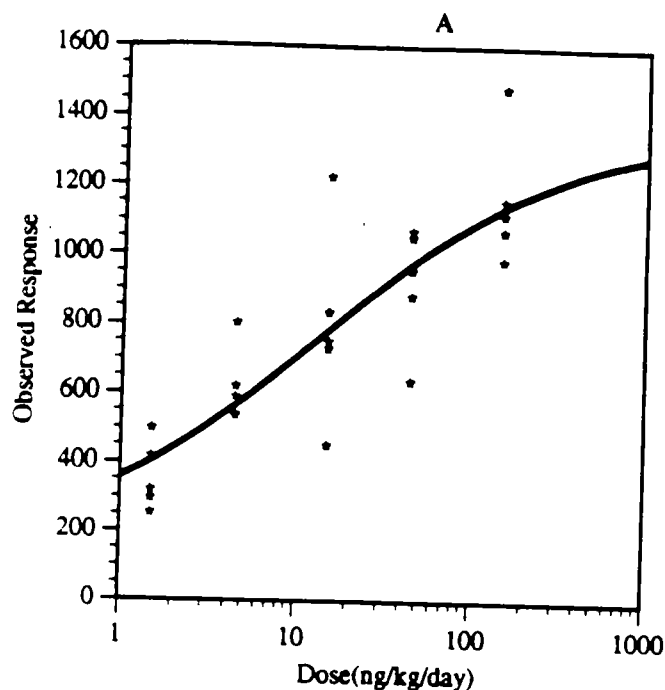
(Graph in Log-Log Coordinates)

Female B6C3F1 mice

(De Vito et al, 1992a)

Dose: 5 X weekly

Response: % increase Cyp1A2 level, Hepatic EROD, Lung EROD and Skin EROD



Functional Form of Sigmoid Dose-Response

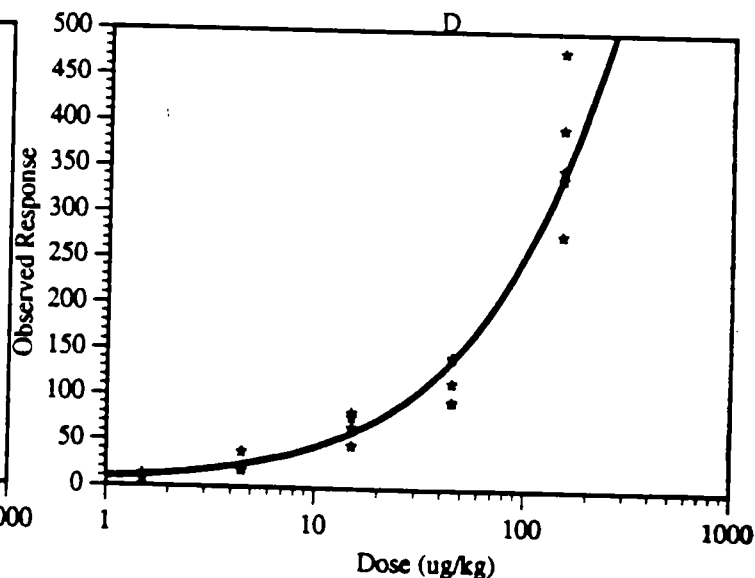
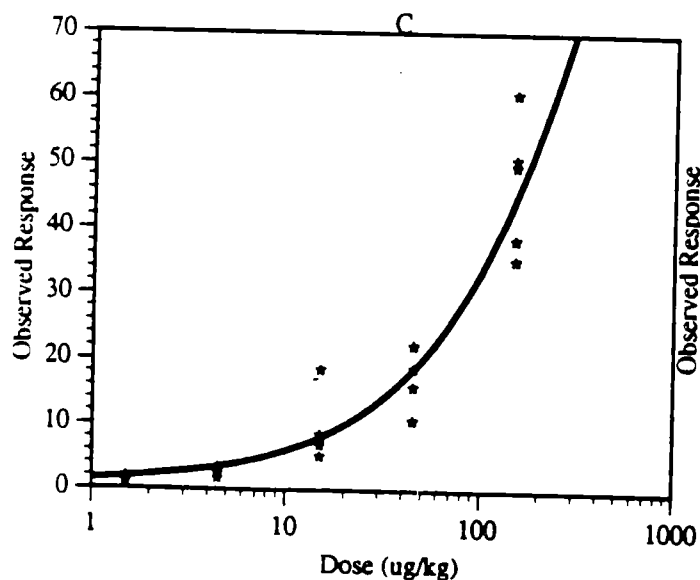
(Best Fit Estimate for "Hill function + background parameter")

$$\text{1A2 levels } f(x) = 1.242448E+3 \cdot x^{5.924782E-1} / 4.586583E+0 + x^{5.924782E-1} + 135$$

Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Hill function" type response)

$$\text{Hepatic EROD } f(x) = 9.115251E+3 \cdot x^{1.115666E+0} / 2.283763E+2 + x^{1.115666E+0} + 80$$



Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Hill Function" type response)

$$\text{Skin EROD } f(x) = 2.397953E+2 \cdot x^{8.615018E-1} / 3.332969E+2 + x^{8.615018E-1} + 0.83$$

Functional Form of Sigmoid Dose-Response

(Best Fit Estimate for "Hill Function" type response)

$$\text{Lung EROD } f(x) = 2.070050E+3 \cdot x^{8.436144E-1} / (3.480857E+2 + x^{8.436144E-1}) + 3.19$$

Graph P4
Comparative Dose-Response

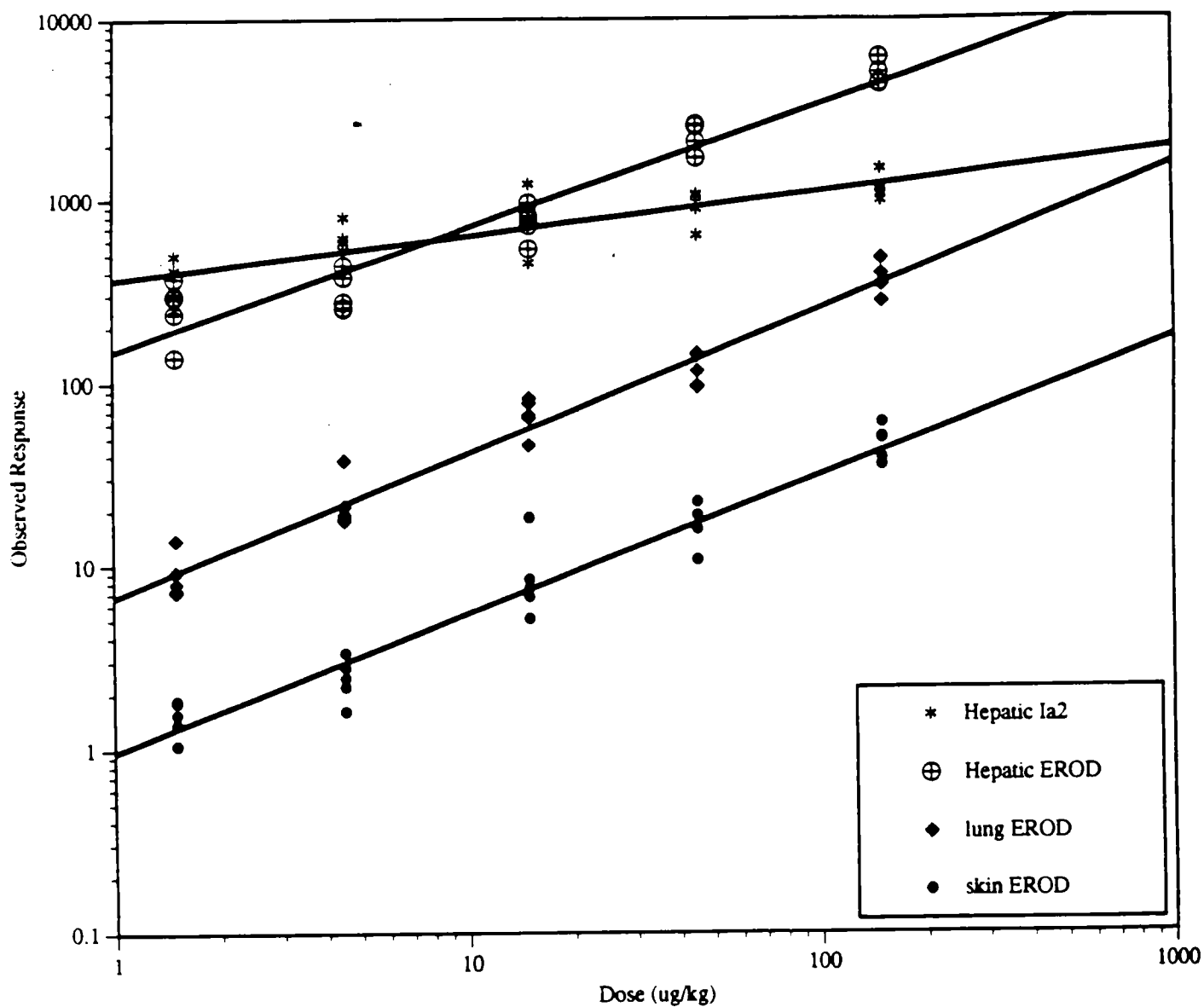
(Graph in Log-Log Coordinates)

Female B6C3F1 Mice

(DeVito et al., 1992a)

Dose: p.o. 5 X per week 13 weeks

Response: % increase Hepatic EROD, Hepatic Ia2, Skin and Lung EROD



Functional Forms of Dose-Response Curves

(Best Fit Estimates for Power Laws)

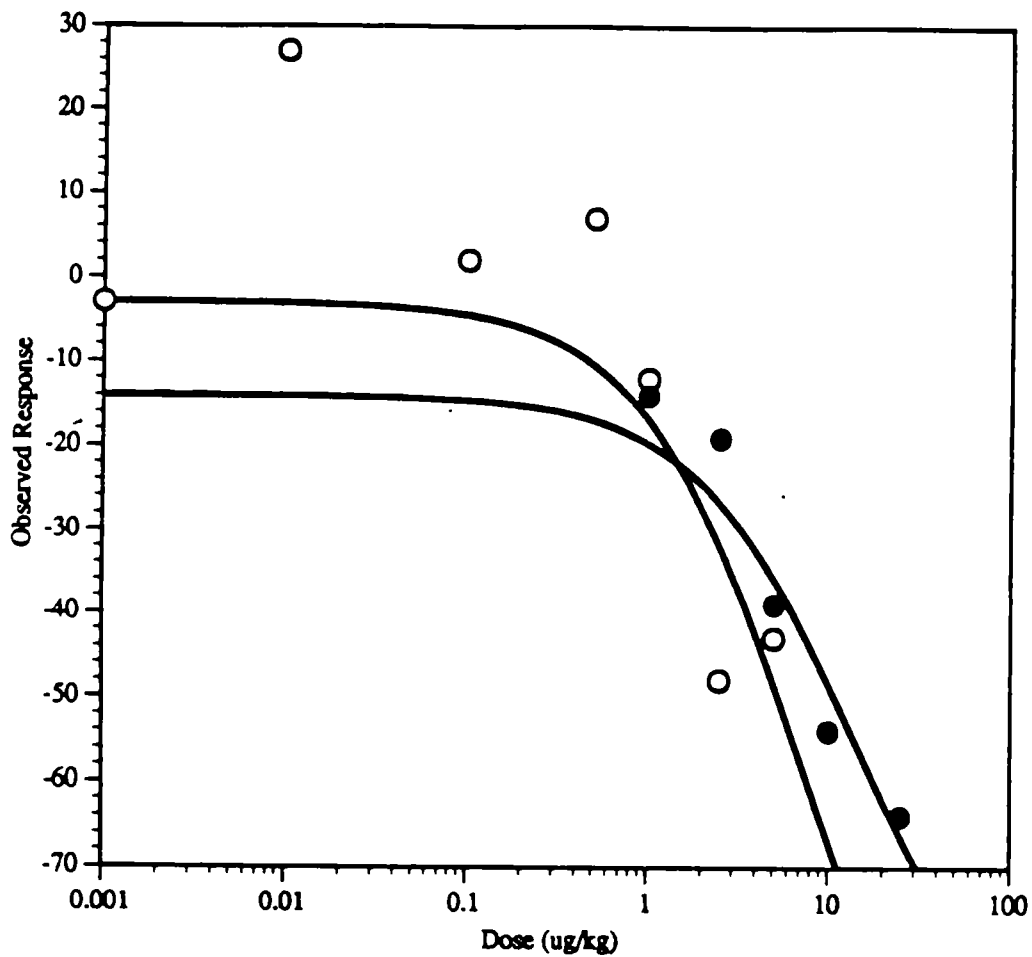
Hepatic EROD: $f(x) = 1.482129E+2 * (x^{6.769077E-1})$, $R^2 = 9.229244E-1$

Hepatic Ia2: $f(x) = 3.650628E+2 * (x^{2.423515E-1})$, $R^2 = 7.277352E-1$

Skin EROD: $f(x) = 9.676016E-1 * (x^{7.538292E-1})$, $R^2 = 9.363354E-1$

Lung EROD: $f(x) = 6.679660E+0 * (x^{7.910685E-1})$, $R^2 = 9.658391E-1$

Graph Q
Dose-Response
 (Graph in Log-Linear Coordinates)
 for
 Sprague-Dawley Rat
 Dose: single oral
 Response: % change in liver EGR receptor binding levels



Functional Form of Sigmoid Dose-Response
 (Best Fit Estimate for "Michaelis-Menten" + initial response parameter)
 Intact $f(x) = -14 + 8.004886E+1 / (1.319891E+1 + x)$
 Adrenalectomized $f(x) = -3 - 1.072898E+2 * x / (6.766685E+0 + x)$