United States
Environmental Protection
Agency

Office of Research and
Development
Washington, DC 20460

EPA/600/AP-92/001h
August 1992
Workshop Review Draft

EPA

Chapter 8. Dose-Response Relationships

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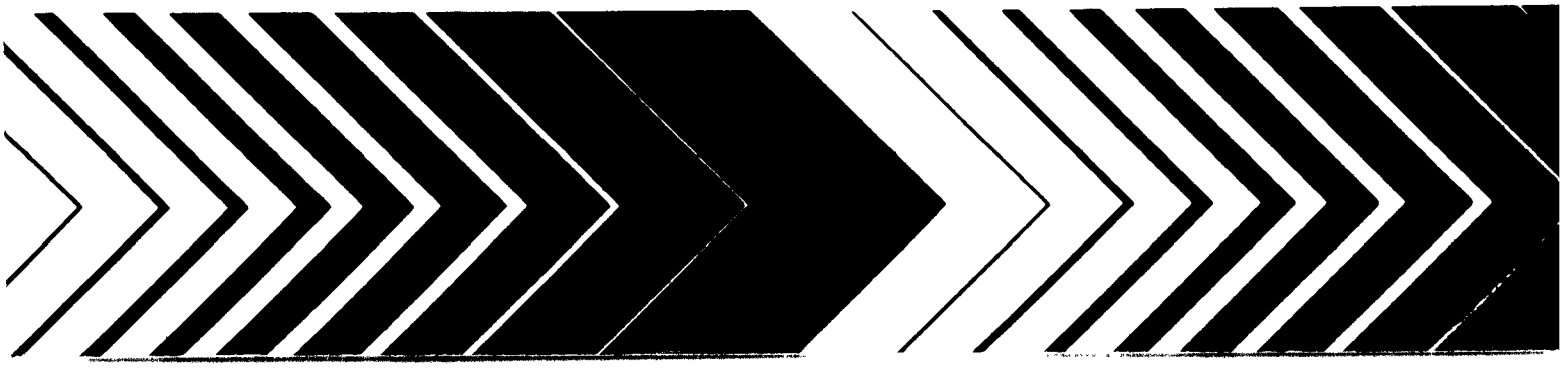

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August 1992
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Chapter 8. Dose-Response Relationships

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

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
Ah	Aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
ALT	L-alanine aminotransferase
AST	L-asparate aminotransferase
BDD	Brominated dibenzo-p-dioxin
BDF	Brominated dibenzofuran
BCF	Bioconcentration factor
BGG	Bovine gamma globulin
bw	Body weight
cAMP	Cyclic 3,5-adenosine monophosphate
CDD	Chlorinated dibenzo-p-dioxin
cDNA	Complementary DNA
CDF	Chlorinated dibenzofuran
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DCDD	2,7-Dichlorodibenzo-*p*-dioxin
DHT	5α-Dihydrotestosterone
DMBA	Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

LIST OF ABBREVIATIONS (cont.)

DRE	Dioxin-responsive enhancers
DTG	Delayed type hypersensitivity
DTH	Delayed-type hypersensitivity
ED_{50}	Dose effective for 50% of recipients
ECOD	7-Ethoxycoumarin-0-deethylase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EROD	7-Ethoxyresurofin 0-deethylase
EOF	Enzyme altered foci
FSH	Follicle-stimulating hormone
GC-ECD	Gas chromatograph-electron capture detection
GC/MS	Gas chromatograph/mass spectrometer
GGT	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
GST	Glutathione-S-transferase
HVH	Graft versus host
HAH	Halogenated aromatic hydrocarbons
HCDD	Hexachlorodibenzo-p-dioxin
HDL	High density lipoprotein
HxCB	Hexachlorobiphenyl

LIST OF ABBREVIATIONS (cont.)

HpCDD	Heptachlorinated dibenzo-p-dioxin
HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HxCDD	Hexachlorinated dibenzo-p-dioxin
HxCDF	Hexachlorinated dibenzofuran
ID_{50}	
I-TEF	International TCDD-toxic-equivalency
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)
LH	Luteinizing hormone
LDL	Low density liproprotein
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MFO	Mixed function oxidase
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK	Natural killer

LIST OF ABBREVIATIONS (cont.)

NOAEL	No-observable-adverse-effect level
NOEL	No-observed-effect level
OCDD	Octachlorodibenzo-p-dioxin
OCDF	Octachlorodibenzofuran
PAH	Polyaromatic hydrocarbon
PB-Pk	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyl
OVX	Ovariectomized
PBL	Peripheral blood lymphocytes
PCQ	Quaterphenyl
PeCDD	Pentachlorinated dibenzo-p-dioxin
PeCDF	Pentachlorinated dibenzo-p-dioxin
PEPCK	Phosphopenol pyruvate carboxykinase
PGT	Placental glutathione transferase
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
ppm	Parts per million
ppq	
ppt	Parts per trillion
RNA	Ribonucleic acid
SAR	Structure-activity relationships

LIST OF ABBREVIATIONS (cont.)

SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SRBC	Sheep erythrocytes (red blood cells)
$t_{½}$	Half-time
TCAOB	Tetrachloroazoxybenzene
TCB	Tetrachlorobiphenyl
TCDD	Tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factors
TGF	Thyroid growth factor
tPA	Tissue plasminogen activator
TNF	Tumor necrosis factor
TNP-LPS	lipopolysaccharide
TSH	Thyroid stimulating hormone
TTR	Transthyretrin
UDPGT	UDP-glucuronosyltransferases
URO-D	Uroporphyrinogen decarboxylase
VLDL	Very low density lipoprotein
v/v	Volume per volume
w/w	Weight by weight

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The Office of Health and Environmental Assessment (OHEA) within the Office of Research and Development was responsible for the preparation of this chapter. The chapter was prepared through Syracuse Research Corporation under EPA Contract No. 68-CO-0043, Task 20, with Carol Haynes, Environmental Criteria and Assessment Office in Cincinnati, OH, serving as Project Officer.

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The two Appendices were not prepared by the Workgroup but are included with the gracious permission from the authors.

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

8.1. INTRODUCTION

Most of the information presented in the Introduction is found in more extensive detail in the other background chapters. We feel that it is useful to summarize the salient features of those papers which impact on the development of dose response models so that readers of this chapter will be able to evaluate the scientific foundation on which our dose response models 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 ß-naphthaflavone. The biologic properties of dioxins have been investigated extensively in over 5000 publications and abstracts since the identification of TCDD as a chloracnegen (Kimming and Schulz, 1957). Much of the biological activity of TCDD follows 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 concentration of several hepatic proteins including the up regulation of P-450IA1 and IA2, and the modulation of the estrogen receptor and EGF receptor. The carcinogenicity of TCDD has been shown in several strains of laboratory mice and rats, and the tumor sites include liver, thyroid and the respiratory tract, as well as others. However, 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 receiving TCDD at the level of 1 ng/kg/day. Male rats were remarkably less susceptible to TCDD action in these studies. However, there is no striking sex specifity in the hepatocarcinogenic actions of TCDD in mice.

The overall hypothesis of TCDD action, put forth by several groups, has been proposed for the effects of TCDD on the transcriptional activities of the cytochrome P-450IA1 gene. The biological basis for this approach is outlined in the chapter by Whitlock. Although substantial gaps in our knowledge remain, it

is accepted by most researchers that all cellular responses to TCDD follow upon the interaction between TCDD and an intracellular macromolecule, the so called Ah or Dioxin receptor (AhR). 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 the analogy of the action of TCDD and estradiol at their respective receptor protein. Each AhR appears to bind one molecule of TCDD, and at low concentrations of ligand (i.e., when [ligand]<<[receptor]), the binding of TCDD to AhR is linearly related to [TCDD]. However, the presence of TCDD in cells induces an increase in the cytosolic concentration of AhR, possibly by increasing receptor synthesis, displacement of an endogenous ligand from the receptor or by movement of "spare" receptors from the nucleus. The increase in available receptors could amplify the signal associated with receptor binding by increasing opportunities for TCDD to bind to AhR.

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 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 nucleus, 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). Some of these elements have now been sequenced and identified upstream of promoter elements in several dioxin responsive genes (Sutter et al., 1992;

Denison, 19??). The association of the activated TCDD complex with the XRE is also reversible, but little is known of its kinetic properties (Gasiewicz et al., 1991). This XRE binding in turn evokes the production (or perhaps the suppression) of several mRNA species. The structure and amino acid sequence of the AhR protein was reported by Poland and co-workers (Burbach et al., 1992; Ema et al., 1992). Both the XRE(s) and the structure of the AhR are analogous to the steroid receptors and the respective genomic response elements. This similarity is important in regard to 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 (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 Ah receptor nuclear translocator protein (ARNT) functions in this fashion (Hoffman et al., 1991). Other receptors are associated with heat shock 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.

The steroid hormone receptors regulate gene transcription through specific DNA sequences near the regulated gene. But the situation is even more 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 regulated gene. These transcription factors may regulate the binding affinity of the steroid hormone receptor itself to DNA (Muller et al., 1991). As

mentioned above, a consensus binding site has been proposed for the Ah receptor by Denison et al. (1988). A final level of 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 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 inter- and independently regulated.

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 to cellular response. Of interest to us is the information that may be available concerning the overall dose:response relationship from 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 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 that 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. Straight transduction implies that the relationship of event to event, for all steps, is invariant.

In considering these possible dose:response relationships, it may 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, or if its affinity is higher or lower than an endogenous

ligand. 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 processess) or by enzymatic degradation, as with peptides. With respect to kinetics of binding of TCDD, its 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 require information on the quantitative relationships between ligand concentration, receptor occupancy and biologic response. Roth and Grunfeld in The Textbook of Endocrinology (1985) state:

> "At very low concentrations of hormone ([H]<<Kd), receptor occupancy occurs but maybe 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. 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.

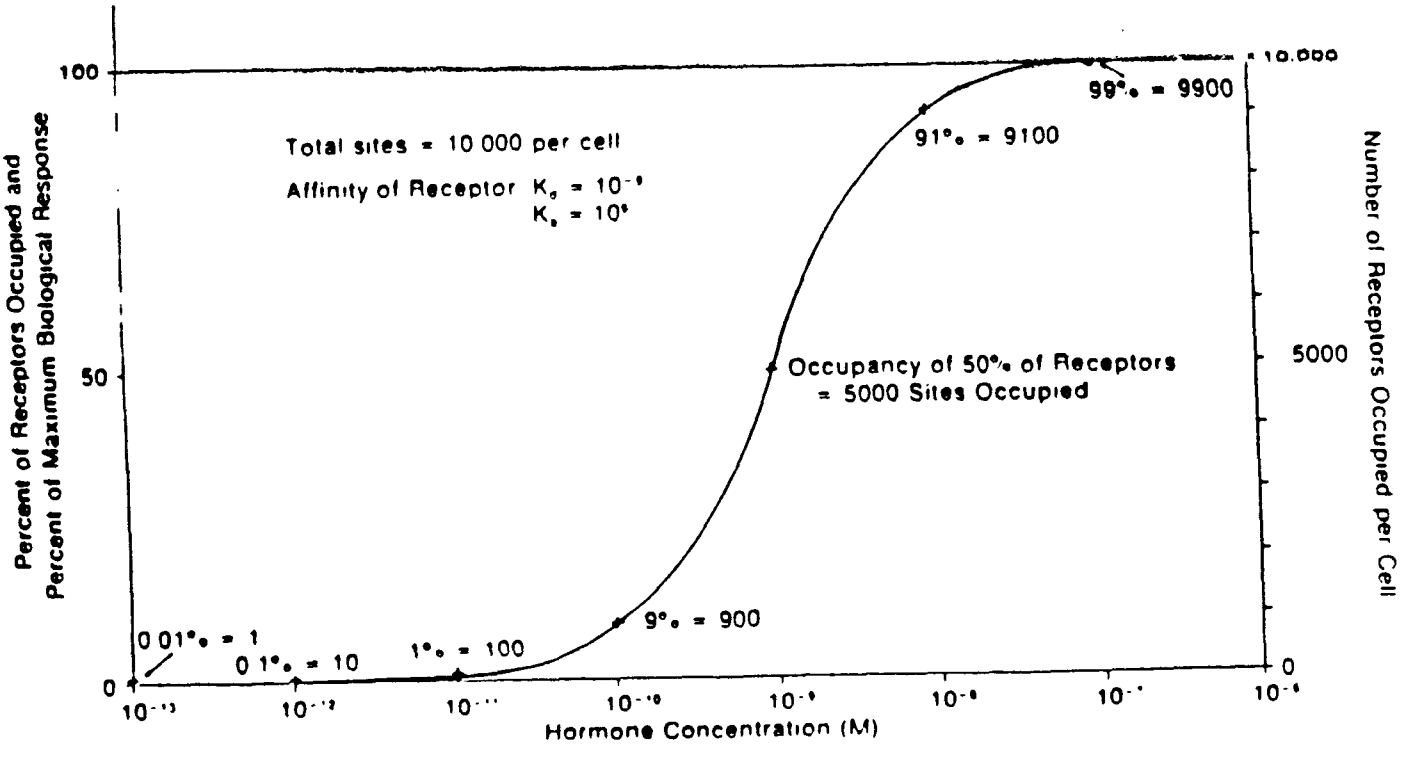

FIGURE 8-1

Dose/Response Graph Showing Proportional Relationship Between Receptor Occupancy and Biological Response (Semilog Scale)

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

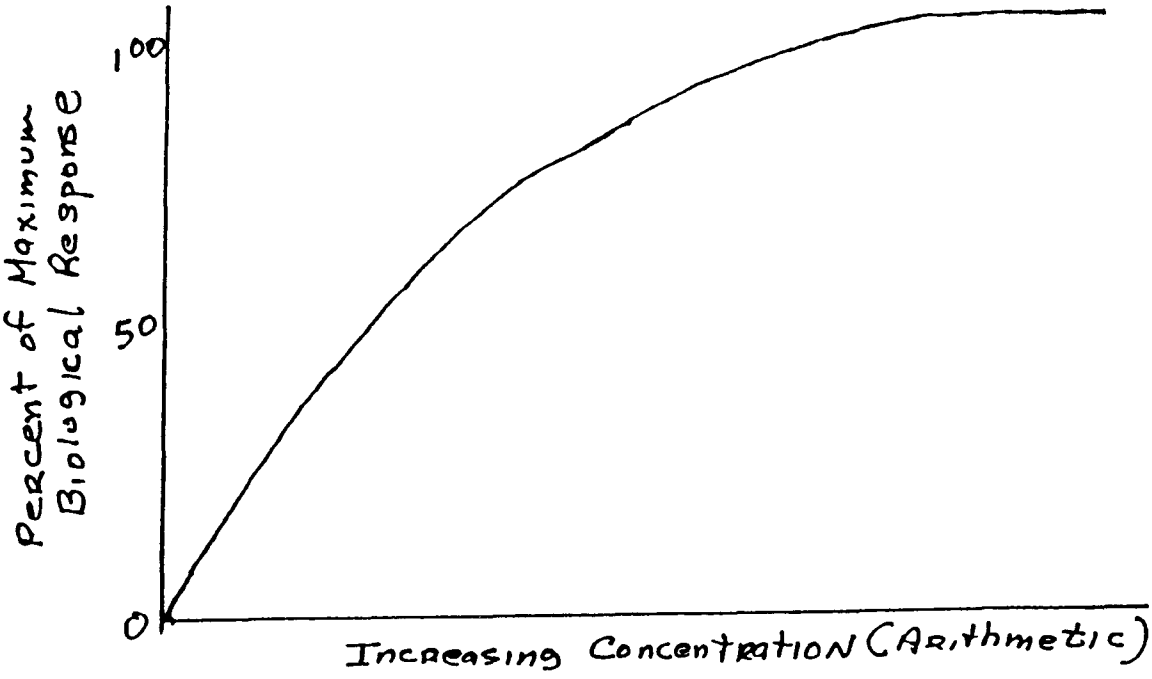

FIGURE 8-2

Dose/Response Graph Showing Proportional Relationship Between Receptor Occupancy and Biological Response (Arithmetic Scale)

Such a simple proportional relationship is not adequate to explain the diverse biological responses caused 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. Therefore, it would be expected that there would be 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., CYPIA1 induction) may not accurately predict dose-response relationships for 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 V "Knowledge Gaps."

Dose-response relationships for TCDD's toxic effects have been established for several endpoints in intact and surgically altered animals. *In vitro* experiments have been used to determine critical concentrations 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. It must also be kept in mind that not all responses will necessarily require binding of the Ah receptor ligand complex to responsive elements on DNA.

Cancer is a multistage disease using a model composed of four or five operationally defined processes: initiation, fixation, promotion and progression (Figure 8-3). In experimental systems, initiation is generally thought to be a DNA damaging or altering event, and fixation is the immortalization of the mutation in clonally expanded progeny. Promotion is the enhancement, via modifications in growth kinetics, of the initiated cell population by an endogenous and/or exogenous factors. Progression refers to the growth of the tumor to an end stage. Tumor promotion in experimental animals is a well

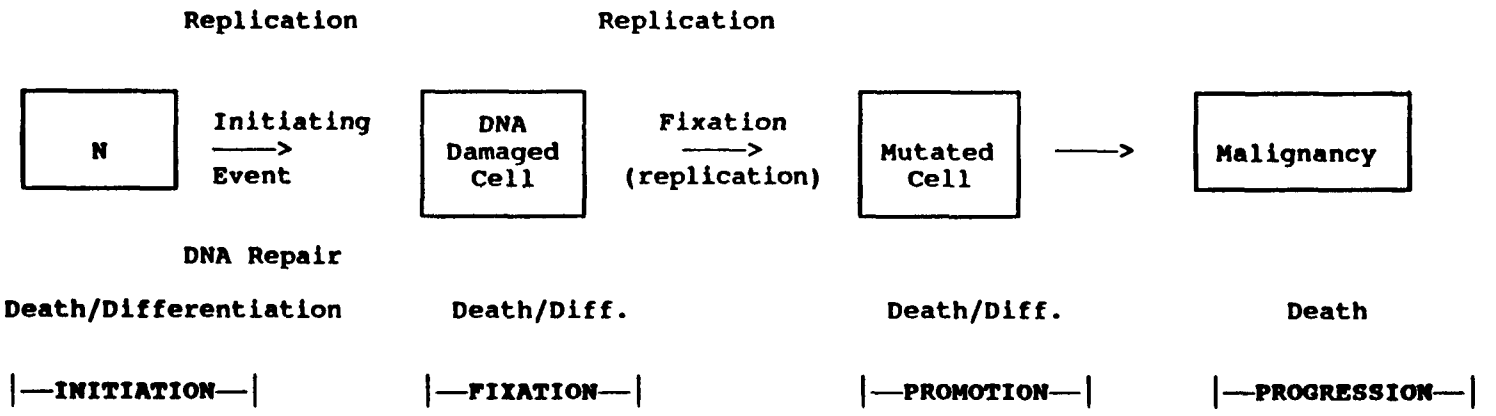

FIGURE 8-3

Multistage Carcinogenesis

Source: Portier and Kopp-Schneider (1991)

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established paradigm that has demonstrated carcinogenicity in many tissues including liver, breast, bladder and skin. Dioxin is a promoter in skin and liver of mice and rats, respectively. However, TCDD promoted, DEN-initiated liver tumors or foci are suppressed in ovariectomized rats. Hence, promotion of DEN initiated hepatocytes by TCDD in rats is dependent on ovarian factors or the receptors for these factors. Articles by Pitot et al. (1987) and Lucier et al. (1991) present data on liver tumor promotion by TCDD.

The general approach of the U.S. EPA to regulation of carcinogens is to use the the Armitage-Doll model of carcinogenesis [Linearized Multistage, LMS] (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 which define the process.

The EPA formulation of this model assumes the mutation rates [u,(d,t)] are a linear function of dose and are constant over time. These assumptions result in a tumor incidence rate which is a polynomial function of dose. In the low dose region, the upper bound on risk is dominated by the linear term in the polynomial (q_1^*). EPA generally uses a upper confidence limit on the linear term of this formulation of the multistage model for cancer risk assessment. 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 shows 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 of a carcinogen can fit the general dose-response shape predicted by the linearized multistage model. Cross species extrapolation is carried out using

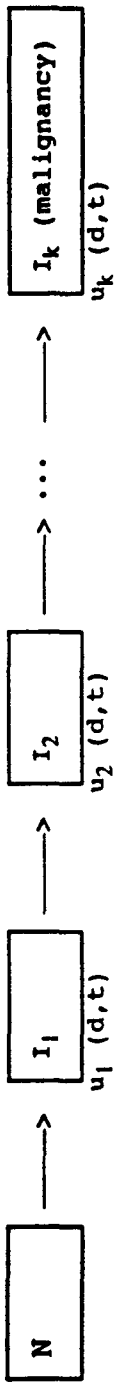

FIGURE 8-4

The Linear Multistage Model of Carcinogenesis

dose expressed as daily exposure per unit surface area, where an allometric scaling factor is applied to body weight to estimate the surface area. The potency of a carcinogen is related to the slope of the dose-response curve (Q*).

For other toxicological endpoints such as terata, organ toxicity, acute toxicity, etc., a threshold has been assumed 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. As discussed above, 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.

Since TCDD action is clearly receptor mediated, it is reasonable to attempt to model the receptor kinetics using equations that have been used for steroid receptor mediated responses. Recent rodent experiments suggest the induction of cytochrome P-450IA1 is linear through zero using long term exposure over a wide dose range (Taitscher et al., 1992; DeVito et al., in press). Exposure data in humans indicates that the general population has a body burden of 5-10 ppt (lipid adjusted). In occupational epidemiology, human disease associated with TCDD exposure has not been detectable until fat levels are one to two orders of magnitude higher than the general population (100-1000 ppt). Thus, in addition to other ligands, one must also be concerned with the present level of TCDD in the general population and the long environmental and biological half-life of this compound.

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 is a special case for a nonmutagenic, receptor mediated carcinogen. The goal of this reassessment is to consider more mechanistically-based models which

are sufficiently credible to the scientific community. Such approaches can be recommended for practical risk assessment needs.

There are several models under consideration at the present time ranging from very simple to complex. What has become obvious over the past year is 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 than dose of subsequent actions as long as the dose-response curves for these subsequent actions are parallel to the receptor binding curves. In general, the data to date indicate receptor mediation or receptor coordination does indeed exist 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 the other factors must be elucidated before there is a complete understanding of TCDD action. However, a relatively complete model can 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 linearized multistage model (LMS) as a function of dose needs to be reevaluated. Three, the biology of receptor mediation should be included in any modeling exercise for TCDD utilizing existing mathematical developments. This is not to say that uncertainties do not remain. However, 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 more biologically-based model will be more or less conservative than LMS. 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:

> "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." (Greenlee et al., 1991)

This chapter presents the current thinking on TCDD mechanistic action. It examines several endpoints and focuses on dose response models for cancer in laboratory animals and man. It also evaluates for use of biomarkers of TCDD action as surrogates for modeling receptor mediated events. In addition the chapter presents a section on "Knowledge Gaps." Critical examination of this section leads to new experiments which will add to the already impressive database on TCDD action. Addition of key molecular, cellular and tissue specific information to the current data base will be important to establish a new risk paradigm based on biological mechanism of action of TCDD and perhaps other receptor-mediated nonmutagenic toxicants. The chapter reviews some of the critical data on noncancer endpoints but does not attempt to model them. These endpoints are clearly important when considering the public health risk of dioxin. However, the lack of key molecular information of action and molecular dosimetry limits mathematical modeling of noncancer endpoints at this time.

8.1.1. Introduction to Modeling for TCDD. Mathematical modeling can be a powerful tool for understanding and combining information on complex biological phenomenon. The development and use of mathematical models is illustrated by

Figure 8-5. In the development of a mechanistically-based mathematical model, the beginning point is generally a series of experiments aimed at 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 which are then validated against the existing knowledge base for the effect under study and the xenobiotic agent. This can then lead to new experiments and further laps through this model development loop. Each time through the loop the model either gains additional validation by predicting the new experimental results or it is modified to encompass the new results without sacrificing the prediction of previous results. In either case, subsequent loops through the model generally increase our confidence in the model (although it may be difficult or impossible to quantify this confidence).

There is no one model development loop for any given compound or effect. Instead, there are always numerous exercises which lead to the development of a mechanistic model. In modeling the effects from exposure to TCDD, there are many smaller model development circles which 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 range of experimental findings.

Confidence in any one model is not only dependent upon the information available for that compound, but is also supported by the information available on other systems which 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, general work in physiologically-based pharmacokinetics models and in tumor incidence modeling.

Risk assessment, however, is another issue. The use of mechanistically-based modeling in extrapolating to exposure patterns and exposure 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

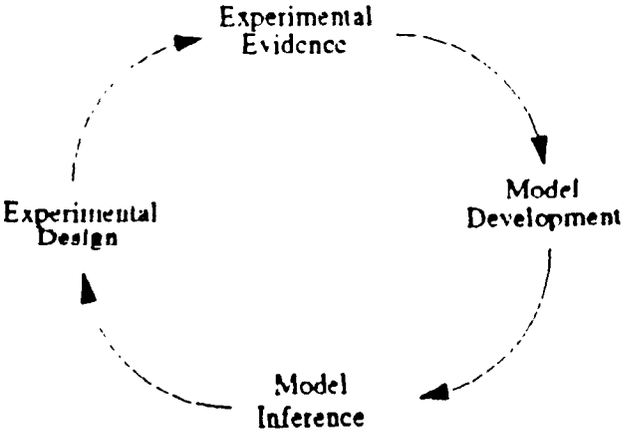

FIGURE 8-5

Developing a Mechanistically-Based Mathematical Model

could be low confidence in the ability of the model to predict outside the range of data. Risk assessment inherently demands a careful scrutiny of the behavior of a mechanistic model under a variety of exposure scenarios, scrutiny which has not generally been applied to the use of mechanistic models in science. Confidence can only be gained by numerous loops through this model development process for the express purposes of risk assessment. Only then will the process be acceptable as a tool for risk extrapolation. It is important to note that mechanistic modeling has a role to aid in explaining and understanding experimental results which is devoid of its proposed use for risk assessment and that our confidence in the methods used in mechanistic modeling will differ upon the history of it's use. For historical perspective, it is important to recognize that this is not the first loop through the cycle of mechanistic modeling for carcinogenesis. Early exercises based upon tolerance distributions used the then current understanding of carcinogenesis to develop statistical models which could be used for risk estimation. Later use of the linearized-multistage model was also based on an understanding of the carcinogenic mechanism. This exercise also benefits from recent attempts to use physiologically-based pharmacokinetic (PB-PK) models in risk estimation. Thus, this exercise is a logical next step in the continuum of mechanistically-based modeling for risk assessment purposes.

In any realistic and practical modeling exercise, the major component of the model revolve around the estimation of parameters in the model 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 intimately tied to the data available to characterize the model. The way in which model parameters are estimated and the data used to estimate those model parameters are the major components in determining the reliability and trust we will place in any mathematical model. Fundamentally, sufficient biological data need to be available to convincingly show that the model correctly represents *in vivo* processes and that the processes modeled are the ones that lead to toxic events.

In modeling biological phenomenon, the data can be divided into 4 broad categories as shown in Table 8-1. At the top are effects on the whole animal. Examples of data included in this category would be data on survival of the organism, its ability to reproduce and it's ability to properly function (e.g. behavioral data). The levels of data then get increasingly specific going from whole organism to tissue/organ system responses to cellular responses down to biochemical responses in the cell. Other groupings are possible and even more detailed categories (e.g. molecular biochemistry); the point is that the data range from data at 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 effectively counting bodies. All of this information is useful and should be incorporated into a mathematical model aimed at understanding some biological response.

Mathematical models which incorporate parameters which are mechanistic in nature do not automatically constitute "mechanistic models." The available data for characterizing the model and the method by which this data is incorporated into the model are important in determining if a model is truly "mechanistic" and soundly based on the biology or is instead, simply a curve fit to 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) is 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 it's 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 the 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

TABLE 8-1

Examples of Levels of Information Available for Estimating Parameters In Dose-Response Modeling

Organism	Morbidity
	Mortality
	Fertility
Tissue	Hyperplasia
	Hypertrophy
	Carcinogenicity
	Chemical Distribution/Disposition
	Improper Development/Function
Cell	Mitosis
	Cell Death
Biochemical	Gene Expression
	Protein Levels
	Receptor Binding
	Adduct Formation

is basically left with a curve fit to the data. The historical applications of the "linearized" multistage model in risk assessment has been in this fashion.

We view true mechanistic modeling 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 which is reasonable in it's 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 divorce mechanistic modeling from curve fitting. At some point in the modeling process, gaps must be filled relating the modeled, mechanistic effects to the observed toxic effects. It is generally at this point that some amount of curve-fitting is necessary to calibrate the mechanistic response to the toxic effect. 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 is 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 a more precise estimate of risk. Without data, the statistical issue of the accuracy of a prediction cannot be easily addressed. Thus, while there may be a greater deal of 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 little 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 will 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 frequency of 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 which may benefit the risk estimation process. Because of this, now is the perfect time to consider the types of designs which are best suited to addressing problems which are specific to risk assessment. In general design situations one would have a mechanism in mind, qualitatively describe that mechanism and form the structure of a mechanistic model, make educated guesses about the parameters of this model, then use the quantitative model to locate designs which are optimal at characterizing the mechanism. For the purpose of risk estimation, the basic outline also holds. There are also some simple design rules which would aid 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 has been chosen as a prototype for exploring and examining the ability of mechanistic modeling to improve the accuracy of quantitative risk assessment. In essence, 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 good human data which is supported by the experimental evidence in animals. On the other hand, some aspects of the mechanism by which TCDD induces it's effects, such as binding to the Ah receptor have not been modeled extensively and, thus, even for scientific purposes are in only their first few loops through the model development cycle shown in Figure 8-5. In this case, several competing mechanistic theories will agree with the existing data adding to the uncertainty in any projected risk estimates. This outcome is

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	Record data on the level of individual animals

inevitable for a novel mechanism and for the application of the technology of mechanistic modeling to a new area. To reiterate an earlier point, mechanistic modeling has a role to aid in explaining and understanding experimental results which is devoid of it's proposed use for risk assessment and our confidence in the methods used in mechanistic modeling will differ depending upon the history of it's 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 TCDD is an early attempt along these lines, we must be cautious in coming to a judgement concerning the overall utility of mechanistic modeling as an important tool for risk assessment based upon this one case study.

8.1.2. Dosimetric Modeling. Dose Delivery and Tissue Modeling and Biochemical Modeling (See Appendix for complete manuscripts by Kohn et al., 1992 and Andersen et al., 1992)

In the NAS report, *Risk Assessment in the Federal Government: Managing the Process* (NRC, 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 related to exposure. This can be due to saturation and activation of metabolic pathways. (Hoel et al., 19??), 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 which can radically modify the homeostatic properties of the tissue. These complex interactions can result in markedly nonlinear dose-response; nonlinearities which could lead to risk estimates which 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 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 which 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 and the extrapolation of dose-response models from test animals to people. These extrapolations will be based upon 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 which 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.

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 which describes the interconnections among these determinants constitutes a mathematical model for the tissue dosimetry of dioxin. The model we will use 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 Galowski and Jain (1983) 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_ℓ represents the concentration of compound in a tissue (ℓ) and C_B the concentration of compound in blood (B), one of the simplest relationships one might use is:

$$\frac{\partial C_\ell}{\partial t} = r_{B\ell}\, C_B - r_{\ell B}\, C_\ell - r_m\, C_\ell \qquad (equation 1)$$

where $\partial C_\ell/\partial t$ represents the change in the concentration in the tissue over time (t), $r_{B\ell}$ is the rate (per unit concentration) of the movement of the compound from blood to tissue, $r_{\ell B}$ 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; Mathews and Dedrick, 1984). King et al. (1983) modeled the kinetics of 2,3 7,8-TCDF in several species and Kissel and 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 upon a finding of hepatocarcinogenicity in female Sprague-Dawley rats, we will focus on the model by Leung et al. (1990a) in this 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 which 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}. They also include 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 upon 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 specific binding of dioxin to a liver protein used by Leung et al. (19??) is an improvement 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 by subcutaneous 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 3-fold increase in the rate of metabolism. There is no evidence to suggest an increase of metabolism in the rat for TCDD; however, there is data supporting small increases in metabolic clearance at high doses for the tetrabrominated analog (Kedderis et al., 1991). The identity of the enzymes responsible for TCDD metabolism are as yet unknown.

Finally, Leung et al. (19??) kept all physiological parameters (flow rates, tissue weights, etc.) 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, 19???; 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 which produces risk which is proportionate to dose for low doses (low-dose linearity). Thus, in discussing the models and submodels which form a basis for a mechanistic model for TCDD, we will focus on aspects of the model which could lead to nonproportional response for low environmental doses. The model of Leung et al. (19??) 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, 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.

Application of this proposed effective dose with the two-stage model of carcinogenesis is presented later in this chapter.

Looking at one small 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 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 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 CYPIA1 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)?? and the Ah receptor (Gasewicz, 1984). 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 which 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 as following Hill kinetics, but with TCDD reducing the binding from the maximum level when no TCDD is present. 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:

$$\frac{\partial P}{\partial t} = B_p + \frac{V_m C^n}{K_d + C^n} - \frac{V_p P}{K_p + P} \qquad (equation2)$$

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 which must be occupied for the effect of the binding reaction to be expressed. When the Hill exponent is not an integer, no real molecular meaning can be attributed to the equation and the model becomes phenomenological (Boeynaems and Dumont, 1980??).

The second model they considered was one in which the basal expression of these proteins was due to an endogenous ligand which competed with TCDD for binding sites. This leads to equations of the form:

$$\frac{\partial P}{\partial t} = \frac{V_m (C+E)^n}{K_d + (C+E)^n} - \frac{V_p P}{K_p + P} \qquad (equation3)$$

where E refers to the concentration of the endogenous 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 upon 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 regards to statistical (Hoel, 19??) and mechanistic (Portier, 1986??) 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 which 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. We do not propose to directly employ the models of Portier et al. (1992) for risk estimation.

Andersen et al. (1992) modified the model of Leung et al., (19??) 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 compounds 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. (19??) 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 constant, 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 1340 hours).

Andersen et al. (19??) 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 1-100 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. (10??) does occur in the data of Kociba et al. (19??) and Tritscher et al. (19??) but in the range of 100 ng/kg/day rather than the 10,000 ng/kg/day predicted by Andersen et al. However, the changes in liver/fat ratio observed by Andersen et al. (19??) and supported by human evidence (Carrier, 1991) are a necessary part of the modeling for TCDD.

Finally, with regards to risk estimation, Andersen et al. (19??) 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 assuming gavage ??? exposure for ??? weeks, they integrated the concentration of TCDD in the liver over time and the concentration of induced protein 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. It has been shown for the tumor incidence data that it is difficult to determine nonlinearity or linearity although it is not inconsistent with a linear response in the low dose region (Portier et al., 1984). In general, one cannot expect a linear correlation between appropriate dose measures and toxic response. In addition, 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 this 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. (19??) 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. (19??) and Andersen et al. (19??) 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 regiments (Kociba et al., 1976, 1978; Krowke et al., 1989). More recent studies in which female Sprague-Dawley rats received TCDD (Tritscher et al., 1992; Sewall et al., 1992) 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. (1992) were concentrations of TCDD in blood and liver, concentrations of hepatic CYP1A1 and CYP1A2 and EGF receptor on the hepatocyte plasma membrane. Kohn et al. (1992) refer to their model as the Kohn model. The tissue dosimetry for the Kohn model were 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 Kohn 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. (19??) 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 Kohn model is dependent upon the body weight of the animal. Body weight changes as a function of dose and age were recorded

by Tritscher et al. (19??) and directly incorporated into the model via a smooth function. Tissue volumes and flows were calculated as a allometric formula based upon recent work by Delp et al. (1991). To allow the model to fit the data of both Rose et al. (1976) and Tritscher (1992), the Kohn 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 Kohn model represents a net contribution of TCDD clearance by TCDD-induced cell death.

In the biochemical effects portion of the Kohn model, the Ah-receptor/TCDD complex up-regulates four proteins; CYP1A1, CYP1A2, the Ah receptor and transforming growth factor-α (see Fig. 1). 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 TGF-α 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 TGFα has not been clearly demonstrated in liver (see Appendix). 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 upon additive induction (equation 3), but again the Hill exponent was estimated to be 1 leading to low-dose linearity under either model (2 or 3). Thus, the Kohn model found that the induction of all gene products appear to be hyperbolic functions 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 Kohn model.

In the Kohn 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 TGF-α. This synergy was introduced to partially account for the observation of reduced TCDD promoting potency in males and ovariectomized females as compared to 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. (19??) (see Appendix).

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), for protein concentrations using data sets which were not included in the original derivation of the model and which 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.

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 upon 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 regards 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., 1992) 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. It is thus appropriate to conclude that independence of the action of TCDD would necessarily imply a nonlinear response at low dose.

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 TGF-α. 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 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., 1992).

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 many molecular/ biological events that lead to toxic responses are not known. However, the mechanistic assertions put forward by the model can be discussed in scientific peer review and, if found valid, can provide additional confidence.

8.2. TOXIC EFFECTS

8.2.1. Modeling Liver Tumor Response for TCDD

[This section has not been reviewed by all members of the Dose Response Committee, so it does not represent a consensus section at this time. There was considerable discussion regarding the appropriateness of applying dose response models for risk assessment in this chapter. The decision was made that we would include progress on the development of biologically-based models using liver cancer in rats as the example. This approach is obviously fraught with uncertain assumptions. Nevertheless, we feel that this information will be of considerable use to the peer review panel in their attempts to generate a risk characterization for dioxin based on an evaluation of dose response relationships.]

Long term carcinogenicity studies in rodents have shown TCDD to be a potent carcinogen (Huff, 1991). The most seriously affected organ has been the liver in female rodents. The lack of any detectable DNA binding and the failure of TCDD to produce positive findings in a battery of short term tests for genotoxicity compels one to conclude that TCDD does not possess direct genotoxic activity. It can only be concluded that the mechanism of action of TCDD is by secondary effects. One possible mechanism of hepatocarcinogenicity from exposure to TCDD is via the promotion of previously incurred genetic damage. This has been illustrated in several initation-promotion studies in rat livers (Pitot, 1987; Clark, 1991a). There is definitely a role of estrogen in the promotional effects of TCDD in rat liver since TCDD enhances hepatocyte proliferation and stimulates the development of enzyme-altered hyperplastic foci in intact female rats but not in ovariectomized rats (Lucier, 1991). The conclusion resulting from these experiments are not definitive, but it has been suggested that the type of promotional effect resulting from TCDD exposure is receptor-mediated mitogenesis. There is also the possibility that additional CYPIA2 mediated metabolism could lead to an increase in the activation of estrogens leading to cell damage (e.g., via free oxygen radicals).

This section will discuss modeling 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 modelling 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 which seem reasonable for this endpoint. Thus, this exercise falls in between curve fitting and pure mechanistic modeling. At the end of this section, we will discuss the data needed to move this approach into a full mechanistic development and discuss tumor responses in other sexes and species at other sites.

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, 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 the Maine Scientific Advisory Panel (PWG, 1990). The data we will concentrate on is this analysis is the incidence of liver adenomas and carcinomas (combined) based upon 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 2-fold change in the low-dose risk estimates ( ). A simple correction for survival differences ( ) was applied to these data to present the risk summaries given in Table 8-3. In the analysis which follows, a more rigorous statistical approach was employed.

8.2.2. Tumor Incidence. In recent years, there has been a resurgence in interest in refining the mechanistic representation of mathematical models of

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 (PWG, 1990)

Original Dose (ng/kg/day)	0.0	1	10	100
Number with Neoplasm	2	1	9	18
Number on Study	86	50	50	50
Survival-adjusted number at risk[a]	57	34	27	31
Lifetime Tumor Risk	0.035	0.029	0.333	0.581

[a]Using the "poly-3" survival adjustment suggested by Portier and Bailer (19??)

carcinogenesis. With few exceptions, the mathematic modeling of carcinogenesis at the cellular level has concentrated 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, deemed the two-stage model which is illustrated in Figure 8-6.

The two-stage models 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 is considerable confusion as to the underlying mathematics in 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 which 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 which 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.

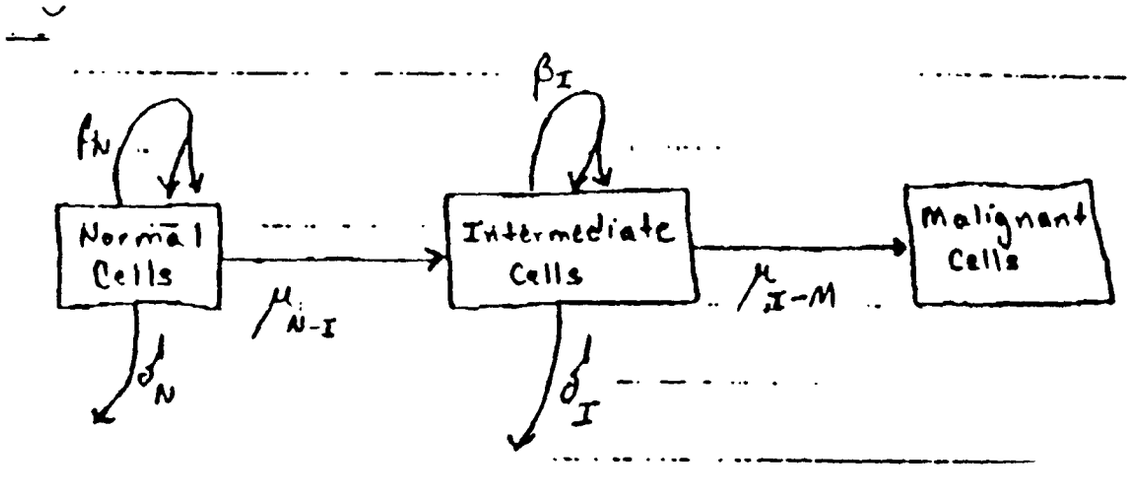

FIGURE 8-6

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 Kohn et al. 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 were assumed constant (this is equivalent to setting $\beta_N = {}_N = 0$ 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 chosen to be 1, 2 or 3 based upon 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 (PWG, 1991). Shown are the number of animals with tumor (row 2), number of animals placed on study (row 3), a survival-adjusted (Portier and Bailer, 19??) 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).

The data is more complex than appears in Table 8-3 since death times were recorded. Since there are no interim sacrifices which would allow for an estimation of tumor-induced mortality (Portier, 1986), the two-stage model was

fit to this data using the assumption of incidental tumors (Hoel and Walbury, 1972??; Dinse and Lagakos, 19??). Since liver tumors induce little, if any, mortality in male and female Fischer 344 rats and B6C3F mice (Portier et al., 1986; Portier and Bailer, 1989), this assumption seems to be warranted.

There are a variety of mathematical formulations which could be used to derive a tumor incidence rate under the two-stage model. As has been assumed by other authors (_____), we assume:

1. all cells act independently of all other cells;
2. the rates in the two-stage model (Figure 8-6) are constant over the life span of the animal; and
3. the tumor incidence rate corresponds to the rate of appearance of the first malignant cells.

All three of these assumptions are likely to be violated in the case of dioxin. 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 which is intractable. 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 upon 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 has been studied (Moolgavkar and Lubeck, 1992) and the assumption (3) was found to have a moderate impact on the tumor incidence rates. This also will be investigated 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.

Under these assumptions, 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). 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% labeling index for a 6 day labeling experiment) suggesting that $\beta_N = 3.333 \times 10^{-3}$. This value was used in the analysis which follows. Other values for $\beta_N = \delta_N$ was specified and assuming changes in β_N do not imply changes in $\mu_{N\text{-}I}$ (Portier and Kopp-Schneider, 1991). The number of normal hepatocytes was assumed to be 6×10^8 for the female rat.

This leaves 16 parameters to be estimated from the tumor incidence data. Table 8-4 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 which give effectively the same answer for this data. The main problem seems to be estimating $\mu_{N\text{-}I}$ and $\mu_{I\text{-}M}$ simultaneously (for example, in the high-dose group, any model with $\mu_{N\text{-}I} \cdot \mu_{I\text{-}M}$ equal to $3.4 \cdot 10^{-15}$, seemed to yield equivalent fits) and with fitting β_I and δ_I separately (no obvious relationship was observed). The main reason for including the "Unrestricted" model in Table 8-4 is that this model represents the best fit we can possibly achieve and the likelihood (Column 6) represents a best possible measure of goodness-of-fit. We will compare the likelihood for other models with this target likelihood.

TABLE 8-4

Fitting the Two-Stage (TS) 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-I}	β_I	δ_I	μ_{1-M}	
Unrestricted	0	1.14×10^{-10}	6.90×10^{-2}	3.14×10^{-2}	1.20×10^{-12}	
	1	9.48×10^{-10}	4.75×10^{-2}	1.58×10^{-2}	9/2-x1-$^{-15}$	
	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}	-58.61
Unrestricted μ_{N-I}	0	2.55×10^{-8}	1.75×10^{-2}	1.06×10^{-2}	1.31×10^{-9}	
	1	1.37×10^{-8}	–	–	–	
	10	1.36×10^{-7}	–	–	–	
	100	5.80×10^{-7}	–	–	–	-64.96
Unrestricted μ_{I-M}	0	1/31x10^{-5}	2.86×10^{-2}	2.16×10^{-2}	2.46×10^{-12}	
	1	–	–	–	1.37×10^{-12}	
	10	–	–	–	1.53×10^{-11}	
	100	–	–	–	5.81×10^{-11}	64.96
Unrestricted β_I	0	2.56×10^{-7}	1.24×10^{-2}	1.79×10^{-2}	3.02×10^{-9}	
	1	–	5.82×10^{-3}	–		
	10	–	2.15×10^{-2}	–		
	100	–	2.46×10^{-2}	–		-67.57
Unrestricted β_I (β_I in low-dose group set to zero)	0	2.56×10^{-7}	6.70×10^{-3}	1.23×10^{-2}	3.02×10^{-9}	
	1	–	0.0	–	–	
	10	–	1.59×10^{-2}	–	–	
	100	–	1.90×10^{-2}	–	–	67.57

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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 $\mu_{I\text{-}M}$ to be constant across all dose groups and allowing $\mu_{N\text{-}I}$ to vary freely as dose changes. The parameter estimates for this model are given in Table 8-4 as model (2). There are nine less 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 8-4. 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 $\mu_{N\text{-}I}$ are held constant over all groups and $\mu_{I\text{-}M}$ 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 $\mu_{N\text{-}I}$ (2) are identical. This indicates that the two models explain the same amount of noise in the data. The second point is that, even thought 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 this data indicates this to be true over a wide range of β_I values. The third point is that, for each dose group, $\mu_{N\text{-}I} \cdot \mu_{I\text{-}M}$ is the same in the two models. Again, use of different fixed values for $\mu_{N\text{-}I}$ in model (3) and $\mu_{I\text{-}M}$ 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 $\mu_{N\text{-}I}$ relative to $\mu_{I\text{-}M}$ 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 8-4. This model is also inconsistent with the data (likelihood = 67.57) and provides a fit which worse than the mutational effect model given by (2). [Technical note: it is difficult to directly compare thee two models since they constitute nonnested models (Becker 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. Firstly, no statistical confidence can be placed on this statement so the observed difference in the two models may solely be due to random change. Secondly, 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 upon 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 8-4. 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 nonidentifiability 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. 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 8-5 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 parameter and the proliferation parameter to change as a function of dose (model (1) in Table 8-4). For this

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TABLE 8-5

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}

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 $25x10^6$-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 (10-fold 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 climbs 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% 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 $8x10^{11}$, ~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 size of $2x10^6$ 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 Rate") of Table 8-5. The pattern in this case is very clear and matches the observed cumulative tumor incidence will 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 ($6.9x10^{-3}$) 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 8-5. 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 which can be used to examine the adequacy of this model for tumor incidence from exposure to TCDD. Lucier and his 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. (reference). If this is true, it is possible to apply the methods of DeWanji et al. (1990) to this 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.

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 8-6 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 index then increases with increasing dose. Under the assumption of a linear birth death process, it is

TABLE 8-6

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	1.67×10^{-13}	1.42×10^{-2}	2.16×10^{-2}	2.34×10^{-2}	3.19×10^{-2}
Using labeling index (Model A)	mutation rate	7.505×10^{-13}	13.98×10^{-13}	8.007×10^{-13}	25.20×10^{-13}	9.912×10^{-13}
	birth rate	1.23×10^{-2}	1.41×10^{-2}	2.14×10^{-2}	2.32×10^{-2}	2.30×10^{-2}
	ratio (δ_1/β_1)	0.01	0.01	0.01	0.01	0.01
Allowing all parameters to vary (Model B)	birth rate	2.60×10^{-2}	3.36×10^{-2}	5.02×10^{-2}	3.47×10^{-2}	3.33×10^{-2}
	ratio (δ_1/β_1)	7.74×10^{-12}	5.28×10^{-2}	3.75×10^{-1}	1.79×10^{-2}	1.00×10^{-5}
	mutation rate	1.507×10^{-12}	1.087×10^{-12}	0.881×10^{-12}	1.896×10^{-12}	1.572×10^{-12}
	hepatocytes initiated	2.0%	4.34%	3.32%	12.84%	8.72%
Constant ratio (δ_1/β_1 estimated 1.52×10^{-2})	birth rate	2.60×10^{-2}	3.20×10^{-2}	3.34×10^{-2}	3.41×10^{-2}	3.32×10^{-2}
	mutation rate	1.49×10^{-12}	1.035×10^{-12}	0.59×10^{-12}	1.875×10^{-12}	1.579×10^{-12}

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

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possible to convert these labeling indices into estimated birth rates for these cells using the formula:

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

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 8-6. 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 8-6).

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 noninitiated group exposed to 125 ng/kg/day of TCDD was 36% which corresponds to a birth rate of 0.0319. The ratio of birth rate in nonfocal cells to birth rate in focal cells is 0.0319/0.00526 = 6.06. 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 show as row 4 in Table 8-6.

Using the same method as that used by Moolgavkar et al. (1991), given these birth rates, it is possible to estimate μ_{N-I} and δ_I for the focal lesion data of Lucier et al. (1991). The resulting parameter estimates are given as rows 5 (μ_{N-I}) and 6 (δ_I/β_I) in Table 8-6. In all cases, the best estimate of the death rate was $\delta_I = 0.01$, an arbitrary lower bound chosen by curve-fitting. This indicates that these birth rates are far too small for the sizes of the lesions observed since death rates of this magnitude would result in almost no loss of initiated cells. (Technical note: the results do not change markedly if the death rate is allowed to be estimated as zero). The estimated mutation rates are also extremely small and have no set pattern to them. Finally, there is considerable lack-of-fit with these parameters of the model to the focal lesion data themselves.

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, starting with the solution given in rows 3 and 4 of Table 8-6 and allowing β_I to roam freely and the ratio δ_I/β_I to be $>10^{-5}$ (rather than 10^{-6}). The resulting parameter estimates are given in rows 5, 6 and 7 of Table 8-6.

The first point to note in this table is that the estimated birth rate in the high dose group (0.0333) is approximately the same as that estimated from the labeling data. This provides some mechanistic support for this particular parameterization of the model. We also see that the death rate of initiated cells (δ_I = ratio · β_I) is near zero in all groups except for the middle dose group. The birth rate is virtually identical in the 3.5, 35.7 and 125 ng/kg/day dose groups corresponding to a labeling index in 7 days of 37.5%. The control group has a birth rate for initiated cells of 0.026 which corresponds to an LI of ~30.5% and the dose group receiving 10.7 ng/kg/day have a birth rate of 0.050 corresponding to an Li of roughly 50%. These values suggest that TCDD may have a small effect on the growth characteristics of initiated cells, but the effect is not as large as might have been expected. There is very little change in the mutation rate as a function of dose level. These small changes do, however, result in changes in the expected number of cells in the initiated state; this is shown in row 11 of Table 8-6. This is calculated as follows: assuming that liver cells have a radius of 0.012 mm, the volume of a single liver cell is 4/3 Π $(.012)^3$ = 1.5079×10^{-3} mm^3. Inverting this gives a total of 663 cells/mm^3. The expected number of initiated cells per mm^3. The expected number of initiated cells per mm^3 is given by the formula:

$$E[X_1] = \frac{X_o \mu_{N-I}}{B_I \delta_I} \left[e^{(B_I - \delta_I) t} - 1 \right]$$

where t = 229 days (the length of the experiment). The percentage is simply $E[X_1]/663 \cdot 100\%$.

The differences observed in the percentage of initiated hepatocytes can be easily explained by examining β_I and μ_{N-I}. For example, the difference in control percentage and high dose group percentage is strictly a change in β_I. Between the high-dose group and the group given 35.7 ng/kg/day, the difference is mutational.

The calculations have hidden assumptions which 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-I} (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 which are initiated can only imply most of these initiated cells are single cells and cannot be observed upon 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.

The parameter estimates given for the model with all parameters estimated (Model B) is still subject to problems of identifiability. One way to limit the impact of this problem is to assume the ratio of death rate to birth rate is constant across all treatment groups as was done by Moolgavkar et al. (1991). The parameter estimates under this restricted model are given at the bottom of Table 8-6. This model is not statistically significant from the unrestricted model and has effectively the same features. The major difference is that for the this model, the parameter estimates follow a clearer pattern. There is a roughly constant birth rate in all dose groups of approximately 3.4×10^{-3} per day, where as dose increase, there is a maximum effect at 35.7 ng/kg/day with a slight decrease in the higher dose group (again, almost to the value observed in the labeling index). The mutation rate seems to reach a minimum at 10.7 ng/kg/day and climbs to slightly higher than control levels in the higher dose group.

As one final exercise with the use of these foci data for risk assessment, we applied the parameters estimated from the liver foci data to the tumor incidence data of Kociba et al. (1978), and only estimated the remaining parameter in the model, $\mu_{I\text{-}N}$. Interpolated values for $\mu_{N\text{-}I}$, β_I and δ_I had to be derived. These are given in Table 8-7.

This resulted estimates of $\mu_{I\text{-}M}$ yielded a model which was not significantly different from the best fitting model ($p>0.10$). This mutation parameter in the second stage shows a dramatic change as a function of dose indicating a strong mutational effect for TCDD in the second stage.

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 which 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. (19??) 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 8-8 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 in rows 2 ("Both"), 3 ("Mut. Rates") and 4 ("Prol. Rates") is shown in the last 3 columns of Table 8-8. It is clear that these 3 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.)

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. (19??), these are integrated over the lifespan of the animal (in this

TABLE 8-7

Using Liver Foci Parameters to Fit Tumor Incidence

Dose	$\mu_{N\text{-}I}$	β_I	δ_I	μ_{IM} (estimated)
Control	1.491×10^{-12}	2.60×10^{-2}	3.95×10^{-4}	1.26×10^{-9}
1 ng/kg/day	1.361×10^{-12}	2.77×10^{-2}	4.21×10^{-4}	1.94×10^{-10}
10 ng/kg/day	0.591×10^{-12}	3.34×10^{-2}	5.08×10^{-4}	2.56×10^{-4}
100 ng/kg/day	1.579×10^{-12}	3.32×10^{-2}	5.05×10^{-4}	4.05×10^{-3}

Like = 66.982

$X^2_{12} = 16.74$

TABLE 8-8

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
Leungg 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[e]	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 EGFR[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

[a]Pearson 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 smaple sizes involved

case, total integrated rather than average, but this will not affect the correlations). As for Leung et al. (19??), 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 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. (19??)], 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. (1992) 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 8-8: 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. (19??) model. Total CYP1A2, which Kohn et al. (19??) 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. (19??) 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 <10-fold change in risk over what would be estimated by applying a one-stage model to the Kociba et al. (19??) 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 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 upon mechanistic considerations. The paper by Kohn et al. (19??) discussing their mechanistic model can be used to provide considerable direction on this topic (see Appendix).

8.2.3. Other Effects 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 occured in the incidence if 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) alteraion 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) and Safe (Romkes and Safe, 1987; Astroff and Safe, 1988, 1990) and Gierthy et al. (1987). This down regulation occurs in a rank order relationship with the affinity of TCDD structural anaolgs 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 cytochromes P-450 is the explanation for the decreased activity of the ER. Safe has reported that the ED_{50} for decreased ER in MCF-7 mice cells is several orders of magnitude below the ED_{50} for induction of EROD in these same cells (Zacharewski et al., 1991). Gierthy's position is supported by Bradlow who suggests that Indolo(3,2ß)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 19unpublished). 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 the Ah locus 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 these is ample evidence that other steroid receptor complexes modify the DNA binding of different members of the steroid receptor super family (Gustafsson et al., 1987; Cutliff et al., 1987).

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-hypothalmus and the estrogen receptors. The ER in liver and uterus is controlled by the pituitary (Lucier et al., 1972) and growth restores the albation 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. (19??) has made similar observations for regulation of testosterone and testicular function by TCDD.

The importance of this subsection is at least 2-fold. The regulation of the estrogen receptor is one of the most sensitive non-P-450 markers of TCDD exposure in 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 by TCDD (Lucier et al., 1991) without altering the ability of TCDD to induce cytochrome P-450IA1 (DeVito et al., 1992).

The modeling of the hepatic tumors clearly shows a biological relationship between the absence of ovaries (which decreases both estrogens and estrogen receptors) and the absence of the hepatic tumors in rats.

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 neurotoxicity which 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 maybe just as sensitive and important in the risk assessment process. For noncancer endpoints, health assessments 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 is available to propose a mechanistic model. Many of the toxic effects of TCDD are well characterized with respect to the dose-response relationship, time-course relationships, species differences and the magnitude for the effects. These type of data can be applied to a variety of curve-fitting models which can be used to describe the responses quantitatively. The development of biologically-based mechanistic models for noncancer endpoints 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 non-cancer endpoints such as TGFα, 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. TCDD affects differentiation, proliferation and programmed cell death. All of these phenomena are involved in the developing nervous system thus making the developing nervous system a potential target for the toxic effects of TCDD. There are only a few studies which have tested the potential neurological and behavioral toxicity of TCDD or other Ah receptor agonists. In primates, prenatal exposure to TCDD produces specific deficits in object learning while not effecting other behavioral tasks such as spatial learning, reflex learning, locomotor activity or fine motor control (Schantz and Bowman, 1989). In rats, prenatal exposure produces a feminization of male sexual behavior but does not alter performance on working or reference memory tasks nor does it affect field locomotor activity (Mably et al., 1992). The potential neurotoxic and behavioral effects of other Ah agonist have also been examined. Prenatal exposure to 3,4,3',4'-tetrachlorobiphenyl

produces permanent alterations in striatal dopaminergic synapses in mice (Agarwal et al., 1981; Chou et al., 1979; Tilson et al., 1979). Several studies have demonstrated that prenatal exposure to PCB mixtures produce a variety of neurological effects in monkeys, rats, mice as well as humans (Tilson et al., 1990).

The neurological and behavioral effects of TCDD and other Ah receptor agonists are not well characterized. The evidence available indicates that TCDD and its congeners can produce behavioral effects in several species and are potentially neurotoxic in humans. Many of the PCBs can decrease dopamine stores in the central nervous system (Seegal et al., 1990). However, structure activity relationships indicate an Ah receptor independent mechanism is involved in the decreased dopamine stores (Shain et al., 1991), and it is unclear if these studies are applicable to TCDD and other Ah receptor agonists. The mechanism of the developmental and behavioral toxicities of TCDD, and its congeners are undetermined. Future studies on the behavioral deficits produced by prenatal exposure to TCDD are warranted to better characterize these effects.

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 phenomenon (Birnbaum et al., 1987a,b, 1991). The doses of TCDD 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 palatal shelves to fuse. Temporal changes in the expression of EGF, TGF-α, TGF-β 1 and TGF-β 2 are critical for the fusion of the palate. Experimental evidence indicates that changes in the 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 [^{3}H]

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-β 1 and TGF-β 2 and decreased TGF-α levels (Abbott and Birnbaum, 1992).

The use of cultured embryo palates (Abbott and Birnbaum, 1990a, 1991; 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 the palate tissue of several species. *In vitro* observations found that the human and rat palates are sensitive to cleft palate formation through the same mechanism 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 ~100-1000 times more sensitive that 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 which increases TGF-ß 1 mRNA and protein, increases in TGF-β 2 and EGF receptor protein levels and decreases TGF-α protein levels (Abbott and Birnbaum, 1992). These changes alter the normal signalling pathways in the medial epithelial cells. In control animals, the interaction between these signalling 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 subsequentially prevents the fusion of the palate (Abbott and Birnbaum ,1989).

This preliminary model for the induction of cleft palate by TCDD requires the better characterization of several steps. Although, structure activity relationships indicate that the Ah receptor is involved, there is no direct 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 are undetermined (Denker and Pratt, 1987). It is presently unknown if the increases in TGF-β 1 mRNA is mediated by the interaction of the Ah receptor with a DRE directly activating transcription of the TGF-β 1 gene or if the increases in TGF-β 1 mRNA is due to the initiation of a cascade of cytosolic-plasma membrane of events by the Ah receptor. Further research is needed into the interaction of the growth factors and their specific roles in palate formation. Much 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 is unavailable at this time.

Cleft palate in rats (Schwetz et al., 1973; Couture et al., 1989) and hamsters (Olson et al., 1990) is induced only at doses that result in significant maternal toxicity and fetal mortality, and maximal induction of cleft palate is between 10% and 20% while in the mouse, cleft palate can reach 100% 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 only occur 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.

8.2.6.3. THYMIC ATROPHY -- Thymic atrophy has been reported in rats, guinea pigs and hamsters, and there has been some suggestion that there are thymic effects in humans. In all mammalian species tested, thymic atrophy is one of the most sensitive clinical indicators of toxicity. Experimental evidence indicates that TCDD induced thymic atrophy segregates with the Ah locus. In human risk assessment TCDD induced thymic atrophy is relevant during fetal development. In animal studies prenatal exposure to TCDD produces thymic atrophy in all species tested and occurred 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 5000-fold difference in the LD_{50} 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 been elucidated sufficiently to incorporate into a biologically based mechanistic model. Current research focuses on the TCDD induced alteration in thymocyte development, and their role in immunotoxicity.

8.2.7. Immunotoxicity. Dioxins are immunotoxic in several species. However, much of the data available on the immunotoxic effects of TCDD are phenomenological and do not provide adequate information to propose a mechanism (Holsapple et al., 1991; Kerkvliet, this document). Decreased T-cell mediated immunity is associated with thymic atrophy which may be due to alterations in the thymic epithelial cells (Greenlee et al., 1985). These cells provide a microenvironment which supports the maturation and differentiation of the T-cells. Dioxin exposure results in the formation of epithelial cell aggregates which may alter the microenvironment in the thymus and inhibit the maturation of T-cells (Vos et al., 1991). However, the mechanism by which this occurs is unclear. While there is ample evidence indicating that these effects are mediated through the Ah receptor, steps beyond the binding of TCDD to the

receptor are unknown. Furthermore, there is evidence that the effects are not solely mediated through changes in the thymic epithelial cells but may involve direct effects on the T-cells (McKonkey et al., 1988) or prothymocytes (Fine et al., 1990). Future studies are required to identify the target tissue involved in the TCDD-mediated thymic atrophy and its relationship to decreases in T-cell mediated immunity. Similarly, the available data on the effects of TCDD on B-lymphocytes are primarily phenomenological. The most consistent effect is the decrease in plaque forming cells in mice in response to sheep red blood cells which have been documented by several laboratories. The ED_{50} of this response ranges from 0.7 to 1.2 μg/kg in female B6C3F/1 mice (Kerkvliet, this document). Inhibition of B cell maturation is thought to be responsible for the suppressed antibody response (Tucker et al., 1986; Luster et al., 1988). Alterations in protein tyrosine phosphorylation have been correlated with inhibition of antibody synthesis *in vitro* (*Clark et al., 1991a). Whether there is a direct cause and effect relationship between changes in phosphorylation and inhibition of antibody synthesis remains to be demonstrated. The suppression of the antibody response appears to be Ah receptor mediated. The lack of information for the mechanism of immunosuppression by TCDD does not allow for mechanistic mathematical modelling of this response.

There are other difficulties in mechanistic modeling of immunosuppressive responses. The quantitative and qualitative relationship between immune function changes and end stage diseases is unclear (Luster et al., 1992). Without increased understanding of these relationships quantitative extrapolation between species remains difficult.

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 at low doses reduces fertility, litter size and uterine weights in some species. TCDD alters menstrual or estrus cycling in monkeys, mice, and rats. These data indicate that TCDD has antiestrogenic effects which may results in decreased reproductive functioning.

The antiestrogenic actions of TCDD could be mediated by either 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 and hepatic estrogen receptors (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 receptor 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 which do not accumulate activated Ah receptors in their nucleus (Zacharewski et al., 1991). These studies provide further evidence that the Ah receptor is directly 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 can be mediated either by decreased transcription of the estrogen receptor gene or 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 2- to 4-fold 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.

8.2.8.2. MALE REPRODUCTIVE TOXICITY -- When administered to adult rats, TCDD decreases testis and accessory sex organ weights, decreases spermatogenesis and reduced 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). While 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.

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). Maternal doses as low as 0.064 μg/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. It is also possible that these effects are due in part to alterations in tissue sensitivity to testosterone (Mably et al., 1991). The exact mechanism of these effects is unknown and the precise target tissue remains undetermined.

In summary, there is ample evidence that noncancer endpoints are extremely sensitive to the toxic effects of TCDD. However, for many of these effects the mechanism by which they occur are unknown and in some cases the target tissue remains undetermined. Furthermore, few if any of the molecular events beyond ligand binding to the Ah receptor are understood. The only information we have to develop mechanistic models is dose-response relationships. The available data do not provide enough information to develop biologically based mechanistic models for noncancer endpoints. Future studies which better characterize target tissues and the molecular mechanisms underlying these events are indicated.

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. There 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.

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 8-9. 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 (footnote), 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.

Secondly, the endpoint being measured (plaque forming cell response) has a greater numerical value that 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 upon the statistical properties of the endpoint being studied and the sensitivity of a particular response.

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 fron control to low dose is 6% 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 (NTP, 1982). Even more

TABLE 8-9

Toxic Endpoints Data

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 pwe 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 form 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 form 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
	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]	

[a]In ng/kg/day
[b]Gavage dosing
[c]Probablility of getting a tumor prior to the end of the study
[d]Number with tumor/number examined for the tumor
[e]Significantly different form control response ($p<0.05$)
[f]TCDD in diet
[g]Surviav-adjusted using poly-3 adjustment of Portier and Bailer (1989)
[h]Number with tumor/poly-3 adjusted number at risk of tumor
[i]Single intraperitoneal injection

dramatic is the infinite change in response from control (0.00) to low dose (0.019) for cleft palate in the Birnbaum et al. (1989) study. Thus, using this measure of relative potency, cleft palate is the most sensitve 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 increases; the one bounded by 0 and the other unbounded (although for practical purposed, this is easily dealt with). Finally, one could also use absolute (rather than relative change over background), 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 (eventually) 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 which 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% at some chosen dose. This would represent a 5% increase in mortality for potential fetuses and an overall loss of 5% of the total available number of animal lifetimes (resulting in a 5% 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% 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%, Thus, on one scale (life expectancy), the two endpoints, resorptions and cancer, produce the same results, wheras on another scale, mortality by age, they are different. This is illustrated in Rogan et al. (1990??). 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 can not 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 is 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 (Okey, ???; Manchester, ???; Cook and Greenlee, ???) 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 8-10. *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, thyroid tumors and

TABLE 8-10

Similiarities Between Laboratory Animals and Humans in Biological Effects of TCDD*

Effect	Laboratory Animals	Human or Human Cells
In vitro		
Presence of Ah receptor	+	+
Enzyme induction	+	+
Altered pattern of growth and differentation		+
Immunosuppression	+	+
Choracnogenic response	+	+
In vivo		
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 nothing is known on the effects of TCDD on the system.

lung tumors (Fingerhut et al., 1991; Manz et al., 1991; Zobel et al., 1991; Saracci et al., 1991; Poole, this document). Recently three large cohort studies performed by IARC and NIOSH have been completed (Boehringer,???). All three studies included individuals who were suspected of exposure to dioxin as a result of occupational exposure. An increase in thyroid tumors was noted in the IARC registry. Increased risk of all cancers was observed in the NIOSH registry as well as increased risk of respiratory tract and lung cancer (Fingerhuut et al., 1991; Manz et al., 1991). Mortality from several cancers in the Seveso, Italy, area included biliary cancer has been reported (Bertazzi et al., 1989). Although several earlier studies showed a lack of liver tumors in humans, the majority of cohorts were male. Based on data obtained in rats, liver tumor formation by TCDD is partially dependent on ovarian hormones since liver tumor incidence in dioxin-exposed rats is markedly decreased in ovariectomized rats (Lucier et al., 1991). However, TCDD promotion of lung tumors occurs in ovariectomized but not intact rats (*Clark et al., 1991b). These results emphasize the importance of TCDD endocrine interactions in the observed site specificity of cancer. The emerging realization from the human cancer studies is that TCDD is a multisite carcinogen, which is not unexpected if we assume that TCDD is acting like a potent and persistent hormone agonist/antagonist. Likewise, TCDD is a multisite carcinogen in animals (Lucier, this document).

Several non-carcinogenic effects of CDDs and 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. In alliance with these results, workers exposed to TCDD 7-8 years previously during the manufacture of trichlorophenol showed elevated total serum triacylglycerides and cholesterol with decreased HDL (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., 1991). Significant associations between serum dioxin levels and several lipid-related variables were found (percent body fat, cholesterol, triacylglycerols and HDL). Another interesting result of these studies was a positive relationship between dioxin exposure and diabetes, to our knowledge the first report of such an association.

The human to experimental animal comparison is confounded by at least two factors: (1) For every toxic effect 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. 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 are sensitive to several of the toxic effects of CDDs and CDFs and that there is good agreement with the effects observed in laboratory species.

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 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 suggest 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 CYPIA1-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 CYPIA1 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 stuctural 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-a (Choi et al., 1991; Hudson et al., 1985). Second, placenta from humans exposed to rice oil contanimated with polychlorinated dibenzofurans, exhibit markedly reduced EGF stimulted autophophorylation of the EGF receptor and this effect occurred with similar sensitivity as observed in rats (Lucier, 1991; Sunahara et al., 1987). 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 that that responsible for liver cancer.

8.5. HUMAN MODELS

8.5.1. Introduction. Unlike animal data where a 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 thyroxin have been documented. These, however, remain efforts for the future. The focus of this section will be on cancer, and 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 sparsity of TCDD dose-response data for enzyme induction in humans and especially in the human liver and lung basically limit analysis to dose-cancer response. For liver cancer, (Section IV), the female rat dose-response is used since there is very little evidence of TCDD induced liver cancer in humans. 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, Section 8.?.?, involves estimating human intake dose associated with cancer response and curve fitting both additive and multiplicative risk models to the data.

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 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 Rombarge (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 (Cbody), the proportion of that body burden associated with the liver (Fh) increases towards a maximum value (Fmax). This has been previous described for rats (Abraham et al., 1988). The data from the Kociba et al. (1976) in rats, monkeys (McNulty et al., 1982) and from people, using toxic equivalency factor conversions (Kuroki and Masuda, 1978) was examined using an empirical, Michaelis-Menton type saturable binding isotherm:

$$\text{Liver fraction (Fh)} = \text{Fmax Cbody} / (\text{Kd} + \text{Cbody})$$

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 Kd was very similar for people and experimental animals, possibly indicative of similar protein induction dynamics in various species. With different dioxin-like isomers Fmax and Kd 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 Cbody and Fmax are difficult to interpret in biological terms. Cbody represents a body burden of chemical rather than a tissue concentration and the term for maximum liver concentration Fmax 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 CYPIA2 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, (Olson, this document), 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 8-11. 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 Andersen et al. (1992) and Kohn et al. (1992), 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 is ~7000 times as high as that currently used by EPA but only 6-7 times higher than that of other western countries (Kociba, 1991). However, estimates derived below based on human studies showing increased mmortality from lung cancer and all cancers combined suggest that in humans other organs may be for more sensitive than the liver.

8.5.3. Lung Cancer and All Cancers Combined. Data from four recent epidemiology retrospective cohort studies provide evidence of 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 showed increased mortality from all cancers combined. The largest study with 18,000 total workers (Saracci, 1991) showed no increase in overall cancer mortality, but those authors have not presented the data allowing for a latent period. In the Fingerhut et al. (19??) study the results showed significance only for the high-exposure, long

TABLE 8-11

Rat and Human Comparison of Daily TCDD Intakes and Body and Liver Concentration for Equitoxic Response

Parameter	Low Intake		High Intake	
	Rat	Human	Rat	Human
Daily 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 $\mu g/kg^2$	24 $\mu g/kg^2$

[a]Source: Carrier, 1991
[b]Rat NOAEL (Kociba et al., 1978)
[c]Marked liver toxicity; tumors in rats

latent period subcohort. Furthermore, the Saracci et al. (19??) 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.

Kuratsune (1988) reported increased lung cancer in male victims (SMR=3.3, based on eight cases) of the Yusho PCB and CDF contamination rice poisonings. While there are serum measurements and 37 TEF estimates available for this cohort, there was no actual TCDD in the contaminants. Thus, this cohort will also not be used in the modeling effort here.

The largest of the three studies used here is the Fingerhut et al. (1991) study of >5000 U.S. workers from 12 plants producing chemicals contaminated with TCDD. Of 1520 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 1200 men in an herbicide manufacturing plant in Hamburg, Germany, also found statistically significant increases in deaths from lung cancer (SMR=167; 95% C.I. 109-244) and all cancers combined (SMR=139; 95% C.I. 110-175). 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. (19??) studied three subcohorts totaling 250 workers exposed 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 SMR's for all cancer deaths not including lung cancer remain statistically significant in all three studies. Further details of these studies are presented in Chapter 7.

These findings are supported by recent animal evidence from Lucier et al. (1991) who found lung tumors in ovariectomized but not intact female Sprague-Dawley rats following administration of TCDD. Other animal data support the tumor-promoting ability of dioxin in the liver and skin (Pitot et al., 1980).

Based on the evidence from these three studies, a quantitative analysis of dioxin's cancer potency will be modeled from these data. All three of the epidemiology 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 both the representativeness of these sampled subjects and the dose-response model used to estimate risk. The details are presented below.

8.5.3.1. DOSE-RESPONSE MODELS -- The following analysis provides maximum likelihood and 95% 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 the cumulative exposure up to that age. In mathematical terms, this is

$$h_1(t) = BX_t$$

where X_t is the cumulative exposure up to age t, and B, 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 a group exposed at a cumulative level X_j, the sum of the h(t) for all individuals in the group yields

$$E_j = \sum_{i=1}^{N_j} h(t_i) = \sum_{i=1}^{N_j} h_0(t_i) + \sum_{i=1}^{N_j} h_1(t_i)$$

or, using summary notation,

$$E_j = E_{0j} + BX_jW_j$$

where E_j is the total number of expected cancer deaths in the observation period from the group exposed to cumulative 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 jth exposure group; and the parameter B represents the slope of the dose-response model. To estimate B, 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 cumulative dioxin exposure.

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

$$L = \prod_{j=1}^{N} [\exp-(E_{0j} + BX_jW_j)] \; [E_{0j} + BX_jW_j]^{Oj}/o_j!$$

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

$$\frac{d \ln L}{dB} = \sum_{j=1}^{N} -X_jW_j + (O_jX_jW_j \; / \; (E_{0j} + bX_jW_j)) = 0$$

The asymptotic variance for the parameter estimate b is

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

where b is the MLE. This variance can then be used to obtain approximate 95% upper and lower bounds for B. 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

$$h(t) = h_0(t)(1 + BX_t)$$

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

$$E_j/E_{0j} = 1 + BX_j$$

Again, to estimate B, 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

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

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 (EPA, 1992) 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 preceding sections). 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, while there is evidence that dioxin causes some human liver toxicity (Di Domenico and Zopponi, 1986) and that the dioxin-like compounds PCBs and dibenzofurans can also cause liver cancer in humans (Kuratsune, 1988) (see Chapter 7), there is no evidence that TCDD induces cancer in human liver. Thus, while Table 8-11 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% and a liver weight of 2.5%, over 90% 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 ~50-90% of TCDD exposure to the Seveso residents following the 1976 accident occurred via the dermal route but they assumed 100% dermal and inhalation absorption. A more likely 1-10% dermal and 75% inhalation absorption estimate (U.S. EPA, 1985) would project that the inhalation route provided the major TCDD exposure.

The data on body concentration levels in the three studies are presented in Table 8-12. 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 21 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 TCDD serum levels. Based on this correlation, they divided the sample into a high-exposure group (those exposed more than 1 year) and a low-exposure group, which was exposed <1 year. The mean TCDD level of the low-exposure group was 69 ppt,

Table 8-12

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 concentration at time of last exposure adjusted for background
			Including Background	Adjusted for background	
Fingerhut et al., 1991	Surviving cohort of workers from Plants 1 and 2 tested approximately 21 years after last occupational exposure.	253			Range 2,000-32,000[d]
	Exposed >1 year, all	119	mean 418 median 231	226	
	Exposed >1 year, ≥20-year latency	95	mean 462	226[c]	1770
	Exposed <1 year, all	134	mean 69 median 24	19	
	Exposed <1 year, ≥20-year latency	81	mean 78	19[c]	150
Zober et al., 1991	Sample of survivors tested 32 years after the accident.	28			
	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
	Either Chloracne or erythema	16	mean 50 median 15	10	230
	Neither Chloracne or erythema	12	mean 25 median 5.8	0.8	20

[a]Background adjustment by subtracting 5 ppt from median value

[b]Estimation based on adjusted medians, since serum levels are not normally distributed

[c]Estimated based on median for all latency periods

[d]Calculated by Fingerhut et al. (1991) not adjusted for background

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TABLE 8-12 (cont.)

Study	Sample	Sample size	Concentration		Estimated Median Concentration at Time of Last Exposure Adjusted for Background (ppt)
			Including Background	Adjusted for Background	
Manz et al., 1991	sample of nonselected members of cohort tested approximately 31 years after 1954 TCP production stopped	48			
	high exposure scenario	37	mean 296 median 137	123	2750[d]
	medium and low exposure scenario	11	mean 83 median 60	55	1150[d]
Schecter et al., 1989	sample of U.S. veterans of Operation Ranch Hand	10	mean 22 median 17 Range 7-55	12	250[e]
Schecter, 1991	sample of U.S. general population	100	mean 5.2	0	5.2[f]

[d]Assumes no TCDD exposure after 1954

[e]Assumes no TCDD exposure after 1969

[f]Continuous background exposure only, no adjustment

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while that of the high exposure group was 418 ppt. Among the 176 workers last exposed >20 years before, "those with under one year of exposure (N=81) had a mean level of 78 ppt, and those with over one year of exposure (N=95) had a mean level of 462 ppt.

For the Zober et al. (19??) 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, respectively. An alternative breakdown 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. (19??) 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 8-12 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 (Schecter et al., 1988). The Fingerhut et al. (19??) referent controls had a mean level of 7 ppt.

Table 8-12 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. In order 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:

$$C_t = C_0 e^{-kt}$$

where

C_t = concentration at time of measurement

C_0 = estimated concentration at time of last exposure

k_e = elimination constant (per year)

t = years since last exposure

In order to back extrapolate from C_0 to intake dose, the conversion formula used is (C. Jenkins, Personal communication)

$$TD_i = D \cdot t = C_0 \cdot f \cdot k_e t / ((1-e^{-ket})(RA))$$

where

D = dose per body weight per year

f = fraction of adipose tissue = 0.20

t = years of exposure in period i

TD_i = total dose during period i

RA = absorption fraction, assumed to be 0.5, based on 75% absorption via inhalation and significantly less through dermal exposure

Then lifetime average daily dose (LADD) is

$$LADD = TD/(70 \text{ years} \times 365 \text{ days/year})$$

where

$$TD = \text{Total Dose} = \sum_i TD_i$$

An example calculation for the high exposure group of Fingerhut et al. (19??) (average exposure = 6.8 years) yields

$$TD = \frac{(1770\ ppt)(0.20)(0.098)}{1-e^{-}(0.098)(6.8)} = 970\ ng/kg$$

For body dose after external exposure to TCDD ends, the long elimination half-life of 7.1 years leads to continuous exposure. Furthermore, it is reasonable to assume that continued dioxin in the body will continue to elicit

biologic effects. Thus, for the Fingerhut et al. (19??) study high exposure group, the median adipose tissue concentration for the 21-year post-exposure period is estimated as:

$$\bar{C}=\frac{1}{21_0}\int^{21}1770e^{-kt}dt=\frac{1770}{21}\left(\frac{1-e^{-(.098x21)}}{.098}\right)=750ppt$$

and average equivalent intake dose per year is:

$$\bar{D}=\bar{C}xf/RA=\frac{(750x.2)}{.5}=300ng/kg\text{-}year$$

Then total body equivalent intake during the post exposure period is

$$TD_1=\overline{Dt}=300x21=6,300ng/kg$$

and the total dioxin dose intake is estimated as

$$TD = 970 + 6,300 = 7,270 \text{ ng/kg}$$

On a per day intake basis, this LADD is (eq 8.4.3-2) 0.284 ng/kg-day. Estimates for the other subcohorts are also presented in Table 8-13.

This conversion from serum ppt to dose essentially uses the body concentration x time dose metric to account for the long duration time of dioxin in the human body. Estimation of risks could also be made on the basis of estimated dose during the exposure period only, but this would probably substantially overstate the risks.

8.5.3.3. CALCULATION OF RISK ESTIMATES -- Table 8-14 presents the LADD's from Table 8-13, 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

TABLE 8-13

Estimate of Total Dose based on Adjusted Median Concentration at Test Times, Estimated Concentrations at Last Exposure, and Average Duration of Exposure, by Study Cohort

Study	Cohort	Adjusted[e] Median Co (ppt)	Average Exposure Furation (years)	Dose During Exposure Period[a] (ng/kg)	Number Years Post-exposure to End of Follow-up	Dose During Post-exposure Period (ng/kg)[a]	Total Dose (ng/kg)	Lifetime[b] Average Daily Dose (ng/kg/day)
Fingerhut et al., 1991	exposed >1 year, all		6.8		21			
	exposed >1 year, 20 year latency	1770	6.8	970	21	6300	7270	0.284
	exposed <1 year, all		0.3		21		680	
	exposed <1 year, ≥ 20 year latency	150	0.3	60	21	530		0.026
Zober et al., 1990	high exposure scenario	450	0.1[c]	180	32	1760	1940	0.076
	medium exposure scenario	105	0.1	40	32	410	450	0.018
	low exposure scenario	100	0.1	40	32	390	430	0.017
	either chloracne or erythema	230	0.1	90	32	900	990	0.039
	neither chloracne nor erythema	20	0.1	8.0	32	80	88	0.003
Manz et al., 1991	high exposure scenario	2750	2[d]	1210	31	10,350	11560	0.452
	medium and low exposure scenario	1150	2	510	31	4330	4840	0.189

[a]Equation 8.4.3-1
[b]Equation 8.4.3-2
[c]Most exposure assumed due to accident
[d]Estimated based on described TCP production period
[e]From Table 6

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TABLE 14

Estimated Lifetime Average Daily Doses and Relative Risks by Individual Study Cohort

Study	Cohort	LADD (ng/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		5172	116,748	96	84.5	1.13	265	229.9	1.15
	exposed <1 year all									
	exposed >1 year, ≥ 20 year latency	0.284	1520	15,136	43	30.2	1.42	114	78.0	1.46
	exposed <1 year, ≥ 20 year latency	0.026	1516	12,299	19	18.4	1.03	48	46.8	1.02
Zober et al. (1990)	(≥20-year latency)									
	high-exposure scenario	0.076	57	673	3	1.25	2.52	7	4.20	1.67
	medium exposure scenario	0.018	74	524	2	1.03	1.94	8	3.37	2.38
	low exposure scenario	0.017	81	676	0	0.97	0	1	3.39	0.29
	either chloracne or erythema	0.039	109	1199	5	2.09	2.39	14	6.96	2.01
	neither chloracne nor erythema	0.003	103	674	0	1.16	0	2	4.00	0.50
Manz et al. (1991)	(≥20-year latency)[b]				N = 1148			(entry before 1955)		
	high-exposure exposure scenario	0.452	96[a]	No Data	26	15.6	1.67	16	5.72	2.77
	medium and low exposure scenario	0.189	200[a]	No Data				27	17.3	1.56

[a]Entry before 1955

[b]Assumed because of entry before 1955; actual data unavailable

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possible with the Fingerhut et al. (19??) cohort. For the Manz et al. (19??) 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 8-7 and indicate trends with increasing LADD's 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 8-15 and 8-16, 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 (19??) and the Manz (19??) study and for all studies combined. While the slope estimates for both the Manz (19??) and Zober (19??) studies are greater than those for the Fingerhut study, the Fingerhut (19??) data provides the bulk of the weight. Thus, the estimates from the combined studies are closer to those based on the Fingerhut (19??) study alone than to the others.

Also shown in Tables 8-15 and 8-16 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 the same results). For lung cancer these unit risk estimates range from $3x10^{-5}$ to $2x10^{-3}$ $(pg/kg-day)^{-1}$, with the estimates for all studies combined between $6x10^{-5}$ and $1x10^{-4}$ $(pg/kg-day)^{-1}$. For all cancers combined the range of MLE estimates is between $3x10^{-4}$ and $1.0x10^{-2}$ $(pg/kg-day)^{-1}$ with the estimates based on all studies combined between $3x10^{-4}$ and $5x10^{-4}$ $(pg/kg-day)^{-1}$. These estimates from all studies combined (both lung cancer and total cancers) range from $6x10^{-5}$ to $6x10^{-4}$ $(pg/kgday)^{-1}$. They border the upper-limit estimate of $1.6x10^{-4}$ $(pg/kg-day)^{-1}$ 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. Using the same Kociba (19??) study and LMS model but with the liver histopathology readings from a recent reanalysis (Sauer and Goodman, 1992) and

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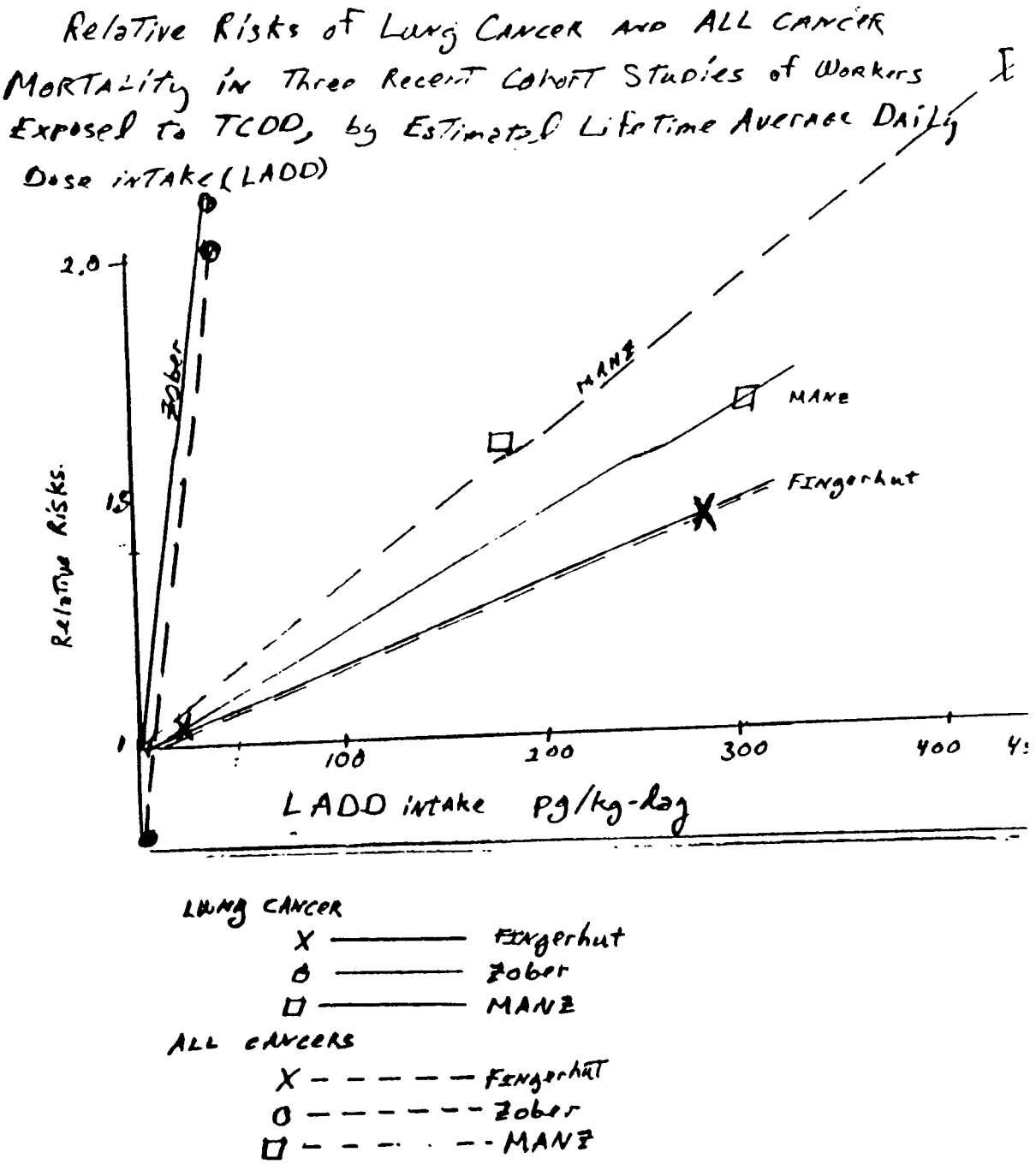

FIGURE 8-7

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.

Source: LADD, 19??

TABLE 15

Calculation of Incremental Unit Cancer Risk Estimates and 95% 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 Estimate[e]	Lifetime Incremental Cancer Risk per 1 pg/kg-day LADD intake 95%	
					Lower Maximum Limit	Likelihood Estimate
Fingerhut et al., 1991	additive	2.96×10^{-3}	2.30×10^{-6}	0.02	0	1.1×10^{-4}
	multiplicative	1.491	0.580	0.02	0	5.7×10^{-5}
Zober et al., 1990	additive					
	H-M-L Scenario	2.78×10^{-2}	8.69×10^{-4}	0.17	0	1.0×10^{-3}
	Chloracne or not	5.78×10^{-2}	2.18×10^{-3}	0.11	0	2.0×10^{-3}
	multiplicative					
	H-M-L Scenario	16.20	269.2	0.16	0	6.1×10^{-4}
	Chloracne or not	33.20	717.0	0.11	0	1.3×10^{-3}
Manz et al., 1991[c]	multiplicative	2.01	1.14	0.03	0	7.6×10^{-5}
All studies combined	additive	3.09×10^{-3}	2.32×10^{-6}	0.02	3.7×10^{-6} 1.5×10^{-6}	1.1×10^{4}
	multiplicative	1.541	0.586	0.02		5.9×10^{-5}

[a]Estimates for additive risk model gives in $(ng/kg\text{-}day)^{-1}$. Estimates for multiplicative risk model given in $(ng/kg\text{-}day)^{-1}/h_o(t)$ where $h_o(t)$ = background age-specific hazard rate

[b]MLE for additive risk model approximated by multiplying by 35 since risk before age 35 is close to zero. MLE for multiplicative risk model approximated by multiplying P_o=0.038

[c]Data set in Table 8.4.3-3 with estimated LADD = 0.3 ng/kg - day

[d]Fingerhut and Zober (chloracne) cohorts only

[e]One sided

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TABLE 16

Calculation of Incremental Unit Risk Estimates and 95% 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[e]	Lifetime Incremental Cancer Risk per 1 pg/kg-day LADD intake 95%	
					Lower Maximum Limit	Likelihood Estimate[b]
Fingerhut et al., 1991	additive	8.32×10^{-3}	6.2×10^{-6}	0.0004	2.4×10^{-4}	5.8×10^{-4}
	multiplicative	1.62	0.230	0.0004	1.3×10^{-4}	3.0×10^{-4}
Zober et al., 1990	additive					
	H-M-L scenario	5.80×10^{-2}	2.4×10^{-3}	0.12	0	4.0×10^{-4}
	chloracne or not	1.44×10^{-1}	6.2×10^{-3}	0.03	0	1.0×10^{-2}
	multiplicative					
	H-M-L scenario	10.11	64.6	0.11	0	1.9×10^{-3}
	chloracne or not	24.70	184.3	0.03	0	4.6×10^{-3}
Manz et al., 1991	multiplicative	3.53	1.22	0.001	5.6×10^{-4}	6.5×10^{-4}
All studies combined	additive[c]	8.57×10^{-3}	6.1×10^{-6}	<0.001	2.6×10^{-4}	6.0×10^{-4}
	multiplicative[d]	2.07	0.198	<0.0001	2.2×10^{-4}	3.4×10^{-4}

[a] Estimates for additive risk model given in $(ng/kg\text{-}day^{-1})$; estimates for multiplicative risk model given in $(ng/kg\text{-}day)^{-1}/h_o(t)$ where $h_o(t)$ = background age specific hazard rate

[b] MLE for additive risk model approximated by multiplying b by 70; MLE for multiplicative risk model approximated by multiplying by P_o=0.185

[c] Fingerhut and Zober (chloracne) cohorts only

[d] Fingerhut, Zober (chloracne) and Manz

[e] One sided

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original Kociba readings for the other tumor sites, the upper-limit estimate is 0.8×10^{-4}.

8.5.3.4. LOW-DOSE DEVIATION FROM LINEARITY -- Based on the LADD's and the relative risk estimates presented in Table 8-14 some idea of the degree of nonlinearity in the dose response for these cancers can be derived. For the Fingerhut et al. (19??) cohort the ratio of 10.9 for high to low LADD's (0.284/0.026) 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. (19??) cohort, the comparisons are also consistent with linearity or slight sublinearity; the LADD ratio of 2.4 (0.452/0.189) corresponds to an increased risk ratio of 3.2 (1.77/0.56). The Zober et al. (19??) chloracne versus no chloracne cohort also suggests some low dose sublinearity, but no quantitative comparisons can be derived, since the relative risk estimates for the low-dose group are <1. For the Saracci et al. (19??) 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 three of the cohorts there is some suggestion of sublinearity in dose response, but for the largest cohort the suggestion is the opposite.

Estimates of LADD's of TCDD intake in the general U.S. population range from 0.3-1.0 pg/kg-day (U.S. EPA, 1992). The LADD's estimated in these studies range from 3-452 pg/kg-day above background with increased risks seen >20 pg/kg-day. The LADD estimates, themselves are just too imprecise for more definitive statements.

8.6. 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. The studies also show increases for all cancers combined. Estimates derived from the data suggest that a range of estimated unit risk for lung cancer is 6×10^{-5} to 1×10^{-4} $(pg/kg\text{-}day)^{-1}$. For all cancers combined the range of unit risk is 3×10^{-4} to 6×10^{-4} $(pg/kg\text{-}day)^{-1}$.

Estimates based on mortality from all cancers suggest that humans are also sensitive to an overall carcinogenic effects of TCDD.

8.7. 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 reflect 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 which 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 which accurately predict risks also require 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 is 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 comprised of numerous cell types but the identity of the target cell(s) for TCDD-mediated lung cancer are not known nor is there much 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 1-3 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 which 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 CYPIA1 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 CYPIA1 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 8-8 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 be comprised of 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 non-cancer 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 which produce diversity of responses for receptor-mediated responses, that is, how does a single receptor and a single ligand produce the wide spectrum of cell 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.

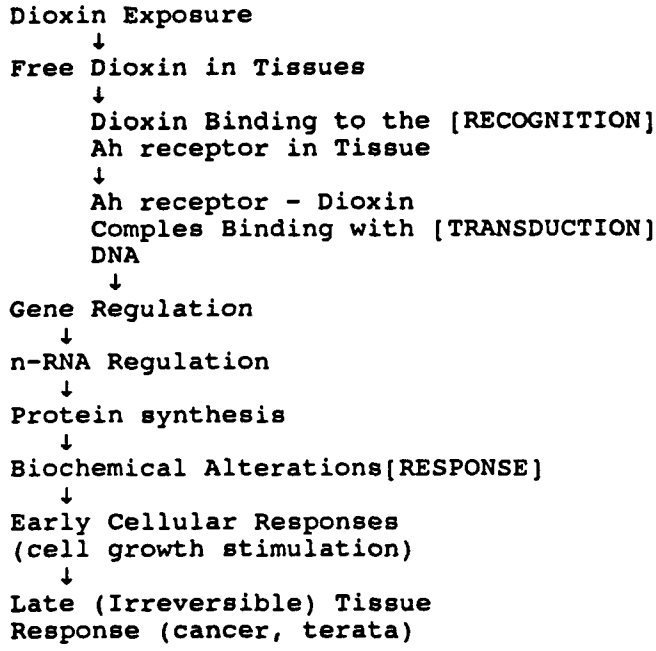

FIGURE 8-8

Biologically Based Risk Assessment Approaches for Dioxin:
Filling the Gaps

Source: Greenlee et al. 1992

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
squelching

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 mechanism 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.

Specific experiments should be developed as part of the peer-review process to fill knowledge gaps that limit application of dose-response models.

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APPENDIX A

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

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Running title: Model of Effects of Dioxin on Hepatic Gene Expression

Abstract

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. (199_) *Toxicol. Appl. Pharmacol.* ___, ___-___. 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-alpha (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.

Introduction

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 hyperplastic 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 ligated 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 keratinocytes (Osborne et al., 1988) or mouse liver (Lin et al., 1991a). 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 ligated receptor.

The liver does not produce EGF, but there is evidence (Mead and Fausto, 1989) that it does produce transforming growth factor-alpha (TGF-α), another ligand of the EGF receptor. 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 tis-

sue 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. 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. (1992). 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 one week following the last dose, and their livers were assayed for TCDD, CYP1A1 and 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, Puastenbach, Murray, and Andersen (1990), hereafter referred to as the LPMA model. To fit the data of Tritscher et al. (1992) and Sewall et al. (1992), the LPMA model has been modified as described below. A flowchart of the resulting model is given in Figure 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 (nmole 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., 1992). 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 g 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

$$\frac{BloodContent \times Flow}{BloodVolume}$$

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

$$\frac{TissueContent \times Flow}{TissueVolume \times PartitionCoeff}$$

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 for 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 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 only binds 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 are included in the NIEHS model. Birnbaum et al. (1980) administered a single oral dose of ^{14}C-labelled 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 hours in their experiments (32 day^{-1}) was an order of magnitude higher than after 3 hours (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 calculated rate constants for clearance of radiolabel from liver and from blood after 3 hours (Birnbaum et al., 1980). The higher rate constant for clearance at early time more likely reflects the rate of uptake of labelled material from the blood by the various tissues, which therefore cannot be rate limiting for clearance. The rates of transport of TCDF's 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 hours. 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 μg/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 ligated 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 nM to 0.012 nM on 32-fold dilution of the Ah receptor protein. Because this protein is induced by TCDD, the K_d in the intracellular milieu should be closer to the value at the higher concentration. Therefore, the model uses the comparable value of 0.27 nM reported by Poland and Knutson (1982) for the K_d of TCDD.

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 hours elapse 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 hours 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 pro-

tein 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., 1992) at a liver TCDD content of 0.063 nmole/g (Tritscher et al., 1992). Ovariectomized rats exhibit only a 19% reduction of EGF B_{max} at a liver TCDD content of 0.106 nmole/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 ten days following gavage with 10 μg TCDD/kg. Madhukar et al. (1984) observed a 45% decline in the B_{max} of the EGF receptor of male rats ten days after a single intraperitoneal injection of 10 μg TCDD/kg. The NIEHS model predicts a liver TCDD content of 0.217 nmole/g for females and 0.223 nmole/g for males at the end of the ten 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 nine-fold rise in CYP1A2 resulting from exposure to TCDD. Only a three-fold 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 Werringloer, 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 were 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 ten 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 amount of estrogen receptor in the livers of ovariectomized or immature female rats is

increased by added 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 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., 1992). 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. (1992). 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 either obtained from the literature, constrained by experimental data, or were 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. (1992) 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. About 2% of the cumulative dose is computed to appear as urinary metabolites. 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 Figures 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 (Figure 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 data of Tritscher et al. (1992). Hill exponents of 1.0 for induction of CYP1A1 (Figure 5) and CYP1A2 (Figure 6) reproduced the observed responses. Because binding of TCDD to 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 expo-

nent 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 Gasewicz (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 consequent 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. (1992) are also shown. Note that the TGF-α concentration is given in pmole/g liver; multiplying by 10 gives the concentration in the interstitial fluid in nM. 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 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 ten days after administration of a single dose of 1 μg 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 two-fold greater responsiveness of the female over the male persisted over all doses in the range of 0.1 μg/kg to 10 μg/kg.

As estrogen plays a major role in regulating TCDD's effects on the EGF receptor, estrogen action was examined in 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 pmole/g to 2.3 pmole/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 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 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 car-

cinogenesis. 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. 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 species (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 two-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, indicating 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 ten 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 posttranscriptional 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 (Figure 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 (Figure 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. (1992) 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. (1992) 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, constitu-

tive 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 (Figure 5). It is also possible that CYP1A1 is constitutively expressed at a low rate by a mechanism that doesn't 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 the ER-E complex induces synthesis of the estrogen receptor protein, a reduced 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 five-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 nmole/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

$$\text{(fraction lipid)} = 0.01865 \times \text{(nmole total liver TCDD)} + 0.1443 \times \text{(kg body weight)}$$

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., 1992). 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 et al., 1991)

StartWeight = 0.237 kg (Tritscher et al., 1992)

BodyWeight = $\text{StartWeight} + 0.219674 \times e^{-0.002859 \times \text{dose}} \times \text{time} / (116.345 + \text{time})$

(*BodyWeight was estimated from the data of* Tritscher et al. (1992) *as a function of dose and time by nonlinear regression*)

BloodVolume = 0.054 × BodyWeight (Delp et al., 1991)

FatVolume = 0.08 × BodyWeight (*Average of literature values*: Leung et al., 1990; Delp et al., 1991)

MuscleVolume = 0.59 × BodyWeight (Delp et al., 1991)

(*This compartment also includes skin and other slowly perfused tissues*)

Viscera Volume = 0.083 × BodyWeight (Delp et al., 1991)

(*This compartment includes all richly perfused tissues*)

LiverVolume = BodyWeight × (0.03324 + 0.01927 × dose / (88.91 + dose))

(*Liver weight was estimated from the data of Tritscher et al. (1992) as a function of dose by nonlinear regression*)

LiverInterstitialVolume = frac_interstitium × LiverVolume

LiverFlow = 0.072 × CardiacOutput (Delp et al., 1991)

FatFlow = 0.07 × CardiacOutput (Delp et al., 1991)

MuscleFlow = 0.36 × CardiacOutput (Delp et al., 1991)

Viscera Flow = 0.40 × CardiacOutput (Delp et al., 1991)

Distribution of TCDD

GutTCDD = GutTCDD + dose(t) × BodyWeight / 322

(*Doses at time t—in ng/kg—were introduced into the gut compartment according to the experimental schedule*; dose(t) = 0 *if no dose is scheduled at time t*)

GutTCDD′ = $-\text{k_absorption} \times \text{GutTCDD} / \text{BodyWeight}^{0.75} - \text{k_feces} \times \text{GutTCDD}$

FecesTCDD′ = k_feces × GutTCDD

cum_exposure′ = LiverTCDD

BloodProtein = MaxBloodProtein × BloodVolume × Ki_BloodProtein / (Ki_BloodProtein + LiverTCDD / LiverVolume)

BloodTCDD′ = k_absorption × GutTCDD / $BodyWeight^{0.75}$ - BloodTCDD × MuscleDiffusion × MuscleFlow / BloodVolume - BloodTCDD × FatDiffusion × FatFlow / BloodVolume - BloodTCDD × VisceraDiffusion × VisceraFlow / BloodVolume - BloodTCDD × LiverDiffusion × LiverFlow / BloodVolume + MuscleTCDD × MuscleDiffusion × MuscleFlow / (MuscleVolume × MusclePartition) + FatTCDD × FatDiffusion × FatFlow / (FatVolume × FatPartition) + VisceraTCDD × VisceraDiffusion × VisceraFlow / (VisceraVolume × VisceraPartition) + LiverTCDD × LiverDiffusion × LiverFlow / (LiverVolume × LiverPartition) - k_binding × BloodTCDD × BloodProtein + k_binding × K_BloodProtein × LiverVolume × BloodBoundTCDD

BloodBoundTCDD′ = k_binding × BloodTCDD × BloodProtein - k_binding × K_BloodProtein × LiverVolume × BloodBoundTCDD

MuscleTCDD′ = BloodTCDD × MuscleDiffusion × MuscleFlow / BloodVolume - MuscleTCDD × MuscleDiffusion × MuscleFlow / (MuscleVolume × MusclePartition)

FatTCDD′ = BloodTCDD × FatDiffusion × FatFlow / BloodVolume - FatTCDD × FatDiffusion × FatFlow / (FatVolume × FatPartition)

VisceraTCDD′ = BloodTCDD × VisceraDiffusion × VisceraFlow / BloodVolume - VisceraTCDD × VisceraDiffusion × VisceraFlow / (VisceraVolume × VisceraPartition)

LiverTCDD′ = BloodTCDD × LiverDiffusion × LiverFlow / BloodVolume - LiverTCDD × LiverDiffusion × LiverFlow / (LiverVolume × LiverPartition) - k_metabolism × LiverTCDD - k_binding × LiverTCDD × LiverCYP1A2 + k_binding × K_TCDD × LiverVolume × LiverCYP1A2_TCDD - k_binding × LiverTCDD × LiverAhReceptor + k_binding × K_AhR × LiverVolume × LiverAhR_TCDD + k_proteolysis × (LiverAhR_TCDD + LiverCYP1A2_TCDD) - k_lysis × cum_exposure / (crit_exposure + cum_exposure) × LiverTCDD

$$\text{LiverCYP1A2_TCDD}' = \text{k_binding} \times \text{LiverTCDD} \times \text{LiverCYP1A2} - \text{k_binding} \times \text{K_TCDD} \times \text{LiverVolume} \times \text{LiverCYP1A2_TCDD}$$

TCDD Metabolite Clearance

$$\text{LiverMetabolite}' = \text{k_metabolism} \times \text{LiverTCDD} - \text{k_bile} \times \text{LiverMetabolite} + \text{BloodMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow} / \text{BloodVolume} - \text{LiverMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow} / (\text{LiverVolume} \times \text{LiverPartition})$$

$$\text{BloodMetabolite}' = - \text{BloodMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow} / \text{BloodVolume} + \text{LiverMetabolite} \times \text{LiverDiffusion} \times \text{LiverFlow} / (\text{LiverVolume} \times \text{LiverPartition}) - \text{k-urine} \times \text{BloodMetabolite}$$

$$\text{UrineMetabolite}' = \text{k_urine} \times \text{BloodMetabolite}$$

$$\text{GutMetabolite}' = \text{k_bile} \times \text{LiverMetabolite} - \text{k_feces} \times \text{GutMetabolite}$$

$$\text{FecesMetabolite}' = \text{k_feces} \times \text{GutMetabolite}$$

Ah Receptor and P450 Induction

$$\text{LiverAhR_TCDD}' = \text{k_binding} \times \text{LiverTCDD} \times \text{LiverAhReceptor} - \text{k_binding} \times \text{K_AhR} \times \text{LiverVolume} \times \text{LiverAhR_TCDD} - \text{k_proteolysis} \times \text{LiverAhR_TCDD}$$

$$\text{LiverAhR_Inducer}' = \text{k_binding} \times \text{LiverVolume} \times \text{Natural_Inducer} \times \text{LiverAhReceptor} - \text{k_binding} \times \text{K_AhR} \times \text{LiverVolume} \times \text{LiverAhR_Inducer} - \text{k_proteolysis} \times \text{LiverAhR_Inducer}$$

$$\text{LiverAhReceptor}' = \text{V_AhRinduction} \times \text{LiverVolume} / (1 + (\text{K_AhRinduction} \times \text{LiverVolume}) / \text{LiverAhR_TCDD}(t - \text{lt})) + \text{AhRexpression} \times \text{LiverVolume} - \text{k_binding} \times \text{LiverTCDD} \times \text{LiverAhReceptor} + \text{k_binding} \times \text{K_AhR} \times \text{LiverVolume} \times \text{LiverAhR_TCDD} - \text{k_binding} \times \text{LiverVolume} \times \text{Natural_Inducer} \times \text{LiverAhReceptor} + \text{k_binding} \times \text{K_AhR} \times \text{LiverVolume} \times \text{LiverAhR_Inducer} - \text{k_proteolysis} \times \text{LiverAhReceptor}$$

$$\text{LiverCYP1A1}' = \text{V_CYP1A1induction} \times \text{LiverVolume} / (1 + (\text{K_CYP1A1induction} \times \text{LiverVolume}) / (\text{LiverAhR_TCDD}(t - \text{lt}) + \text{LiverAhR_Inducer}(t - \text{lt}))) - \text{k_proteolysis} \times \text{LiverCYP1A1} - \text{k_binding} \times \text{LiverCYP1A1} \times \text{LiverP450Reductase} + \text{k_binding} \times \text{K_Reductase} \times \text{LiverVolume} \times \text{LiverCYP1A1_R}$$

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 × LiverPartition) - 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_CYP1A1induction × 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

$$\text{LiverCYP1A2_R_E2}' = \text{k_binding} \times \text{LiverCYP1A2_E2} \times \text{LiverP450Reductase} - \text{k_binding} \times \text{K_Reductase} \times \text{LiverVolume} \times \text{LiverCYP1A2_R_E2} + \text{k_binding} \times \text{LiverCYP1A2_R} \times \text{LiverE2} - \text{k_binding} \times \text{K_E2} \times \text{LiverVolume} \times \text{LiverCYP1A2_R_E2} - \text{V_E2H} \times \text{LiverCYP1A2_R_E2}$$

$$\text{LiverE2OH}' = \text{V_E2H} \times \text{LiverCYP1A2_R_E2} - \text{k_binding} \times \text{LiverEReceptor} \times \text{LiverE2OH} + \text{k_binding} \times \text{K_ER_E2OH} \times \text{LiverVolume} \times \text{LiverER_E2OH} - \text{k_conjugation} \times \text{LiverE2OH}$$

$$\text{LiverEReceptor}' = \text{V_ERinduction} \times \text{LiverVolume} / ((1 + \text{K_ERinduction} \times \text{LiverVolume} / (\text{LiverER_E2}(t - \text{lt}) + \text{LiverER_E2OH}(t - \text{lt}))) \times (1 + \text{LiverAhR_TCDD}(t - \text{lt}) / (\text{Ki_ERinduction} \times \text{LiverVolume}))) + \text{ERexpression} \times \text{LiverVolume} - \text{k_proteolysis} \times \text{LiverEReceptor} - \text{k_binding} \times \text{LiverEReceptor} \times \text{LiverE2} + \text{k_binding} \times \text{K_ER_E2} \times \text{LiverVolume} \times \text{LiverER_E2} - \text{k_binding} \times \text{LiverEReceptor} \times \text{LiverE2OH} + \text{k_binding} \times \text{K_ER_E2OH} \times \text{LiverVolume} \times \text{LiverER_E2OH}$$

$$\text{LiverER_E2}' = - \text{k_binding} \times \text{LiverEReceptor} \times \text{LiverE2} + \text{k_binding} \times \text{K_ER_E2} \times \text{LiverVolume} \times \text{LiverER_E2} - \text{k_proteolysis} \times \text{LiverER_E2}$$

$$\text{LiverER_E2OH}' = - \text{k_binding} \times \text{LiverEReceptor} \times \text{LiverE2OH} + \text{k_binding} \times \text{K_ER_E2OH} \times \text{LiverVolume} \times \text{LiverER_E2OH} - \text{k_proteolysis} \times \text{LiverER_E2OH}$$

EGF Receptor and TGF-a

$$\text{LiverEGFReceptor}' = \text{EGFRexpression} \times \text{LiverVolume} - \text{k_endocytosis} \times \text{LiverEGFReceptor}$$

$$\text{LiverTGF}' = \text{V_TGFinduction} \times \text{LiverVolume} / (1 + \text{K_TGFinduction} \times \text{LiverVolume} / (\text{LiverAhR_TCDD}(t - \text{lt}) \times (1 + \text{Ka_TGFinduction} \times \text{LiverVolume} / (\text{LiverER_E2}(t - \text{lt}) + \text{LiverER_E2OH}(t - \text{lt}))))) - \text{k_proteolysis} \times \text{LiverTGF} - \text{k_binding} \times \text{LiverEGFReceptor} \times \text{LiverTGF} + \text{k_binding} \times \text{K_TGF} \times \text{LiverVolume} \times \text{LiverInternalEGFR}$$

$$\text{LiverInternalEGFR}' = \text{k_binding} \times \text{LiverEGFReceptor} \times \text{LiverTGF} - \text{k_binding} \times \text{K_TGF} \times \text{LiverVolume} \times \text{LiverInternalEGFR} - \text{k_endocytosis} \times \text{LiverInternalEGFR}$$

Initial Conditions

LiverAhReceptor	=	0.017806 nmole (Sloop and Lucier, 1987)
LiverAhR_Inducer	=	0.000674 nmole[b]
LiverCYP1A1	=	0.063174 nmole (Tritscher et al., 1992)
LiverCYP1A1_R	=	0.094902 nmole[b]
LiverCYP1A2	=	2.4199 nmole (Tritscher et al., 1992)
LiverCYP1A2_R	=	1.9799 nmole[a]
LiverP450Reductase	=	0.602536 nmole (Miwa et al., 1978)
LiverE2	=	0.00123 nmole[b]
LiverEReceptor	=	0.01495 nmole (Clark et al., 1991)
LiverER_E2	=	0.02325 nmole[a]
LiverER_E2OH	=	0.0066792 nmole[a]
LiverEGFReceptor	=	0.022563 nmole (Sewall et al., 1992)

(*All other state variables start at zero.*)

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a.. Determined from conservation of mass

b. Assumed at equilibrium with blood

Appendix 2

Physiological parameters

frac_interstitium = 0.1

lt = 0.25 day (Sloop and Lucier, 1987)

(*delay between binding of ligated receptor to DNA and appearance of induced proteins in the liver cell*)

ConcBloodE2 = 0.185 nmole/L (Shiverick and Muther, 1983)

crit_exposure = 0.3 nmole

Production of blood proteins[a]

MaxBloodProtein = 5 nmole/L

Ki_BloodProtein = 0.1 nM

K_BloodProtein = 0.4 nM

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 et al., 1987)

K_ER_E2OH = 1.3 nM (Li et al., 1985)

K_TGF = 0.28 nM (Hudson et al., 1985)

K_TCDD = 30 nM (Poland et al., 1989; Voorman and Aust, 1989)

Constitutive expression rates[b]

AhRexpression = 1.4553 nmole/L/day

ERexpression = 0.67273 nmole/L/day

CYP1A2expression = 213.4 nmole/L/day

P450Reductase_expression = 47.45 nmole/L/day

EGFRexpression = 0.69376 nmole/L/day

Endogenous ligand of Ah receptor

Natural_Inducer = 0.0015 nmole/L[a]

(*assumed same K_d as for TCDD*)

Gene induction parameters

V_AhRinduction = 7.2 nmole/L/day (*Lower bound of* 4 nmole/L/day: Sloop and Lucier, 1987)

K_AhRinduction = 4 nM (Sloop and Lucier, 1987)

V_CYP1A1induction = 3319 nmole/L/day[a]
K_CYP1A1induction = 4.279 nM[a]
V_CYP1A2induction = 4197 nmole/L/day[a]
K_CYP1A2induction = 7.458 nM[a]
V_ERinduction = 3.2488 nmole/L/day (Romkes et al., 1987)[c]
K_ERinduction = 0.35 nM[c]
Ki_ERinduction = 3.1 nM[c]
V_TGFinduction = 1.5 nmole/L/day[d]
K_TGFinduction = 9 nM[d]
Ka_TGFinduction = 0.75 nM[d]

Metabolic parameters

V_E2H = 8496 day^{-1} (Graham et al., 1988)
K_E2 = 9400 nM (Voorman and Aust, 1987)
K_Reductase = 83.5 nM (Miwa et al., 1987)

Rate constants

k_absorption = 4.8 $kg^{0.75}$/day (Leung et al., 1990)
k_binding = 10^5 $nmole^{-1}day^{-1}$
(value selected to ensure that binding reactions are always close to equilibrium)
k_proteolysis = 0.693 day^{-1} (Lucier et al., 1972)
k_endocytosis = 0.271 day^{-1} [d]
k_metabolism = 2.75 day^{-1}
k_urine = 5.36 day^{-1} (Birnbaum et al., 1980)
k_bile = 3.81 day^{-1} (Birnbaum et al., 1980)
k_feces = 1.152 day^{-1} (Lutz et al., 1977)
k_conjugation = 56.693 day^{-1} (Lucier and McDaniel, 1977)
k_lysis = 9.5 day^{-1}

Partition and diffusion coefficients

FatPartition = 350 (Leung et al., 1990)
MusclePartition = 40 (Leung et al., 1990)
VisceraPartition = 20 (Leung et al., 1990)
LiverPartition = 20 (Leung et al., 1990)
FatDiffusion = 0.1[e]
MuscleDiffusion = 0.5[e]
VisceraDiffusion = 0.5[e]
LiverDiffusion = 0.8[e]

a. Determined from the data of Tritscher et al. (1992)

b. Determined from conservation of mass

c. Determined from the data of Clark et al. (1991)

d. Determined from the data of Sewall et al. (1992)

e. Selected to fit the data of Abraham et al. (1988)

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Table 1: Computed TCDD distribution

Dose, ng/kg/day	Blood, nM[a]	Liver, nmole/g[b]	Fat, nmole/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. A.M. Tritscher, unpublished results

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

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 nmole/g tissue; observed values (Rose et al., 1976) in parentheses

Table 3: Fit of model to observed TCDD concentrations following single doses[a]

Dose, ng/kg	Liver, nmole/g	Fat, nmole/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 in parentheses
b. Rose et al. (1976)
c. J. Vanden Heuvel, NIEHS, personal communication
d. Abraham et al. (1988)

Table 4: Ah receptor and induced cytochrome P450 isozymes

Dose, ng/kg/day	Ah Receptor, pmole/g	CYP1A1, nmole/g[a]	CYP1A2, nmole/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

Table 4: Ah receptor and induced cytochrome P450 isozymes

Dose, ng/kg/day	Ah Receptor, pmole/g	CYP1A1, nmole/g[a]	CYP1A2, nmole/g[a]
115.0	8.24	4.612	4.899
125.	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) given in parentheses
b. Poland and Knutson (1982)
c. Sloop and Lucier (1987)
d. Maximal induction from Poland and Knutson (1982)

Table 5: Effects of TCDD on the EGF receptor

Dose, ng/kg/day	EGF Receptor, pmole/g[a]	TGF-α, pmole/g	Internalized EGF Receptor, pmole/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. (1992) given in parentheses

Table 6: Effects of TCDD on estrogen metabolism in liver

Dose, ng/kg/day	Estrogen Receptor, pmole/g[a]	Estradiol, pmole/g	Catechol Estrogen, pmole/g	Estradiol 2-Hydroxylase, nmole/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) given in parentheses

Figure Captions

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

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

Figure 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, squares are for observations in fat.

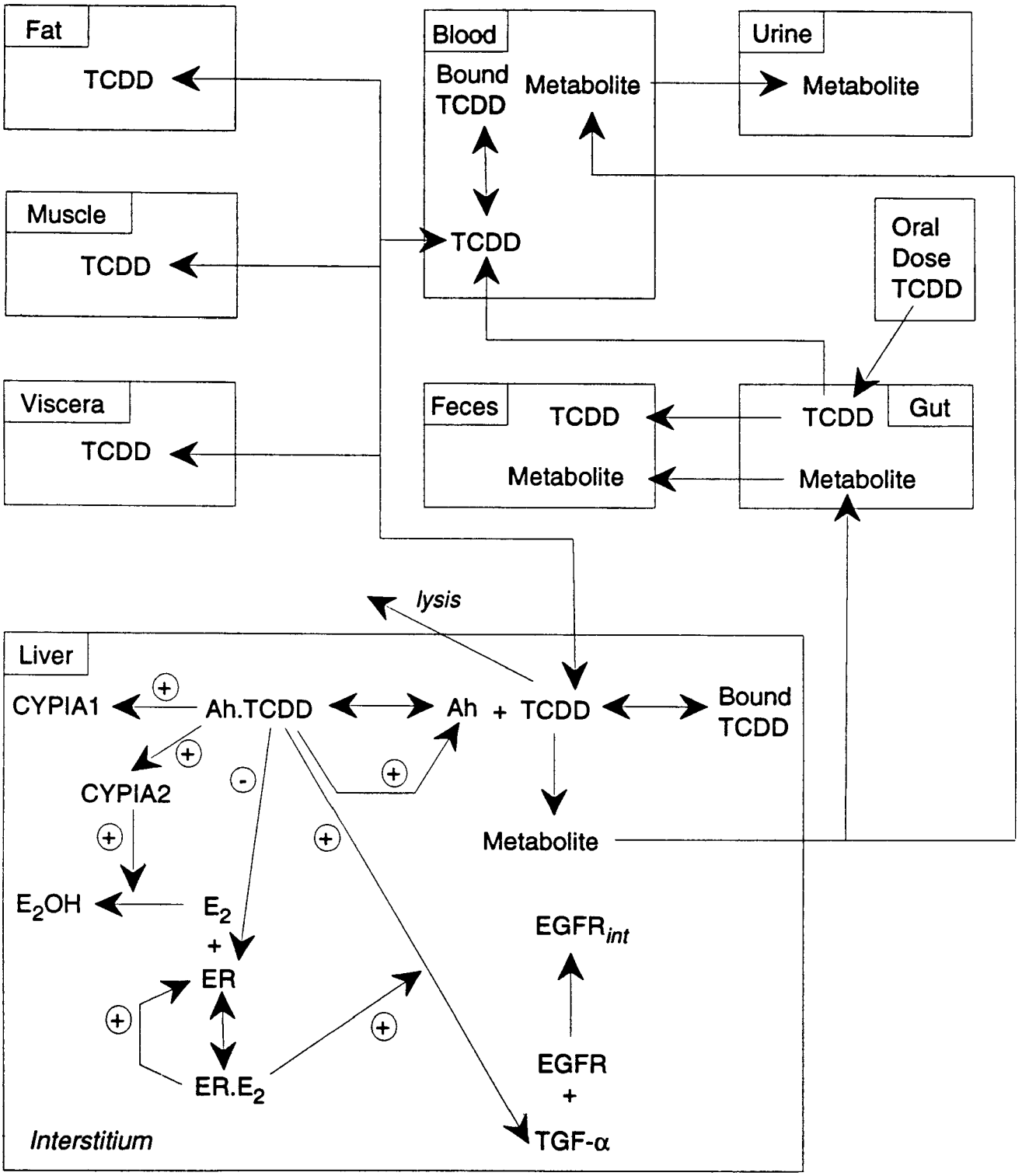
Figure 4. Computed relationship between liver TCDD concentration and administered dose. Experimental data points (filled circles) are the average values of Tritscher et al. (1992). Error bars denote the range of observed values.

Figure 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.

Figure 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.

Figure 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. (1992). Error bars denote the range of observed values.

Figure 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. (1992).


Fat
TCDD
Blood
Bound TCDD
Metabolite
Urine
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Muscle
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Oral Dose TCDD
Viscera
TCDD
Feces
TCDD
Metabolite
TCDD
Gut
Metabolite
lysis
Liver
CYPIA1
Ah.TCDD
Ah + TCDD
Bound TCDD
CYPIA2
Metabolite
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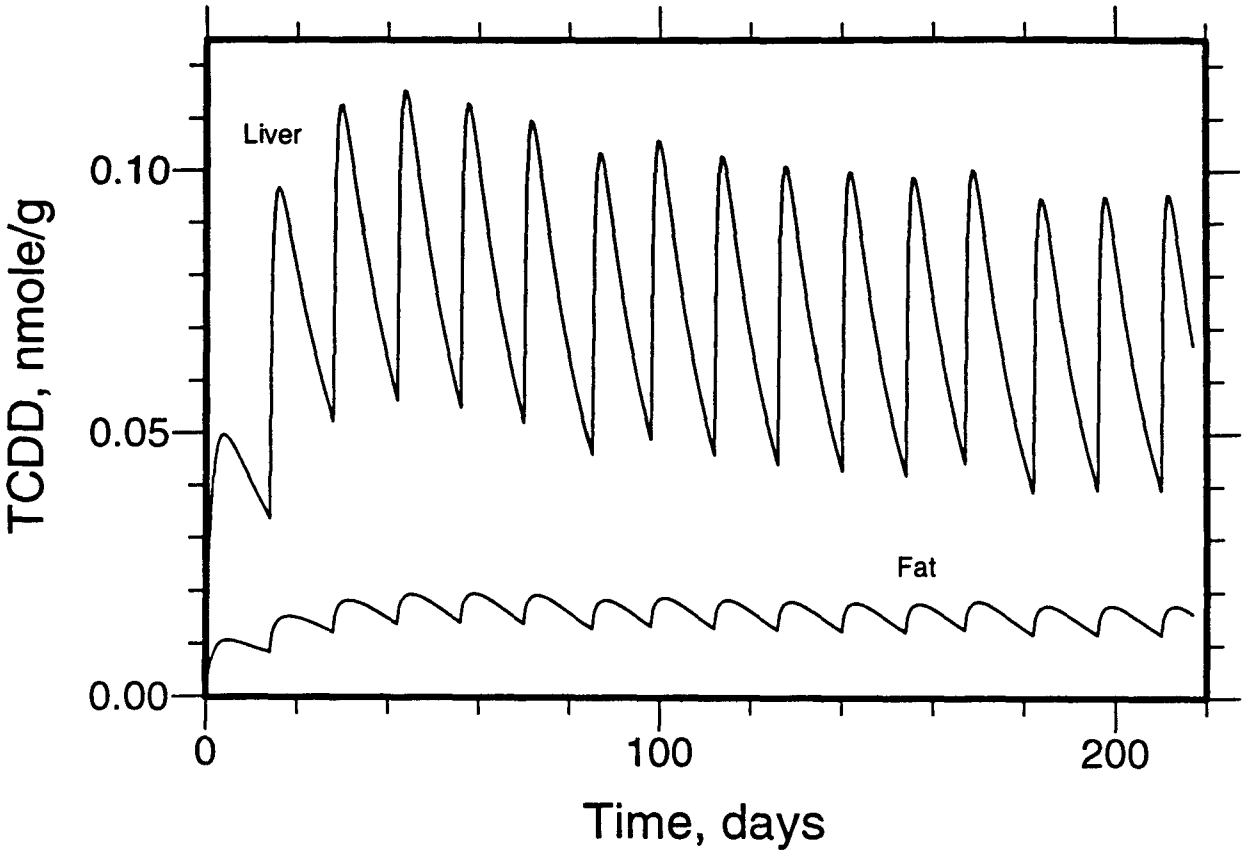
+
ER
ER.E2
EGFRint
EGFR
+
TGF-α
Interstitium


Liver
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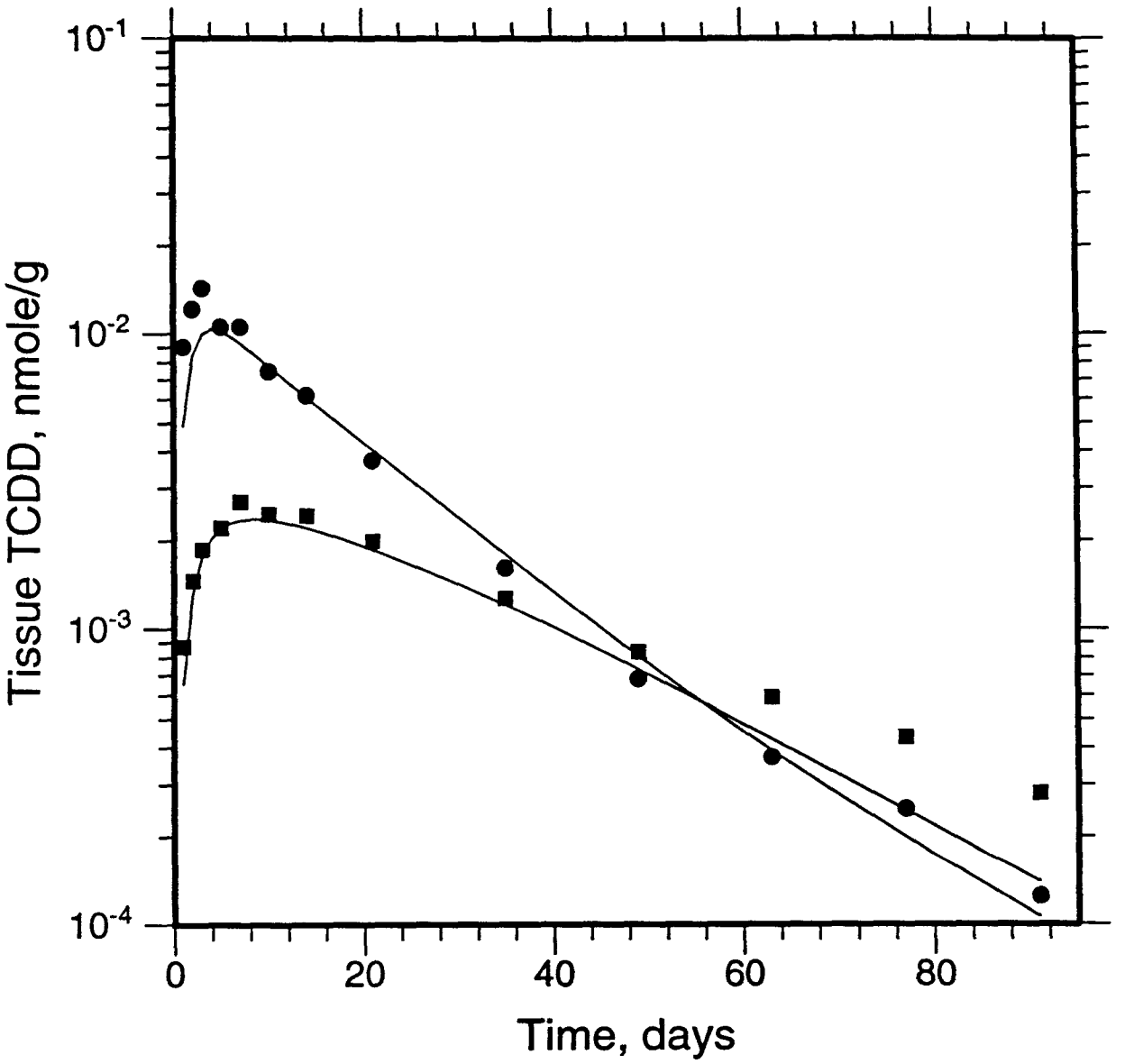
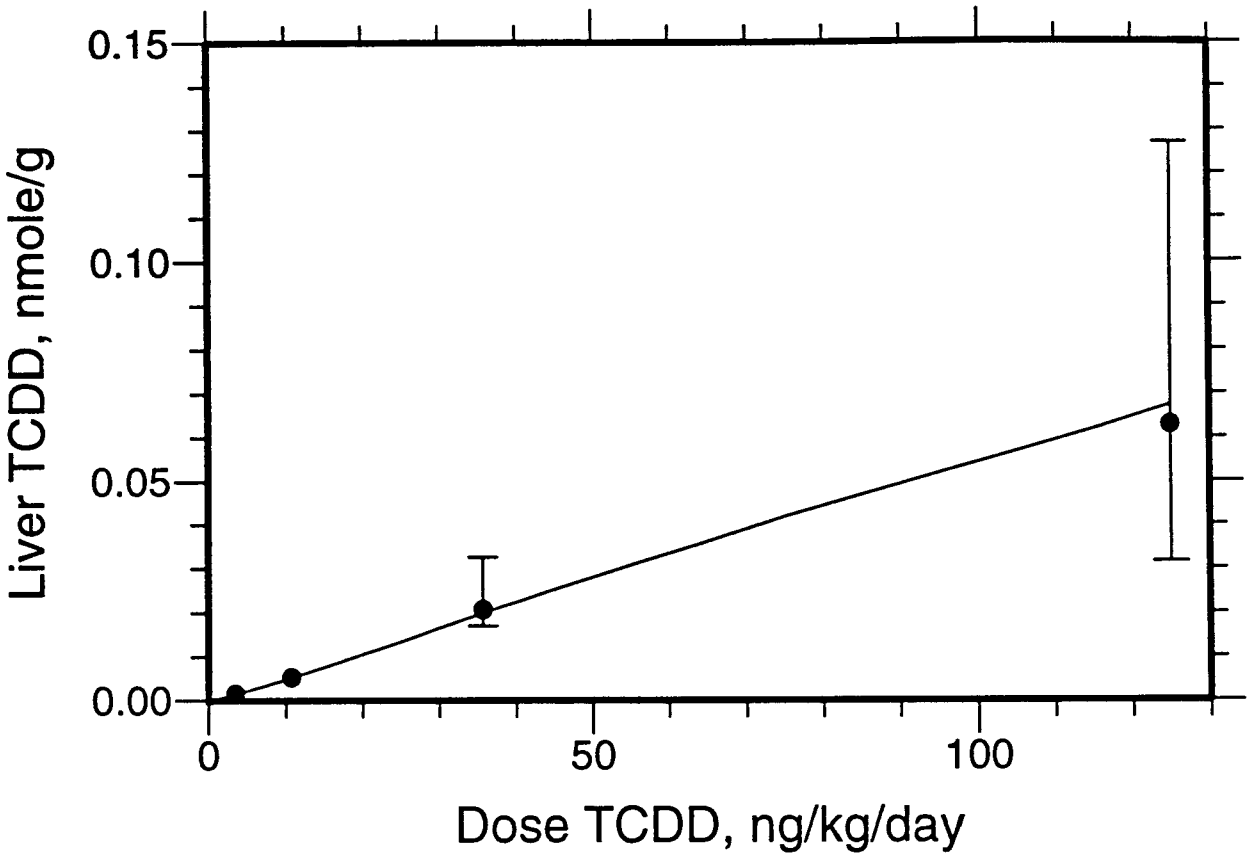
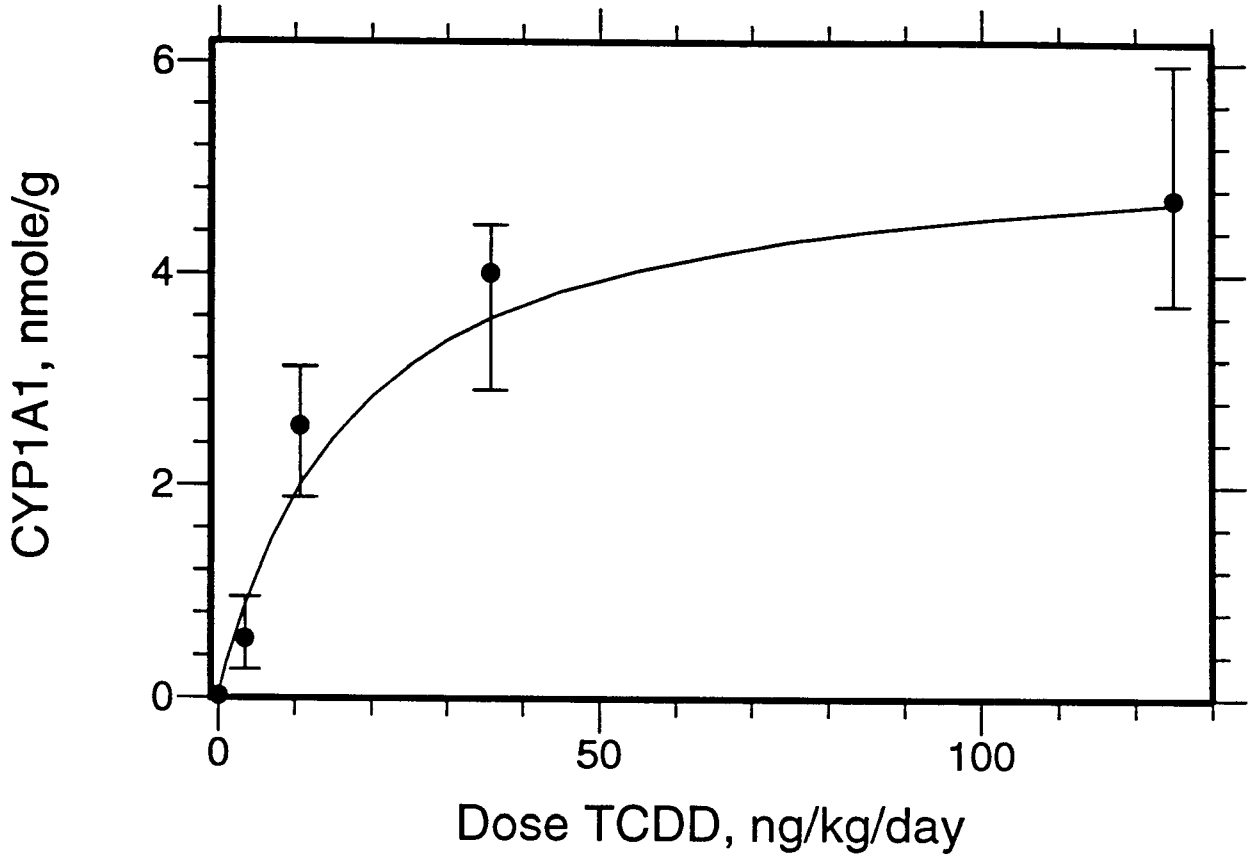
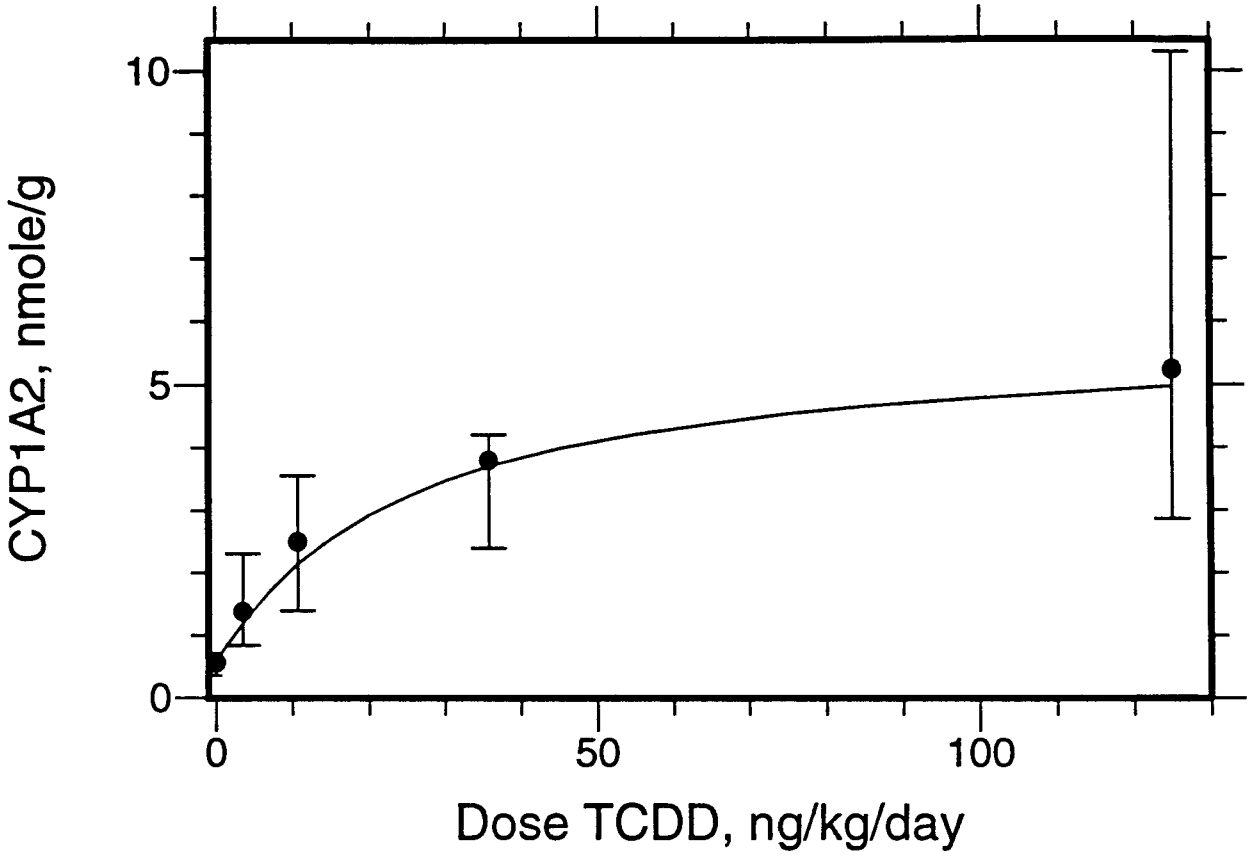
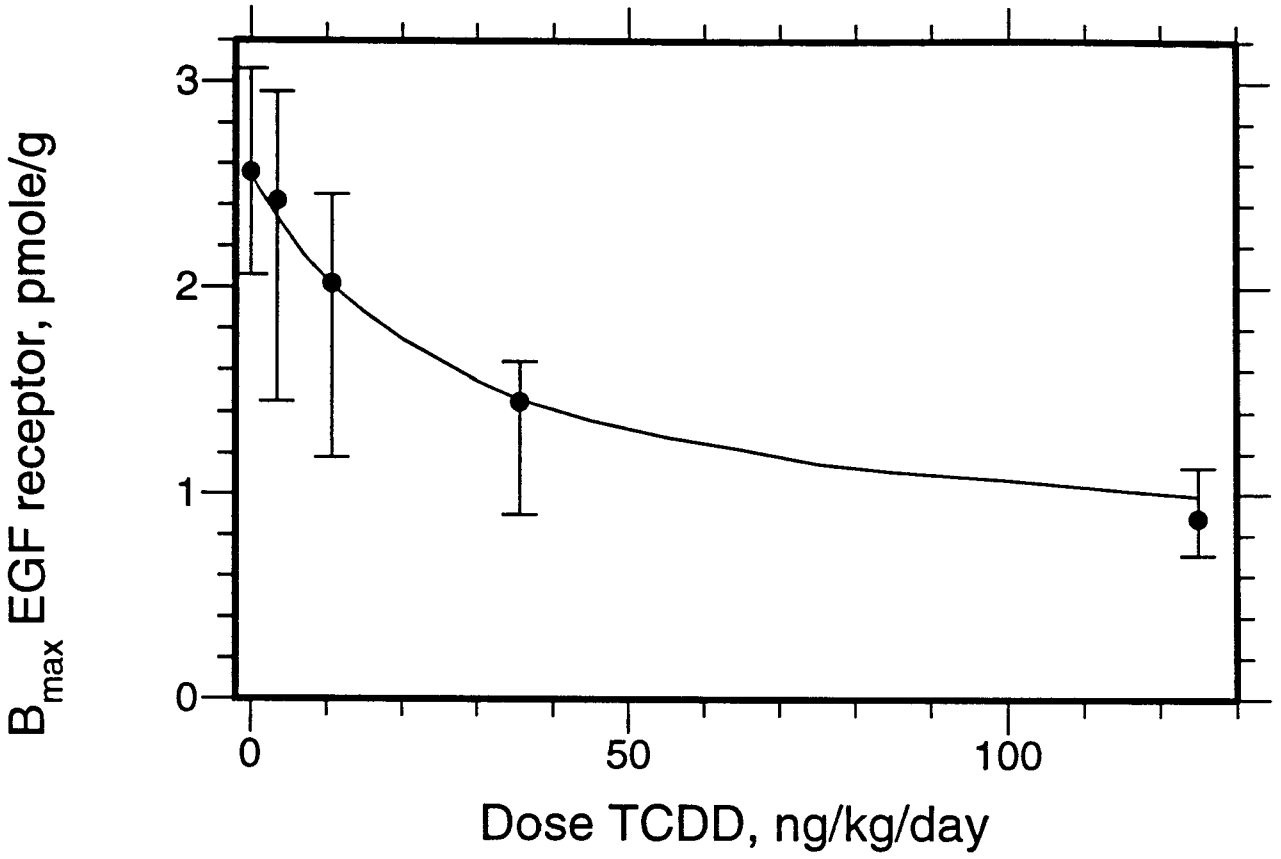
200
TCDD, nmole/g
Time, days

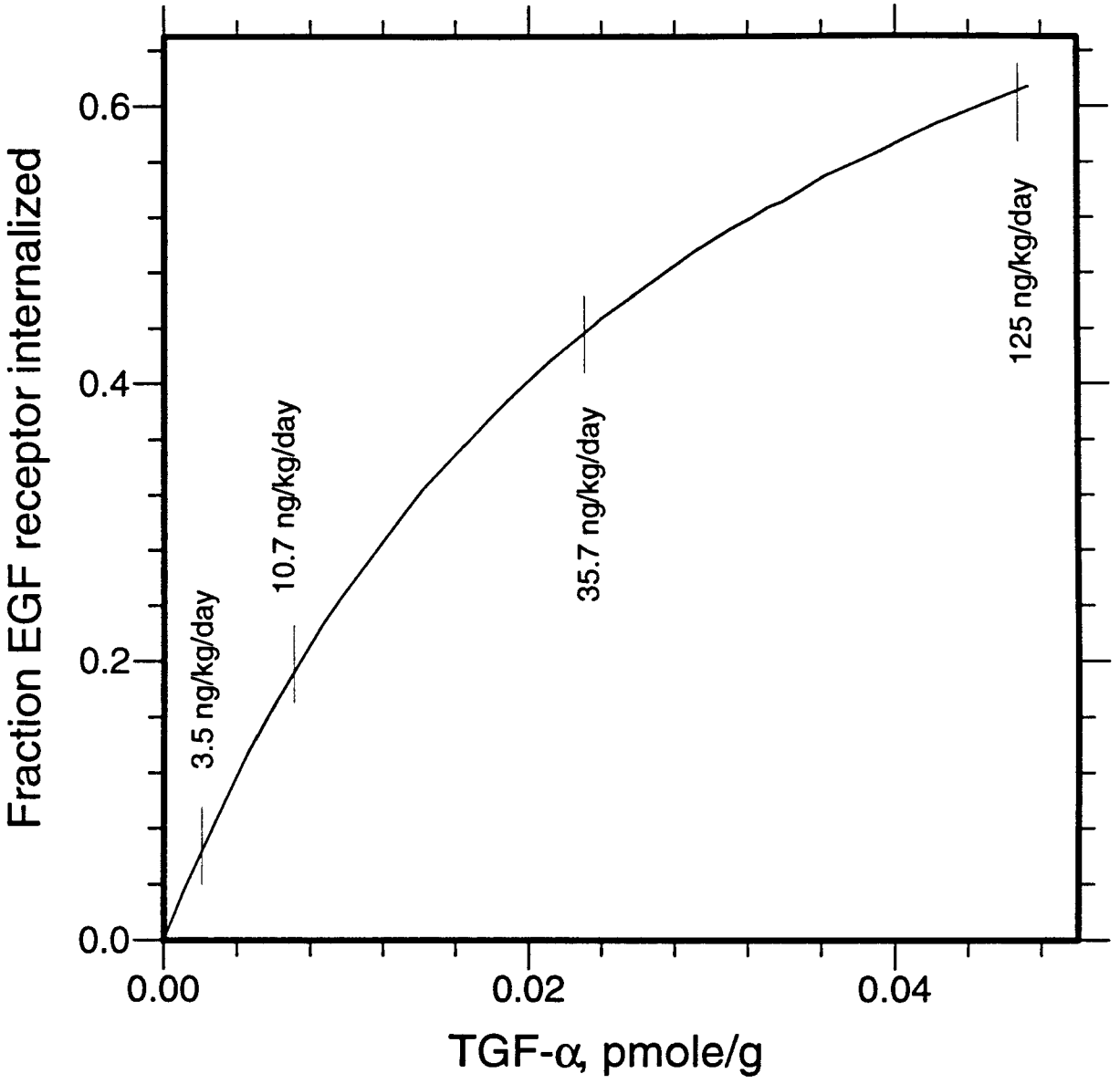

10^{-1}
10^{-2}
10^{-3}
10^{-4}
Tissue TCDD, nmole/g
0
20
40
60
80
Time, days


0.15
0.10
0.05
0.00
Liver TCDD, nmole/g
0
50
100
Dose TCDD, ng/kg/day


CYP1A1, nmole/g
6
4
2
0
0
50
100
Dose TCDD, ng/kg/day


CYP1A2, nmole/g
10
5
0
0
50
100
Dose TCDD, ng/kg/day


B_{max} EGF receptor, pmole/g
3
2
1
0
0
50
100
Dose TCDD, ng/kg/day


Fraction EGF receptor internalized
0.0
0.2
0.4
0.6
0.00
0.02
0.04
TGF-α, pmole/g
3.5 ng/kg/day
10.7 ng/kg/day
35.7 ng/kg/day
125 ng/kg/day

APPENDIX B

MODELLING RECEPTOR-MEDIATED PROCESSES WITH DIOXIN: IMPLICATIONS FOR PHARMACOKINETICS AND RISK ASSESSMENT

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Abbreviated title: PB-PK Modelling with Dioxin

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To be published in Risk Analysis

Abstract:

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 μg/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 US EPA is re-evaluating 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 between 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 casually 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.

Keywords:
PB-PK modelling, 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 rodents, also causes immunotoxic[4], reproductive[5], and teratogenic[6] effects. The present US EPA cancer risk assessment, based on the incidence of liver tumors in female rats dosed with dioxin for two-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 US[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* (*a*ryl *h*ydrocarbon) receptor. The *Ah* receptor-dioxin complex binds to 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 US 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:

a. Model Structure: The dioxin PB-PK model (Fig. 2) consists of five compartments - liver, fat, slowly perfused tissues (*e.g.*, muscle, skin, etc.) , richly perfused tissues (*e.g.*, kidney, brain, etc.), and blood. The relevant mass-balance differential equations appear in Appendix 1 and the terms are defined in Table 1. Each of the 4 tissue compartments (denoted by subscript, t) has both a specified blood flow (Q_t), tissue compartment volume (V_t) and a tissue blood volume (V_{tb}). The tissue blood volumes were estimated from Bischoff and Brown[16]. Movement of chemical from the tissue blood into the tissue is modelled to be proportional to a permeation coefficient-surface area cross-product (PA) for the tissue (t). Tissue uptake is diffusion-limited when $PA_t < Q_t$. Each tissue has a specified volume, blood flow, PA product, and partition coefficient (P_t). 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 compartment 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 (equation 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 (equation 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.

b 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, Kd. 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 concentration than the *Ah*-TCDD complex. The induction is modelled with a Hill plot binding relationship - equation 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 (Fig. 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}) - equation 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 modelled as if it were instantaneously altered by the *Ah* receptor-dioxin-DNA interactions, (equation 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 Kd 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.

c. 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, MA.

3. RESULTS:

a. 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/gm 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 Kd 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 (KB2) 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 µg/kg bw is commented on more fully in 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 (Fig. 5 and 6). Analysis of these results were especially useful for setting the rate constant for metabolism of dioxin and the PA_t 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.

b. 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 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 (15 µg/kg) followed by a five-fold 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 (Fig. 3-6). Small differences in fat and slowly perfused tissue compartment parameters are noted in the figure legends.

c. Correlation with Responses: The present US 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 sub-chronic exposure. The measures of tissue dose associated with enhanced 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 μg/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 μg/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:

a. 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 2 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 labelled 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 Fig. 3 and Fig. 8b). These low dose effects can now be readily evaluated by use of high specific activity ^{3}H-labelled 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 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 non-specific, 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 µg/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 to 2.7 as the body burden fell from 600 to 100 µg/kg[22], consistent with the predictions in Fig. 3 for the dose range above 10 µg/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 modelling 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).

b. Protein Binding/Induction: The challenge in providing a biologically-realistic PB-PK model for dioxin is the need to not only 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 (BM1) and binding affinity (KB1) for TCDD binding to the *Ah* receptor have been previously reported. The values used in our model differ significantly: our estimate of BM1 is only about 10% of the value determined by Gasiewicz and Rucci[30] and our KB1 is also much lower than the value reported by Bradfield and Poland[31]. The discrepancy in KB1 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 BM1 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 non-inducing levels, shown in Fig. 3. The liver concentration at doses below 0.01 μg/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 BM1 were increased to the literature value, the liver concentration of TCDD in the low dose region (*i.e.* non-induced 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 Kd, 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 examined, induction of liver sequestration (i.e., 1A2) shows higher affinity (lower Kd 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 4 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.

c. 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 P450 1A1 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 modelling 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.

d. 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 causal 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 modelled 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 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 co-operativity 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, co-operative 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 within 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 newly identified 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.

Acknowledgments: The work on dioxin pharmacokinetics at CIIT has been generously sponsored by a grant from the American Paper Industry. We also thank Drs. R. Conolly and R.J. Preston for helpful comments and discussions.

Although the research described in this article has been supported by the United States Environmental Protection Agency, it has not been subjected to agency review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use.

Appendix I:

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

$$dA_{tb}/dt = Q_t (C_a - C_{vt}) + PA_t (C_t/P_t - C_{vt}) \quad (1)$$

$$dA_t/dt = PA_t (C_{vt} - C_t/P_t) \quad (2)$$

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) - equations (1) and (2). The liver equation -equation (5)- also contains a term for loss due to metabolism:

$$dA_l/dt = PA_l (C_{vl} - C_{lf}) - V_l\, kf\, (1 + fold\, (C_{lf} / (C_{lf} + Kb_1))\, C_{lf} \quad (5)$$

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.

$$A_l = P_l V_l C_{lf} + [BM_1 C_{lf}/(KB_1 + C_{lf})] + [BM_{2t} C_{lf}/(KB_2 + C_{lf})] \quad (6)$$

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).

$$d(P4501A1_t)/dt = K_0 (1 + K_{0max} (Ah\text{-TCDD})^{n1}/((Ah\text{-TCDD})^{n1} + Kd_1^{n1})) - k_1 P4501A1_t \quad (7)$$

The concentration of P4501A2 at any time, $BM2_t$, was calculated assuming an instantaneous adjustment of binding protein concentrations and *Ah*-TCDD levels,

$$BM2_t = BM2_0 + BM2_I (Ah\text{-TCDD})^n/((Ah\text{-TCDD})^n + Kd^n)) \quad (8)$$

where $BM2_I$ 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

Table 1

Parameters in the Physiological Dosimetry Model for Dioxin[1]

Model Parameters	Abbreviations	Wistar Rat[2]
Body weight (kg)	bw	0.215
Volumes[3] (Liters)		
Liver	V_l	0.0375 x bw
Fat	V_f	0.07 x bw
Richly perfused (Viscera)	V_r	0.0525 x bw
Slowly perfused (Muscle/skin)	V_s	0.75 x bw
Blood	V_b	0.05 x bw
Tissue blood volumes		
Liver	V_{lb}	0.01 x V_f
Fat	V_{fb}	0.05 x V_f
Richly Perfused	V_{rb}	0.01 x V_r
Slowly perfused	V_{sb}	0.05 x V_s
Cardiac output (liters/hr)	Q_c	4.4
Tissue blood flow (% Qc)		
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 x Q_l
Fat	PA_f	0.2 x Q_f
Richly perfused	PA_r	0.5 x Q_r
Slowly perfused	PA_s	0.5 x Q_s
Metabolic Constants		
Metabolism (hr^{-1})	kfc	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 (pmoles/liver)	BM_1	3.75
Ah affinity (pM)	KB_1	35
1A2 basal level (nmoles/liver)	$BM2_0$	10
1A2 maximum (nmoles/liver)	$BM2_l$	85
1A2 affinity (nM)	KB_2	6.5
1A2 - Hill term	n	1.0
1A2 - Hill binding constant (pM)	Kd	50
1A1 - Hill term	n_1	2.3
1A1 - Hill binding constant (pM)	Kd_1	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_{0max}	50

<----------->

[1] - Deviations from this base parameter set are noted in the legends to each Figure.

[2] - Parameters are for the 215g rat at the start of the dose-response study in Fig.

[3] - 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)

Table II

RELATIONSHIP BETWEEN HEPATIC EXPOSURE TO DIOXIN, HEPATIC PROTEIN INDUCTION, AND HEPATIC TOXICITY DURING SUBCHRONIC EXPOSURES

Dose[1]	Altered Foci[2] Volume	Tumors[3] (Liver)	AUCL-free[4] C_{lf}	AUCL-total[5] C_l	AU-1A2[6] ($BM2_l$)	AU-1A1[7] (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^{5}
0.1	2.8	11/49	4.2×10^{2}	1.2×10^{5}	3.2×10^{5}	2.0×10^{6}
1	-	-	3.8×10^{3}	1.0×10^{6}	3.5×10^{5}	3.0×10^{6}
10	-	-	3.8×10^{4}	5.4×10^{6}	3.5×10^{5}	3.1×10^{6}

[1]- Biweekly doses used; dose expressed as average dose per day in μg/kg.

[2] - Volume as percent of liver occupied by altered cells, from Pitot et al.[18]

[3] - Liver tumors observed in female rats in the bioassay study of Kociba et al.[1].

[4] - Total area under the curve for free dioxin in the liver, units are nmoles-hr/liters.

[5] - Total area under the curve for total dioxin in the liver, units are nmoles-hr/liter.

[6] - 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 equation (6).

[7] - 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.

FIGURE CAPTIONS

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, metabolizing enzymes, and growth factors. The change in concentration of certain of these proteins is believed to be associated with the various 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 sub-cutaneous administration was described as a first-order process with the chemical appearing in mixed venous blood. Symbol definitions are in Table 1.

Fig. 3: Dose-dependence of dioxin tissue disposition in female Wistar rats 7 days following single sub-cutaneous doses: Tissue concentrations from Abraham et al.[10] are expressed normalized to dose, as %dose/gram tissue. Model parameters are given in Table 1.

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

Fig. 5: Time course of liver and fat tissue dioxin concentrations following a single, sub-cutaneous 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 & 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: Pf, Ps, Pafc, and kfc were, respectively, 250, 50, 0.1, and 1.5. These contrast to values of 375, 30, 0.20, and 1.65.

Fig. 8 : Low dose extrapolation of measures of tissue dose for dioxin in sub-chronic exposures. Panel A: Dose measures related to induction of cytochrome P4501A1 and the liver binding protein. The steeper line is for enhanced P4501A1 which has a co-operativity 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. Panel 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.

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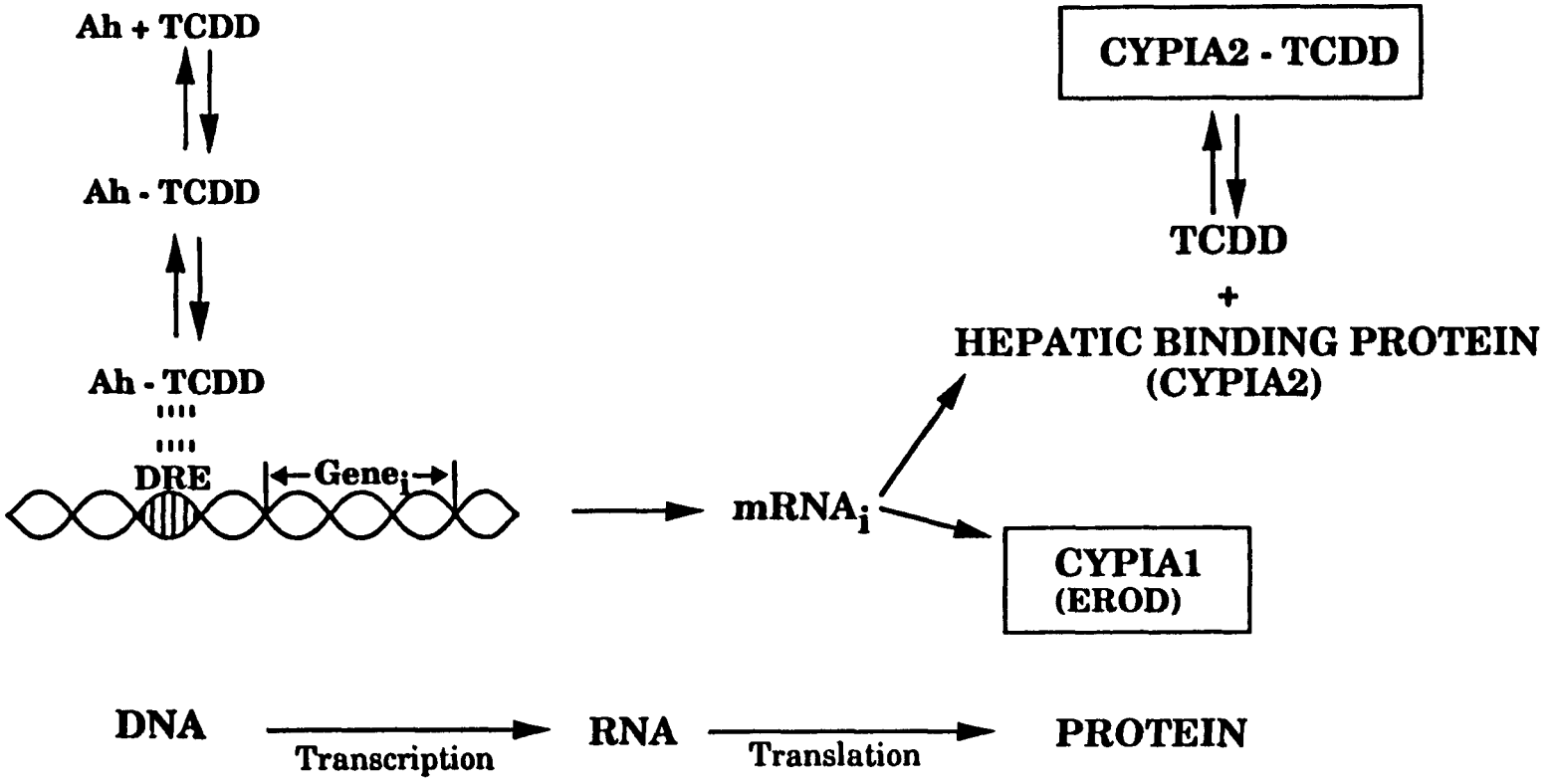
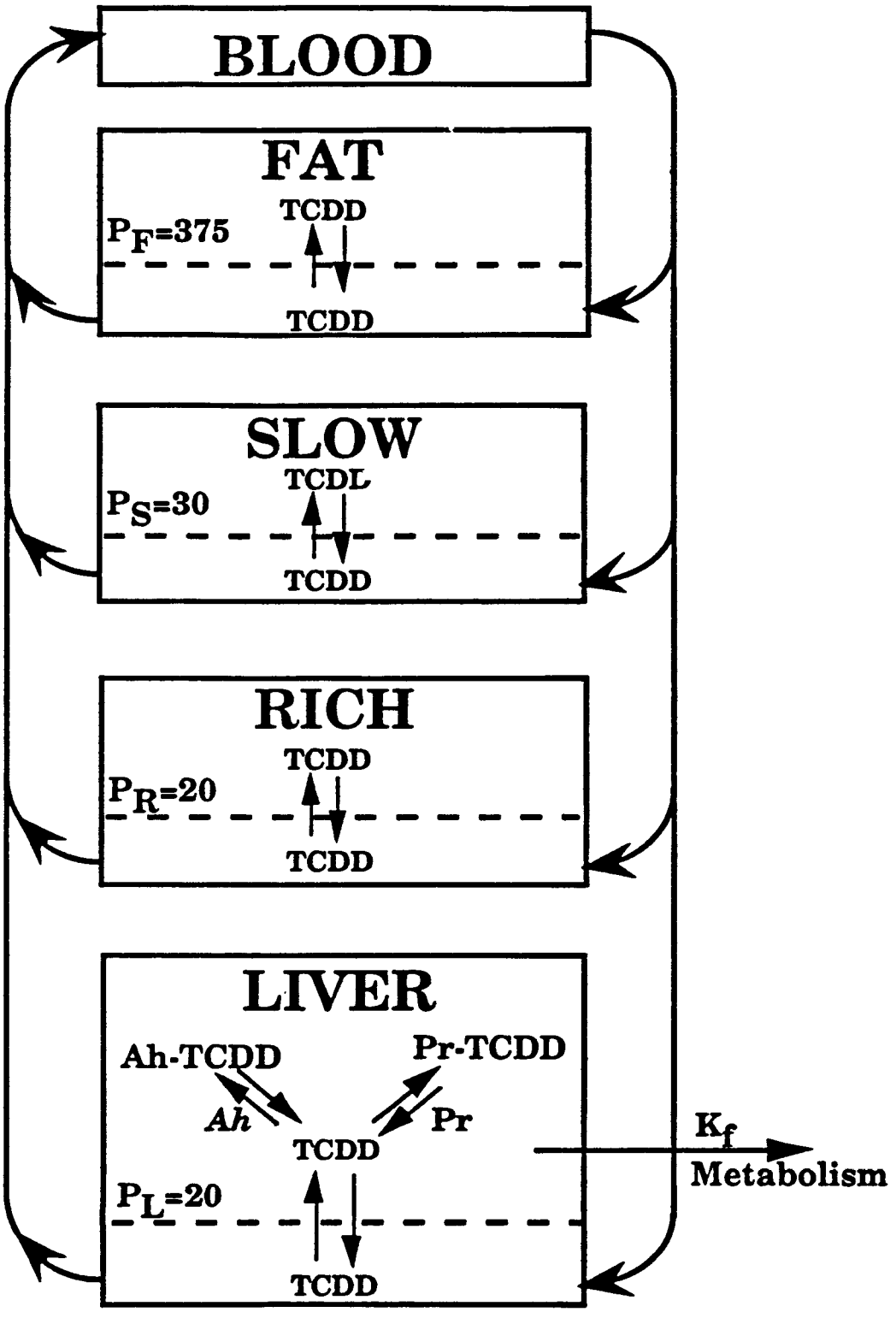

Ah + TCDD
Ah - TCDD
Ah - TCDD
DRE
Gene$_i$
mRNA$_i$
CYPIA2 - TCDD
TCDD
+
HEPATIC BINDING PROTEIN
(CYPIA2)
CYPIA1
(EROD)
DNA
Transcription
RNA
Translation
PROTEIN

Fig 1.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TCDD
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TCDD
SLOW
TCDD
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TCDD
RICH
TCDD
P_R=20
TCDD
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Ah-TCDD
Pr-TCDD
Ah
Pr
TCDD
K_f
Metabolism
P_L=20
TCDD

Fig. 2

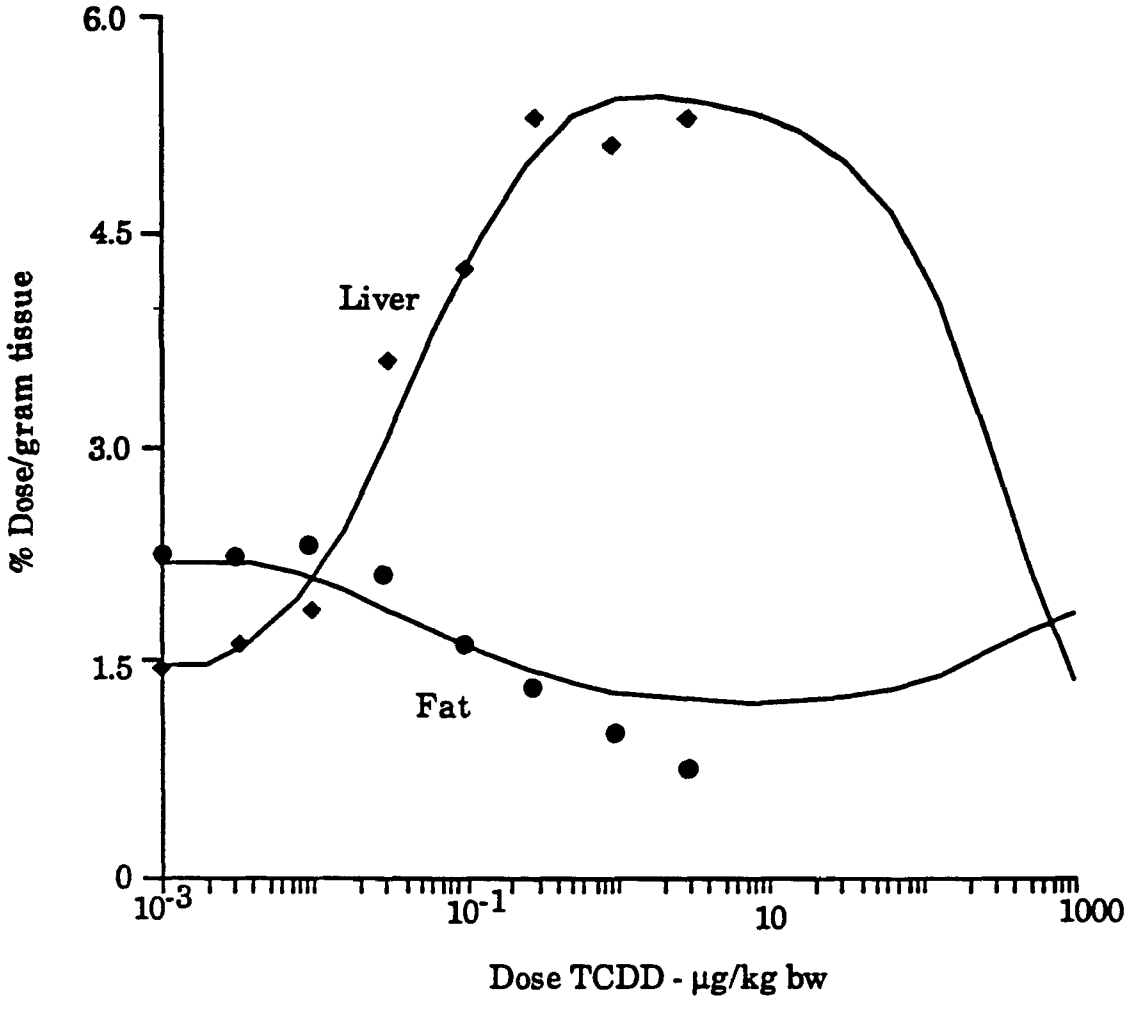
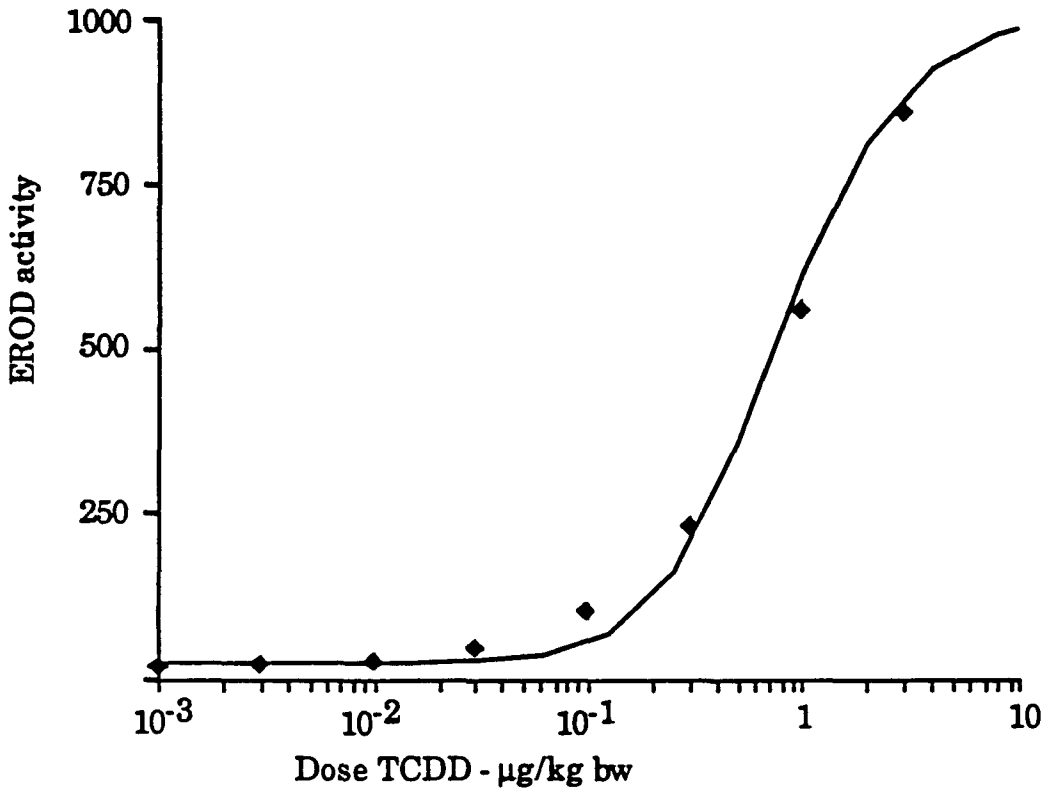

6.0
4.5
3.0
1.5
0
% Dose/gram tissue
Liver
Fat
10^{-3}
10^{-1}
10
1000
Dose TCDD - µg/kg bw

Fig. 3


1000
750
500
250
EROD activity
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10^{-2}
10^{-1}
1
10
Dose TCDD - µg/kg bw

Fig. 4

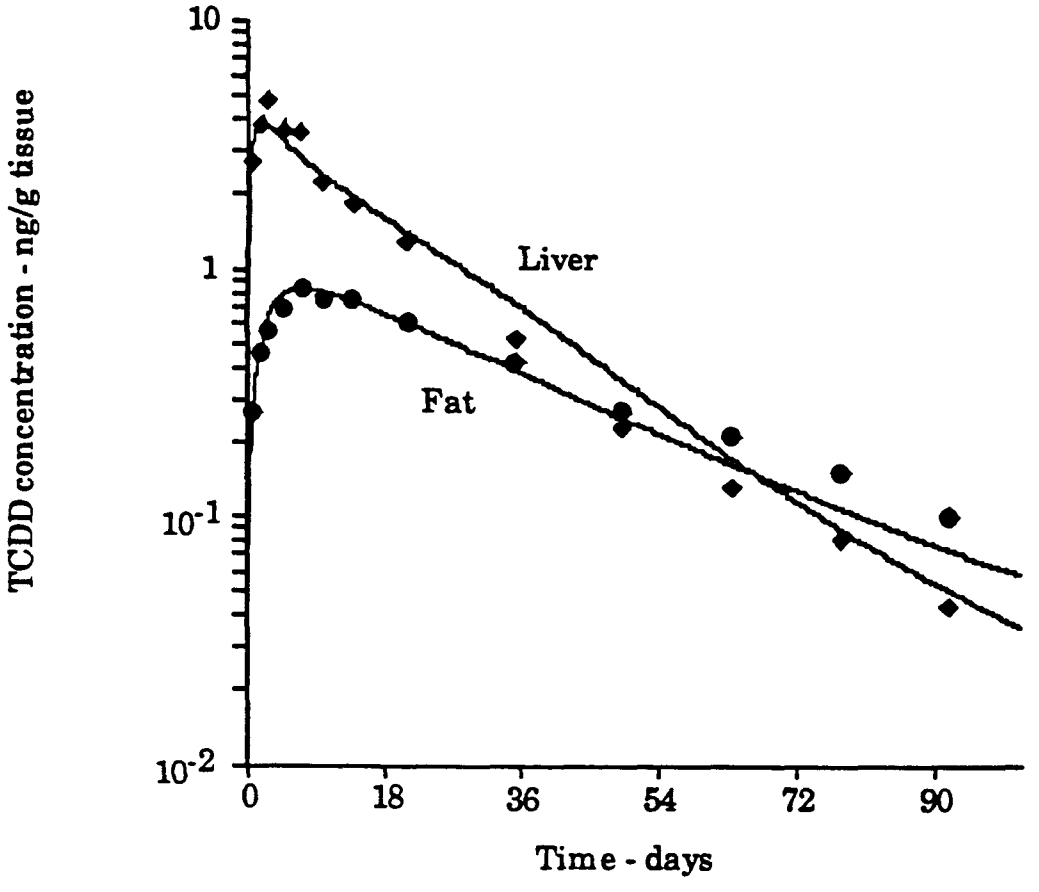
TCDD concentration - ng/g tissue
10
1
10^{-1}
10^{-2}
Liver
Fat
0
18
36
54
72
90
Time - days

Fig. 5

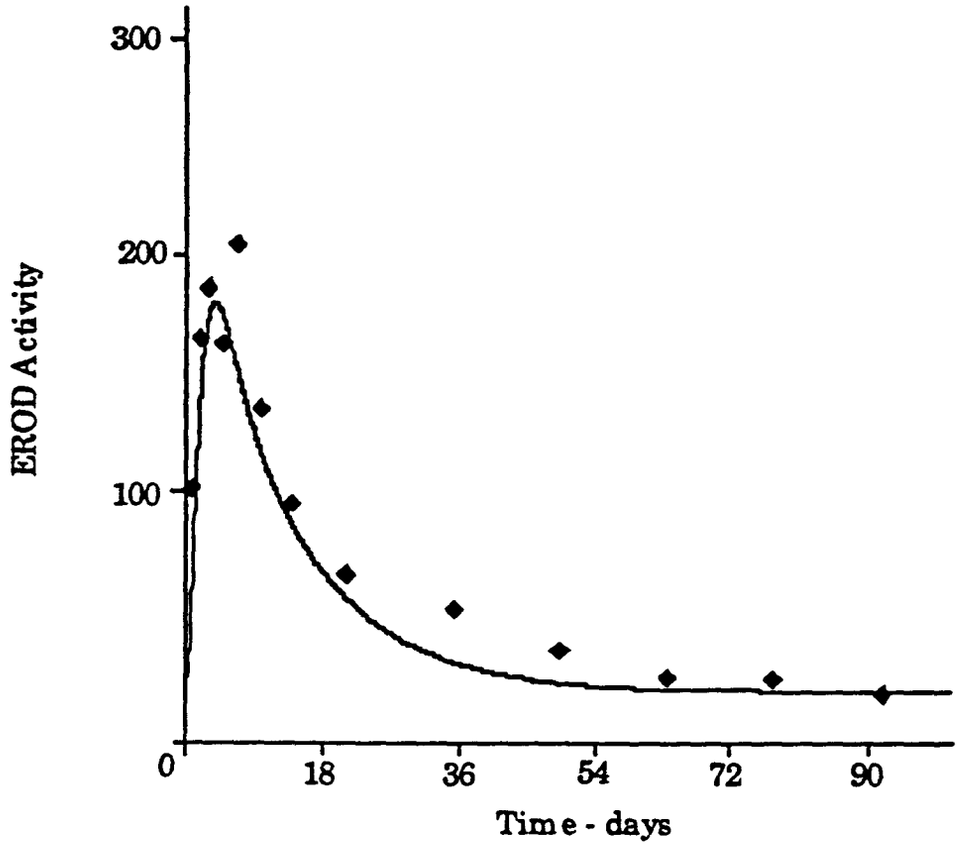

Fig. 6

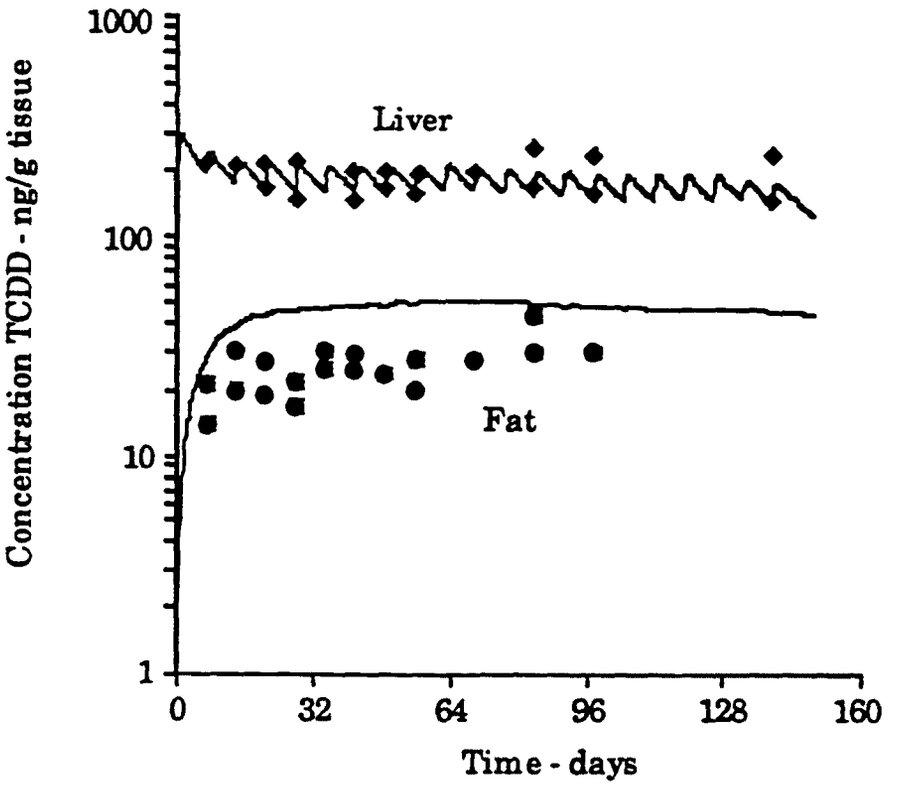
Concentration TCDD - ng/g tissue
1000
100
10
1
Liver
Fat
0
32
64
96
128
160
Time - days

Fig. 7

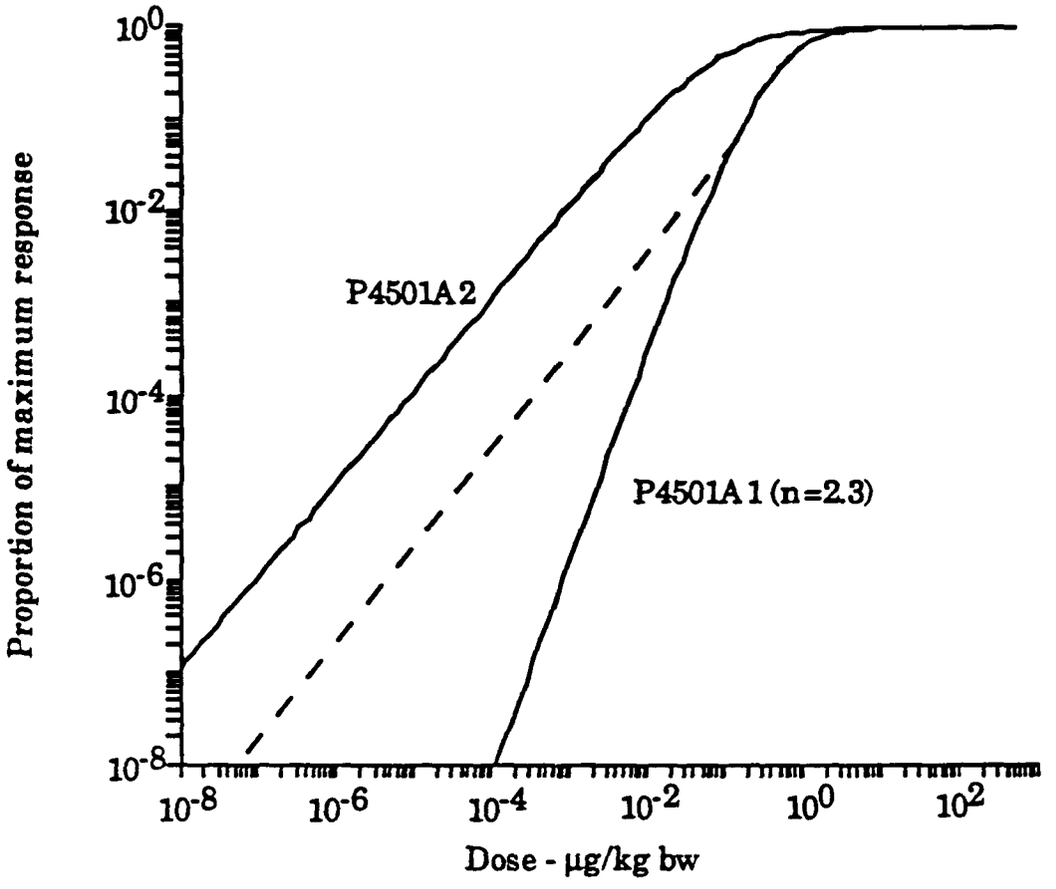
Proportion of maximum response
10^0
10^{-2}
10^{-4}
10^{-6}
10^{-8}
P4501A2
P4501A1 (n=2.3)
10^{-8}
10^{-6}
10^{-4}
10^{-2}
10^0
10^2
Dose - µg/kg bw

Fig. 8a

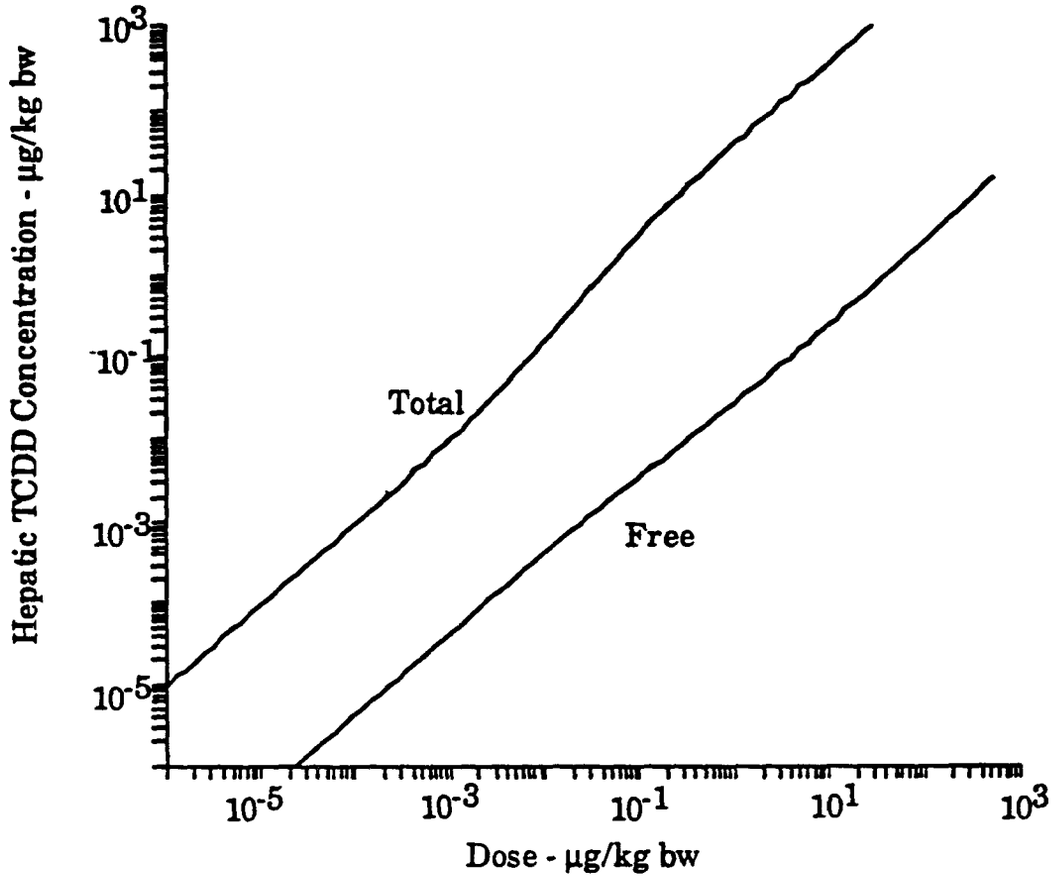
Hepatic TCDD Concentration - µg/kg bw
10^3
10^1
10^-1
10^-3
10^-5
Total
Free
10^-5
10^-3
10^-1
10^1
10^3
Dose - µg/kg bw

Fig. 8b