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EPA

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

Review Draft (Do Not Cite or Quote)

Volume I of III

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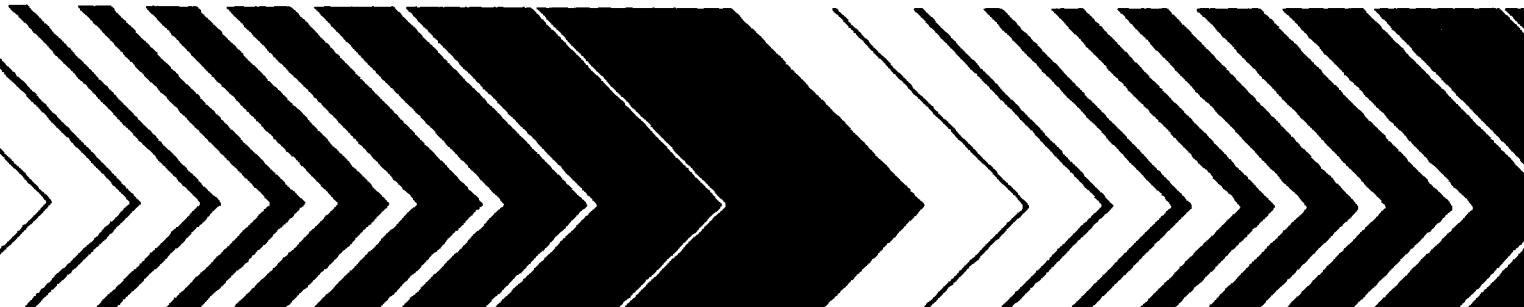

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Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, D.C.

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

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)
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LIST OF ABBREVIATIONS AND ACRONYMS

ACTH	Adrenocorticotrophic hormone
Ah receptor	Aryl hydrocarbon receptor
AHH	Aryl hydrocarbon hydroxylase
ALA	Aminolevulinic acid
ALT	L-alanine aminotransferase
AOR	Adjusted odds ratio
APC	Antigen-presenting cells
AST	L-aspartate aminotransferase
ATPase	Adenosine triphosphatase
BDD	Brominated dibenzo-*p*-dioxin
BDF	Brominated dibenzofuran
BCF	Bioconcentration factor
BGG	Bovine gamma globulin
bHLH	Basic helix-loop-helix
bw	Body weight
cAMP	Cyclic 3,5-adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CDD	Chlorinated dibenzo-*p*-dioxin
CDF	Chlorinated dibenzofuran
cDNA	Complementary DNA
cl	Confidence level
CMI	Cornell Medical Index
CNS	Central nervous system
CSM	Cerebrospinal malformation
CTL	Cytotoxic T lymphocyte
DCDD	2,7-Dichlorodibenzo-*p*-dioxin

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

DEN	Diethylnitrosamine
DHT	5α-Dihydrotestosterone
DIS	Diagnostic Interview Schedule
DMBA	Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin-responsive enhancers
DTH	Delayed-type hypersensitivity
EC_{50}	Concentration effective for 50% of organisms tested
EC_{100}	Concentration effective for 100% of organisms tested
ED_{50}	Dose effective for 50% of recipients
ECOD	7-Ethoxycoumarin-O-deethylase
EEG	Electroencephalogram
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EROD	7-Ethoxyresorufin-O-deethylase
EOF	Enzyme altered foci
EOI	Exposure opportunity index
FEV	Forced expiratory volume
FIQ	Full-scale IQ
FSH	Follicle-stimulating hormone
FTI	Free thyroxine index
FVC	Forced vital capacity
GC-ECD	Gas chromatograph-electron capture detection
GC/MS	Gas chromatograph/mass spectrometer

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

GGT	Gamma glutamyl transpeptidase
GnRH	Gonadotropin-releasing hormone
GST	Glutathione-S-transferase
GVH	Graft versus host
HAH	Halogenated aromatic hydrocarbons
HCB	Hexachlorobenzene
HCDD	Hexachlorodibenzo-*p*-dioxin
HDL	High density lipoprotein
HLH	Helix-loop-helix
HPAH	Halogenated polycyclic aromatic hydrocarbon
HpCDD	Heptachlorinated dibenzo-*p*-dioxin
HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRB	Halstead-Reitan Battery
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HTL	Human tonsillar lymphocytes
HxBB	Hexabrom-biphenyl
HxCB	Hexachlorobiphenyl
HxCDD	Hexachlorinated dibenzo-*p*-dioxin
HxCDF	Hexachlorinated dibenzofuran
ICD-9	International Classification of Diseases 9
ID_{50}	Dose infective to 50% of recipients
I-TEF	International TCDD-toxic-equivalency
KVK	Kemisk Vaerk Køge
LADD	Lifetime average daily dose
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

LDH	L-lactate dehydrogenase
LH	Luteinizing hormone
LDL	Low density liproprotein
LMS	Linearized multistage
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
LPS	Lipopolysaccharide
MACDP	Metropolitan Atlanta Congenital Defects Program
3-MC	3-Methylcholanthrene
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MCF-7	(breast cancer cell)
MCMI	Millon Clinical Multiaxial Inventory
MCPA	(4-Chloro-2-methylphenoxy)acetic acid
MCPB	2-Methyl-4-chlorophenoxybutyric acid
MCPP	2-(4-Chloro-2-methylphenoxy)-propanoic acid
MFO	Mixed function oxidase
MMPI	Minnesota Multiphase Personality Inventory
MLE	Maximum likelihood estimate
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaTCP	Sodium 2,4,5-trichlorophenate
NHL	Non-Hodgkin's lymphoma
NIEHS	National Institute of Environmental Health Sciences

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

NIOSH	National Institute for Occupational Safety and Health
NK	Natural killer
NOAEL	No-observable-adverse-effect level
NOEL	No-observed-effect level
NTP	National Toxicology Program
OCDD	Octachlorodibenzo-*p*-dioxin
OCDF	Octachlorodibenzofuran
OR	Odds ratio
OVX	Ovariectomized
PAA	Phenoxyacetic acid
PAH	Polyaromatic hydrocarbon
PBA	Phenoxybutyric acid
PBB	Polybrominated biphenyl
PBF	Percent body fat
PBL	Peripheral blood lymphocytes
PB-PK	Physiologically based pharmacokinetic
PCB	Polychlorinated biphenyl
PCBA	Phenoxybutyric acid
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PCP	Pentachlorophenol
PCPA	Parachlorophenoxyacetic acid
PCQ	Quaterphenyl
PCT	Porphyria cutanea tarda
PeCDD	Pentachlorinated dibenzo-*p*-dioxin
PeCDF	Pentachlorinated dibenzo-*p*-dioxin

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

PEPCK	Phosphoenol pyruvate carboxykinase
PFC	Plaque-forming cell
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGST	Placental glutathione-S-transferase
PGT	Placental glutathione transferase
PHA	Phytohemagglutinin
PIQ	Performance IQ
PKC	Protein kinase C
PNS	Peripheral nervous system
POMS	Profile of Mood States
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
PRR	Prevalence risk ratio
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
RR	Relative risk
SAR	Structure-activity relationships
SB-IQ	Standford Binet IQ
SCL-90-R	Self-Report Symptom Checklist-90-Revised
SD	Standard deviation
SE	Standard error
SEA	Southeast Asia
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

SIR	Standard incidence ratio
SMR	Standardized mortality ratio
SRBC	Sheep erythrocytes (red blood cells)
STS	Soft tissue sarcoma
$t_{½}$	Half-time
TBB	Tetrabromobiphenyl
TBDD	Tetrabrominated dibenzo-*p*-dioxin
TBDF	Tetrabrominated dibenzo-*p*-furan
TBG	Thyroxine-binding globulin
TBP	Thyroxine-binding protein
TCAOB	Tetrachloroazoxybenzene
TCB	Tetrachlorobiphenyl
TCDD	Tetrachlorodibenzo-*p*-dioxin
TCDF	Tetrachlorodibenzofuran
TCP	Trichlorophenol
TEF	Toxic equivalency factors
TEQ	Toxic equivalents
TGF	Thyroid growth factor
TI	T helper cell independent
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TPA	Tetradecanoyl phorbol acetate
TSH	Thyroid-stimulating hormone
TT	Tetanus toxoid
TTR	Transthyretrin
TxB_2	Thromboxane B_2

LIST OF ABBREVIATIONS AND ACRONYMS (continued)

UDP	Uridine diphosphate
UDPGT	UDP-glucuronosyltransferase
URO-D	Uroporphyrinogen decarboxylase
VIQ	Verbal IQ
VLDL	Very low density lipoprotein
v/v	Volume per volume
w/w	Weight by weight
WAIS	Wechsler Adult Intelligence Scale
WISC-R	Wechsler Intelligence Scale for Children, Revised

PREFACE

In April 1991, the U.S. Environmental Protection Agency (EPA) announced that it would conduct a scientific reassessment of the health risks of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and chemically similar compounds collectively known as dioxin. The EPA has undertaken this task in response to emerging scientific knowledge of the biological, human health, and environmental effects of dioxin. Significant advances have occurred in the scientific understanding of mechanisms of dioxin toxicity, of the carcinogenic and other adverse health effects of dioxin in people, of the pathways to human exposure, and of the toxic effects of dioxin to the environment.

In 1985 and 1988, the Agency prepared assessments of the human health risks from environmental exposures to dioxin. Also, in 1988, a draft exposure document was prepared that presented procedures for conducting site-specific exposure assessments to dioxin-like compounds. These assessments were reviewed by the Agency's Science Advisory Board (SAB). At the time of the 1988 assessments, there was general agreement within the scientific community that there could be a substantial improvement over the existing approach to analyzing dose response, but there was no consensus as to a more biologically defensible methodology. The Agency was asked to explore the development of such a method. The current reassessment activities are in response to this request.

The scientific reassessment of dioxin consists of five activities:

1. Update and revision of the health assessment document for dioxin.
2. Laboratory research in support of the dose-response model.
3. Development of a biologically based dose-response model for dioxin.
4. Update and revision of the dioxin exposure assessment document.
5. Research to characterize ecological risks in aquatic ecosystems.

PREFACE (continued)

The first four activities have resulted in two draft documents (the health assessment document and exposure document) for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. These companion documents, which form the basis for the Agency's reassessment of dioxin, have been used in the development of the risk characterization chapter that follows the health assessment. The process for developing these documents consisted of three phases which are outlined in later paragraphs.

The fifth activity, which is in progress at EPA's Environmental Research Laboratory in Duluth, Minnesota, involves characterizing ecological risks in aquatic ecosystems from exposure to dioxins. Research efforts are focused on the study of organisms in aquatic food webs to identify the effects of dioxin exposure that are likely to result in significant population impacts. A report titled, *Interim Report on Data and Methods for the Assessment of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) Risks to Aquatic Organisms and Associated Wildlife* (EPA/600/R-93/055), was published in April 1993. This report will serve as a background document for assessing dioxin-related ecological risks. Ultimately, these data will support the development of aquatic life criteria which will aid in the implementation of the Clean Water Act.

The EPA had endeavored to make each phase of the current reassessment of dioxin an open and participatory effort. On November 15, 1991, and April 28, 1992, public meetings were held to inform the public of the Agency's plans and activities for the reassessment, to hear and receive public comments and reviews of the proposed plans, and to receive any current, scientifically relevant information.

In the Fall of 1992, the Agency convened two peer-review workshops to review draft documents related to EPA's scientific reassessment of the health effects of dioxin. The first workshop was held September 10 and 11, 1992, to review a draft exposure assessment titled, *Estimating Exposures to Dioxin-Like Compounds*. The second workshop was held September 22-25, 1992, to review eight chapters of a future draft *Health Assessment Document for*

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and Related Compounds. Peer-reviewers were also asked to identify issues to be incorporated into the risk characterization, which was under development.

In the Fall of 1993, a third peer-review workshop was held on September 7 and 8, 1993, to review a draft of the revised and expanded Epidemiology and Human Data Chapter, which also would be part of the future health assessment document. The revised chapter provided an evaluation of the scientific quality and strength of the epidemiology data in the evaluation of toxic health effects, both cancer and noncancer, from exposure to dioxin, with an emphasis on the specific congener, 2,3,7,8-TCDD.

As mentioned previously, completion of the health assessment and exposure documents involves three phases: Phase 1 involved drafting state-of-the-science chapters and a dose-response model for the health assessment document, expanding the exposure document to address dioxin related compounds, and conducting peer review workshops by panels of experts. This phase has been completed.

Phase 2, preparation of the risk characterization, began during the September 1992 workshops with discussions by the peer-review panels and formulation of points to be carried forward into the risk characterization. Following the September 1993 workshop, this work was completed and was incorporated as Chapter 9 of the draft health assessment document. This phase has been completed.

Phase 3 is currently underway. It includes making External Review Drafts of both the health assessment document and the exposure document available for public review and comment.

Following the public comment period, the Agency's Science Advisory Board (SAB) will review the draft documents in public session. Assuming that public and SAB comments are positive, the draft documents will be revised, and final documents will be issued.

PREFACE (continued)

The *Health Assessment Document for 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and Related Compounds* has been prepared under the direction of the Office of Health and Environmental Assessment, Office of Research and Development, which is responsible for the report's scientific accuracy and conclusions. A comprehensive search of the scientific literature for this document varies somewhat by chapter but is, in general, complete through January 1994.

AUTHORS, CONTRIBUTORS, AND REVIEWERS

This draft Health Assessment Document was prepared under the leadership and direction of the Office of Health and Environmental Assessment (OHEA) within EPA's Office of Research and Development (ORD). The overall coordination and leadership of the activities associated with EPA's reassessment of dioxin, which includes the development of this draft document, is Dr. William H. Farland, Director of OHEA.

Authors and chapter managers for the Health Assessment Document are listed below. Early drafts of some chapters were prepared by Syracuse Research Corporation under EPA Contract No. 68-CO-0043. Other chapters were authored totally or in part by scientists within EPA and other agencies within the federal government. The ORD chapter managers were responsible for providing oversight, review, and technical editing of successive drafts, and incorporating comments from reviewers to develop a comprehensive and consistent document. In some cases, the chapter managers also authored sections or parts of the chapter.

AUTHORS AND CHAPTER MANAGERS

Chapter	EPA Chapter Manager/Author	Outside Author
1. Disposition and Pharmacokinetics	Jerry Blancato U.S. EPA Environmental Monitoring Systems Laboratory Las Vegas, NV	James Olson Department of Pharmacology and Therapeutics State University of New York Buffalo, NY
2. Mechanism(s) of Action	William H. Farland U.S. EPA Office of Health and Environmental Assessment (OHEA) Washington, DC	James Whitlock, Jr. Department of Molecular Pharmacology Stanford University School of Medicine Stanford, CA
3. Acute, Subchronic, and Chronic Toxicity	Debdas Mukerjee Environmental Criteria and Assessment Office/OHEA Cincinnati, OH	Ulf G. Ahlborg Karolinska Institute Stockholm, SWEDEN

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

Chapter	EPA Chapter Manager/Author	Outside Author
4. Immunotoxicity	Ralph Smialowicz U.S. EPA Health Effects Research Laboratory Research Triangle Park, NC Gary R. Burleson* U.S. EPA Health Effects Research Laboratory Research Triangle Park, NC	Nancy Kerkvliet Agricultural Chemistry Oregon State University Corvallis, OR
5. Developmental and Reproductive Toxicity	Gary Kimmel U.S. EPA Human Health Assessment Group OHEA Washington, DC	Richard Peterson School of Pharmacy University of Wisconsin Madison, WI
6. Carcinogenicity of TCDD in Animals	Charalingayya B. Hiremath U.S. EPA Human Health Assessment Group OHEA Washington, DC	George Lucier National Institute of Environmental Health Sciences Research Triangle Park, NC
7. Epidemiology/Human Data Part A. Cancer Effects Part B. Effects Other Than Cancer	David L. Bayliss U.S. EPA Human Health Assessment Group OHEA Washington, DC	Charles Poole* Epidemiology Research Institute Cambridge, MA Marie Haring-Sweeney National Institute for Occupational Safety and Health Cincinnati, OH

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

Chapter	EPA Chapter Manager/Author	Outside Author
8. Dose-Response Modeling	Steven P. Bayard U.S. EPA Human Health Assessment Group OHEA Washington, DC	**Dioxin Dose-Response Modeling Workgroup** Michael Gallo (Co-chair), Keith Cooper, Panos Georgopolous, and Lynne McGrath UMDNJ-Robert Wood Johnson Medical School Environmental and Occupational Health Sciences Institute (EOHSI) Piscataway, NJ George Lucier (Co-chair) and Christopher Portier National Institute of Environmental Health Sciences Research Triangle Park, NC Melvin Andersen and Michael DeVito U.S. EPA Health Effects Research Laboratory Research Triangle Park, NC Steven Bayard and Paul White U.S. EPA Office of Health and Environmental Assessment Washington, DC Lorrene Kedderis University of North Carolina Chapel Hill, NC Jeremy Mills Chemical Industry Institute of Toxicology Research Triangle Park, NC Ellen Silbergeld University of Maryland Baltimore, MD
9. Risk Characterization of 2,3,7,8-TCDD and Related Compounds	William H. Farland (See Chapter 2)	

*Involved with an early draft, but no longer working on the reassessment project.

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

CONTRIBUTORS

Linda Birnbaum	Director, Environmental Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC
Marilyn Fingerhut	Chief, Industry-wide Studies Branch, National Institute for Occupational Safety and Health, Cincinnati, OH
Dorothy Patton	Executive Director, Risk Assessment Forum, Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC
Peter W. Preuss	Director, Office of Science, Planning, and Regulatory Evaluation, U.S. Environmental Protection Agency, Washington, DC
Dwain Winters	Office of Prevention, Pesticides, and Toxic Substances, U.S. Environmental Protection Agency, Washington, DC

REVIEWERS

Early drafts of Chapters 1 through 8 of this health assessment were reviewed by a panel of experts at a peer-review workshop held September 22-25, 1992. Members of the Peer Review Panel for this workshop were as follows:

Edward Bresnick	Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH
M. Judith Charles	Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC
Michael Denison	Department of Biochemistry, Michigan State University, East Lansing, MI
Phillip Enterline	Emeritus Professor of Biostatistics, University of Pittsburgh, Pittsburgh, PA

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

Mark Feeley	Toxicity Evaluation Division, Bureau of Chemical Safety, Health, and Welfare, Ottawa, Ontario, Canada
Thomas A. Gasiewicz	Department of Biophysics, University of Rochester, Rochester, NY
James Gillette	Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD
Claude Hughes	Duke University Medical Center, Durham, NC
Curtis D. Klaassen	Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, Kansas City, KS
Daniel Krewski	Biostatistics and Computer Applications, Environmental Health Centre, Ottawa, Ontario, Canada
Suresh Moolgavkar	Professor of Epidemiology and Biostatistics, The Fred Hutchinson Cancer Research Center, Seattle, WA
Jay Silkworth	Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY
Thomas Webster	Center for the Biology of Natural Systems, Queens College, City University of New York, Flushing, NY

On September 7 and 8, 1993, a peer-review workshop was held to review a greatly revised and expanded draft Chapter 7 (Epidemiology/ Human Data). Members of the Peer Review Panel for this workshop are as follows:

John Andrews	Associate Administrator for Science, Agency for Toxic Substances and Disease Registry, Atlanta, GA
Germaine Buck	Clinical Assistant Professor, Department of Social and Preventive Medicine, State University of New York, Buffalo, NY

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

Harvey Checkoway	Professor, Department of Environmental Health, University of Washington, Seattle, WA
Phillip Enterline	Emeritus Professor of Biostatistics, University of Pittsburgh, Pittsburgh, PA
M. Gerald Ott	Director of Epidemiology, BASF Corporation, Parsippany, NJ
Allan H. Smith	Professor of Epidemiology, University of California, Berkeley, CA
Anne Sweeney	Assistant Professor of Epidemiology, School of Public Health, University of Texas, Houston, TX
Karen Webb	Medical Director, HealthLine Corporation Health, St. Louis, MO

In addition, during the development of this draft Health Assessment Document, selected sections, chapters, or volumes were peer reviewed by scientists and experts within EPA and other federal agencies, as well as by experts in academia and the private sector.

A draft of Chapter 9, the risk characterization, was reviewed by an interagency workgroup comprising scientists from the following agencies of the federal government:

Department of Agriculture

Department of Defense

Department of Health and Human Services*

Department of Labor (Occupational Safety and Health Administration)

Department of Veterans Affairs

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*Drafts of Chapters 7 and 9 have been reviewed by the Subcommittee on Risk Assessment of the Committee to Coordinate Health and Environmental Related Programs (CCEHRP) under the direction of Bryan D. Hardin of the National Institute for Occupational Safety and Health, Centers for Disease Control, Department of Health and Human Services, and Ron Coene, Executive Secretary of CCEHRP.

AUTHORS, CONTRIBUTORS, AND REVIEWERS (continued)

Executive Office of the President

Office of Science and Technology Policy

Council of Economic Advisors

Domestic Policy Council

1. DISPOSITION AND PHARMACOKINETICS

The disposition and pharmacokinetics of 2,3,7,8-TCDD and related compounds have been investigated in several species and under various exposure conditions. Several reviews on this subject focus on 2,3,7,8-TCDD and related halogenated aromatic hydrocarbons (Neal et al., 1982; Gasiewicz et al., 1983b; Olson et al., 1983; Birnbaum, 1985; Van den Berg et al., 1994). During the past 6 years, considerably more data have been published on this class of compounds, which includes 2,3,7,8-substituted CDDs, BDDs, CDFs, BDFs, and the coplanar PCBs and PBBs. This chapter reviews the disposition and pharmacokinetics of these agents and identifies congener- and species-specific factors that may have an impact on the dose-related biological responses of these compounds.

1.1. ABSORPTION/BIOAVAILABILITY FOLLOWING EXPOSURE

Gastrointestinal, dermal, and transpulmonary absorptions represent potential routes for human exposure to this class of persistent environmental contaminants. Parenteral absorption is a route of exposure that has been used to generate disposition and pharmacokinetic data on these compounds.

1.1.1. Oral

1.1.1.1. *Gastrointestinal Absorption in Animals*

A major source of human exposure to 2,3,7,8-TCDD and related compounds is thought to be through the diet. Experimentally, these compounds are commonly administered in the diet or by gavage in an oil vehicle. Gastrointestinal absorption is usually estimated as the difference between the administered dose (100%) and the percent of the dose that was not absorbed. The unabsorbed fraction is estimated as the recovery of parent compound in feces within 24 to 48 hours of a single oral exposure by gavage. Table 1-1 summarizes gastrointestinal absorption data on 2,3,7,8-TCDD and related compounds.

In Sprague-Dawley rats given a single oral dose of 1.0 μg [^{14}C]-2,3,7,8-TCDD/kg bw in acetone:corn oil (1:25, v/v), the fraction absorbed ranged from 66% to 93%, with a mean of ~84% (Rose et al., 1976). With repeated oral dosing of rats at 0.1 or 1.0 μg/kg/day (5

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Table 1-1. Gastrointestinal Absorption of 2,3,7,8-TCDD and Related Compounds Following a Single Oral Exposure by Gavage

Chemical	Species (Sex)	Dose (μmol/kg)	Dose (μg/kg)	Vehicle	% Administered Dose Absorbed[a] [Mean (Range)]	Reference
CDDs						
2,3,7,8-TCDD	Sprague-Dawley rat (M)	0.16	50	acetone:corn oil (1:7)	70	Piper et al., 1973
2,3,7,8-TCDD	Sprague-Dawley rat (M/F)	0.003	1.0	acetone:corn oil (1:25)	84 (66-93)	Rose et al., 1976
2,3,7,8-TCDD	Hartley guinea pig (F)	0.005	1.45	acetone:corn oil (1:45)	50	Nolan et al., 1979
2,3,7,8-TCDD	Golden Syrian hamster (M)	2.0	650	olive oil	74	Olson et al., 1980
2,3,7,8-TCDD	Human (M)	0.000003	0.001	corn oil	87	Poiger and Schlatter, 1986
1,2,3,7,8-PeCDD	Sprague-Dawley rat (M/F)	0.03	9.2	corn oil	NR (19-71)	Wacker et al., 1986
OCDD	Fischer 344 rat (M)	0.11 1.1 1.1 11	50 500 500 5000	o-dichlorobenzene:Emulphor (1:1) o-dichlorobenzene:corn oil (1:1) corn oil suspension corn oil suspension	12 15 2 5	Birnbaum and Couture, 1988

Table 1-1 (continued)

Chemical	Species (Sex)	Dose (μmol/kg)	Dose (μg/kg)	Vehicle	% Administered Dose Absorbed[a] [Mean (Range)]	Reference
BDDs						
2,3,7,8-TBDD	Fischer 344 rat (M)	0.001 0.01 0.1 0.5	0.5 5 50 500	Emulphor:ethanol:water (1:1:3)	78 82 60 47	Diliberto et al., 1990
CDFs						
2,3,7,8-TCDF	Fischer 344 rat (M)	0.1 1.0	30.6 306	Emulphor:ethanol (1:1)	90 90	Birnbaum et al., 1980
2,3,7,8-TCDF	Hartley guinea pig (M)	0.02	6	Emulphor:ethanol:water (1:1:8)	90	Decad et al., 1981a
2,3,4,7,8-PeCDF	Fischer 344 rat (M)	0.1 0.5 1.0	34 170 340	corn oil	~70 ~70 ~70	Brewster and Birnbaum, 1987
PCBs						
3,3'4,4'-T4CB	C57BL mouse (F)	34.5	10,000	corn oil	77	Wehler et al., 1989

[a]Absorption is generally estimated as the difference between the administered dose (100%) and the percent of the dose that was not absorbed.
The unabsorbed fraction is estimated as the recovery of parent compound in feces within 48 hours of exposure.
NR = Not reported.

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days/week for 7 weeks), gastrointestinal absorption of 2,3,7,8-TCDD was observed to be approximately that observed for the single oral exposure (Rose et al., 1976). Oral exposure of Sprague-Dawley rats to a larger dose of 2,3,7,8-TCDD in acetone:corn oil (50 μg/kg) resulted in an average absorption of 70% of the administered dose (Piper et al., 1973).

One study in the guinea pig reported that ~50% of a single oral dose of 2,3,7,8-TCDD in acetone:corn oil was absorbed (Nolan et al., 1979). The gastrointestinal absorption of 2,3,7,8-TCDD was also examined in the hamster, the species most resistant to the acute toxicity of this compound (Olson et al., 1980). Hamsters were given a single, sublethal, oral dose of [1,6-^{3}H]-2,3,7,8-TCDD in olive oil (650 μg/kg), and an average of 75% of the dose was absorbed. When 2,3,7,8-TCDD was administered to rats in the diet at 7 or 20 ppb (0.5 or 1.4 μg/kg/day) for 42 days, 50% to 60% of the consumed dose was absorbed (Fries and Marrow, 1975). These findings indicate that oral exposure to 2,3,7,8-TCDD in the diet or in an oil vehicle results in the absorption of >50% of the administered dose.

The intestinal absorption of [^{3}H]-2,3,7,8-TCDD has also been investigated in thoracic duct-cannulated rats (Lakshmanan et al., 1986). The investigators concluded that 2,3,7,8-TCDD was absorbed into chylomicrons and transported through the lymphatic system before entering the systemic circulation.

The absorption of 2,3,7,8-TBDD in male Fischer 344 rats was studied after oral exposure by gavage at 5 μg/kg in Emulphor:ethanol:water (1:1:3) (Diliberto et al., 1990). The percent of the dose absorbed for this study was defined as 100% (% total oral dose in feces on days 1 and 2 — % total intravenous dose in feces on days 1 and 2) using the intravenous pharmacokinetic data of Kedderis et al. (1991a).

The relative absorbed dose or bioavailability of 2,3,7,8-TBDD after oral exposure was estimated at 78, 82, 60, and 47% at dose levels of 0.001, 0.01, 0.1, and 0.5 μmol/kg, respectively. These results suggest nonlinear absorption at the higher doses, with maximal oral absorption at an exposure of ≤0.01 μmol/kg (5 μg/kg).

The absorption of 2,3,7,8-TCDF has been investigated after oral exposure by gavage. Approximately 90% of the administered dose (0.1 and 1.0 μmol/kg) of 2,3,7,8-TCDF in Emulphor:ethanol (1:1) was absorbed in male Fischer 344 rats (Birnbaum et al., 1980). (Emulphor EL-620 is a polyoxyethylated vegetable oil preparation [GAF Corp., New York,

NY]). Similarly, >90% of the administered dose (0.2 μmol/kg, 6 μg/kg, and 1-15 μg/kg) of 2,3,7,8-TCDF in Emulphor:ethanol:water (1:1:8) was absorbed in male Hartley guinea pigs (Decad et al., 1981a; Ioannou et al., 1983). Thus, 2,3,7,8-TCDF appears to be almost completely absorbed from the gastrointestinal tract. This may be related to the greater relative solubility of 2,3,7,8-TCDF compared with that of 2,3,7,8-TCDD or 2,3,7,8-TBDD.

The oral bioavailability of 2,3,4,7,8-PeCDF and 3,3′,4,4′-TCB in corn oil was similar to that of 2,3,7,8-TCDD (Brewster and Birnbaum, 1987; Wehler et al., 1989; Clarke et al., 1984). Furthermore, 2,3,4,7,8-PeCDF absorption was independent of the dose (0.1, 0.5, or 1.0 μmol/kg). Incomplete and variable absorption of 1,2,3,7,8-PeCDD was reported in rats, with 19% to 71% of the dose absorbed within the first 2 days after oral exposure (Wacker et al., 1986).

Early studies on the pharmacokinetic behavior of OCDD by Williams et al. (1972) and Norback et al. (1975) demonstrated that OCDD was poorly absorbed after oral exposure. More recently, Birnbaum and Couture (1988) also found that the gastrointestinal absorption of OCDD in rats was very limited, ranging from 2% to 15% of the administered dose. Lower doses (50 μg/kg) in an o-dichlorobenzene:corn oil (1:1) vehicle were found to give the best oral bioavailability for this extremely insoluble compound.

1.1.1.2. *Gastrointestinal Absorption in Humans*

Poiger and Schlatter (1986) investigated the absorption of 2,3,7,8-TCDD in a 42-year-old man after ingestion of 105 ng [^{3}H]-2,3,7,8-TCDD (1.14 ng/kg bw) in 6 mL corn oil and found that >87% of the oral dose was absorbed from the gastrointestinal tract. Following absorption, the half-life for elimination was estimated to be 2,120 days.

The above data indicate that gastrointestinal absorption of 2,3,7,8-TCDD and related compounds is variable, incomplete, and congener and vehicle specific. More soluble congeners, such as 2,3,7,8-TCDF, are almost completely absorbed, while the extremely insoluble OCDD is poorly absorbed. In some cases, absorption has been found to be dose dependent, with increased absorption occurring at lower doses (2,3,7,8-TBDD, OCDD). The limited database also suggests that there are no major interspecies differences in the gastrointestinal absorption of these compounds.

Because CDDs, CDFs, and PCBs are present in human milk, McLachlan (1993) investigated the net absorption of these compounds in a nursing infant. The contaminant input, through the ingestion of mother's milk, and the contaminant output in the feces were measured to estimate the digestive tract absorption of these compounds. For almost all congeners, more than 90% of the ingested compound was absorbed, indicating that the common assumption of 100% absorption of CDDs, CFFs, and PCBs in nursing infants is reasonable.

1.1.1.3. *Bioavailability Following Oral Exposure*

Oral exposure of humans to 2,3,7,8-TCDD and related compounds usually occurs as a complex mixture of these contaminants in food, soil, dust, water, or other mixtures that would be expected to alter absorption.

The influence of dose and vehicle or adsorbent on gastrointestinal absorption has been investigated in rats by Poiger and Schlatter (1980), using hepatic concentrations 24 hours after dosing as an indicator of the amount absorbed (Table 1-2). Administration of 2,3,7,8-TCDD in an aqueous suspension of soil resulted in a decrease in the hepatic levels of 2,3,7,8-TCDD as compared with hepatic levels resulting from administration of 2,3,7,8-TCDD in 50% ethanol. The extent of the decrease was directly proportional to the length of time the 2,3,7,8-TCDD had been in contact with the soil. When 2,3,7,8-TCDD was mixed in an aqueous suspension of activated carbon, absorption was almost totally eliminated (<0.07% of the dose in hepatic tissues).

Philippi et al. (1981) and Huetter and Philippi (1982) have shown that radiolabeled 2,3,7,8-TCDD becomes progressively more resistant with time to extraction from soil. Similarly, the feeding of fly ash, which contains CDDs, to rats in the diet for 19 days resulted in considerably lower hepatic levels of CDDs than did the feeding of an extract of the fly ash at comparable dietary concentrations of CDDs (Van den Berg et al., 1987a). The CDDs were tentatively identified as 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD, and 1,2,3,7,8,9-HxCDD, and the difference in hepatic levels noted between fly ash-treated and extract-treated rats was greater for the more highly chlorinated isomers than for 2,3,7,8-TCDD. These results indicate the importance of the formulation or vehicle containing the

Table 1-2. Percentage of 2,3,7,8-TCDD in the Liver of Rats 24 Hours After Oral Administration of 0.5 mL of Various Formulations Containing TCDD*

Formulation	TCDD Dose (ng)	No. of Animals	Percentage of Dose in the Liver
50% ethanol	14.7	7	36.7±1.2
Aqueous suspension of soil (37%, w/w) that had been in contact with TCDD for:			
10-15 hours	12.7, 22.9	17	24.1±4.8
8 days	21.2, 22.7	10	16.0±2.2
Aqueous suspension of activated carbon (25%, w/w)	14.7	6	≤0.07

*Source: Poiger and Schlatter, 1980.
w/w = Weight by weight.

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toxin(s) on the relative bioavailability of 2,3,7,8-TCDD, PeCDD, and HxCDDs after oral exposure.

Because 2,3,7,8-TCDD in the environment is likely to be absorbed to soil, McConnell et al. (1984) and Lucier et al. (1986) compared the oral bioavailability of 2,3,7,8-TCDD from environmentally contaminated soil with that from 2,3,7,8-TCDD administered in corn oil in rats and guinea pigs and rats, respectively. As indicated by biological effects and the amount of 2,3,7,8-TCDD in the liver, the intestinal absorption from soil from Times Beach and Minker Stout, Missouri, was ~50% less than that from corn oil. Shu et al. (1988a) reported an oral bioavailability of ~43% in the rat dosed with three environmentally contaminated soil samples from Times Beach, Missouri. This figure did not change significantly over a 500-fold dose range of 2-1,450 ng 2,3,7,8-TCDD/kg bw for soil contaminated with ~2, 30, or 600 ppb of 2,3,7,8-TCDD. In studies of other soil types, Umbreit et al. (1986a,b) estimated an oral bioavailability in the rat of 0.5% for soil at a New Jersey manufacturing site and 21% for a Newark salvage yard. These results indicate that bioavailability of 2,3,7,8-TCDD from soil varies between sites and that 2,3,7,8-TCDD content alone may not be indicative of potential human hazard from contaminated environmental materials. Although these data indicate that substantial absorption may occur from contaminated soil, soil type and duration of contact, as suggested from the data that demonstrated decreased extraction efficiency with increasing contact time between soil and 2,3,7,8-TCDD (Philippi et al., 1981; Huetter and Philippi, 1982), may substantially affect the absorption of 2,3,7,8-TCDD from soils obtained from different contaminated sites.

1.1.2. Dermal Absorption

Brewster et al. (1989) examined the dermal absorption of 2,3,7,8-TCDD and three CDFs in male Fischer 344 rats (10 weeks old; 200-250 g). The fur was clipped from the intrascapular region of the back of each animal. A single compound in 60 μL of acetone was then applied over a 1.8 cm^2 area of skin, which then was covered with a perforated stainless steel cap. Table 1-3 summarizes data on the absorption of each compound at 3 days after a single dermal exposure. At an exposure of 0.1 μmol/kg, the absorption of 2,3,7,8-TCDF (49% of administered dose) was greater than that of 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF,

Table 1-3. Dermal Absorption of 2,3,7,8-TCDD and Related Compounds in the Rat[a]

Chemical	Dose		% Administered Dose	
	(μmol/kg)	(μg/kg)	Skin Site[b]	Absorbed
2,3,7,8-TCDD	0.00015	0.05	61.73±4.37	38.27±4.37
	0.001	0.32	59.71±1.90	40.29±1.89
	0.01	3.2	72.60±0.41	27.40±0.41
	0.1	32	82.21±2.85	17.78±2.85
	0.5	160	80.92±2.74	19.08±2.74
	1.0	321	82.68±3.69	17.30±3.67
2,3,7,8-TCDF	0.1	31	51.18±11.95	48.84±11.95
	0.5	153	82.14±11.22	17.86±11.22
	1.0	306	88.70±5.17	11.32±5.17
1,2,3,7,8-PeCDF	0.1	34	74.72±3.58	25.27±3.58
	0.5	170	91.67±2.46	8.33±2.46
	1.0	340	84.23±5.44	15.76±5.44
2,3,4,7,8-PeCDF	0.1	34	65.77±4.80	34.19±4.78
	0.5	170	75.50±1.81	24.50±1.80
	1.0	340	81.84±1.67	18.16±1.67

[a]Source: Brewster et al., 1989.
[b]Values are the mean±SD of three to four animals and represent the amount of administered dose of radiolabeled congener remaining at the application site 3 days after dermal exposure.

and 2,3,7,8-TCDD. For each compound, the relative absorption (percentage of administered dose) decreased with increasing dose, while the absolute absorption (μg/kg) increased nonlinearly with dose. Results also suggest that the majority of the compound remaining at the skin exposure site was associated with the stratum corneum and did not penetrate through to the dermis. In a subsequent study, Banks and Birnbaum (1991a) examined the rate of absorption of 2,3,7,8-TCDD over 120 hours after the dermal application of 200 pmol (1 nmol/kg) to male Fischer 344 rats. The absorption kinetics appeared to be first order, with an absorption rate constant of 0.005 hour^{-1}. With a similar exposure protocol, the dermal absorption of 2,3,7,8-TCDF was found to follow a first-order process, with a rate constant of 0.009 hour^{-1} (Banks and Birnbaum, 1991b). Together, these results on dermal absorption indicate that at lower doses ($\leq$0.1 μmol/kg), a greater percentage of this administered dose of 2,3,7,8-TCDD and three CDFs was absorbed. Nonetheless, the rate of absorption of 2,3,7,8-TCDD is still very slow (rate constant of 0.005 hour^{-1}) even following a low-dose dermal application of 200 pmol (1 nmol/kg). Results from Table 1-3 also suggest that the dermal absorption of 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF, and 1,2,3,7,8-PeCDF, occurs at a very slow rate. Using a similar exposure protocol, the dermal absorption of 2,3,7,8-TBDD was only 30% to 40% of that observed for 2,3,7,8-TCDD (Diliberto et al., 1993a).

The dermal absorption of several polyhalogenated aromatic hydrocarbons in male F344 rats was also compared with estimates of their respective octanol-water partition coefficients (Jackson et al., 1993). Inverse correlations were found between octanol-water partition coefficient estimates and the single dose ($\sim$1.0 nmol/kg and $\sim$0.1 μmol/kg) dermal absorption for most of the compounds studied. The differential dermal absorption of 2,3,7,8-TCDD and 2,3,7,8-TBDD may result, in part, from the diminished ability of the more lipophilic 2,3,7,8-TBDD to partition out of the stratum corneum and into the underlying epidermal and dermal layers.

Rahman et al. (1992) and Gallo et al. (1992) compared the in vitro permeation of 2,3,7,8-TCDD through hairless mouse and human skin. In both species, the amount of 2,3,7,8-TCDD permeated increased with the dose, but the percentage of the dose permeated decreased with increasing dose. The permeability coefficient of 2,3,7,8-TCDD in human

skin was about one order of magnitude lower than that in mouse skin. The hairless mouse skin does not appear to be a suitable model for the permeation of 2,3,7,8-TCDD through human skin because the viable tissues were major barriers to 2,3,7,8-TCDD permeation in hairless mouse skin, while the stratum corneum layer provided the greater resistance in human skin. A significant increase in 2,3,7,8-TCDD permeation through human skin was observed when the skin was damaged by tape-stripping. Gallo et al. (1992) suggested that washing and tape-stripping of the exposed area might remove most of the 2,3,7,8-TCDD and reduce the potential for systemic exposure and toxicity because most of the 2,3,7,8-TCDD remained within the horny layer of human skin even at 24 hours following exposure.

Weber et al. (1991) also investigated the penetration of 2,3,7,8-TCDD into human cadaver skin at concentrations of 65-6.5 ng/cm^2. This study also found that the stratum corneum acted as a protective barrier, as its removal increased the amount of 2,3,7,8-TCDD absorbed into layers of the skin. With intact skin and acetone as the vehicle, the rate of penetration of 2,3,7,8-TCDD into the dermis ranged from 6 to 170 pg/hour/cm^2, while penetration into the dermis and epidermis ranged from 100 to 800 pg/hour/cm^2. With mineral oil as the vehicle, there was about a 5- to 10-fold reduction in the rate of penetration of 2,3,7,8-TCDD into the intact skin.

Wester et al. (1993a) studied the dermal permeation potential of two PCBs, Aroclor 1242 and Aroclor 1254, from soil. Soil is the most common medium of contact for humans; hence, any permeation potential is of great interest for risk assessment purposes. This study consisted of experiments conducted in rhesus monkeys (in vivo), in vitro human skin, and powdered human corneum. The monkeys were exposed topically for a 5-week period. Percutaneous absorption was determined by urinary and fecal [^{14}C]-PCB excretion. The percutaneous absorption in the monkey was 13.8% ($\pm$2.7%) for Aroclor 1242 and 14.1% ($\pm$1.0%) for Aroclor 1254. The authors report that these absorption rates for soil are similar to those from vehicles such mineral oil, trichlorobenzene, and acetone. These rates are somewhat higher than those for other compounds such as the pesticide butachlor, which was about 5.0% when applied to human skin (Ademola et al., 1993). On the other hand, Wester et al. (1993b) report a much higher absorption for pentachlorophenol (PCP) (24.4% from soil and 29.2% from acetone) in the rhesus monkey. The authors report that PCP is

one of the more extensively absorbed compounds they have studied. Thus, at least for these two PCBs (Aroclor 1242 and Aroclor 1254), the absorption in the rhesus monkey appears to be intermediate between that of other compounds tested. In summary, these studies indicate that these two PCBs show significant partitioning into the stratum corneum from soil, a common environmental matrix.

1.1.2.1. *Bioavailability Following Dermal Exposure*

Dermal exposure of humans to 2,3,7,8-TCDD and related compounds usually occurs as a complex mixture of these contaminants in soil, oils, or other mixtures, which would be expected to alter absorption. Poiger and Schlatter (1980) presented evidence that the presence of soil or lipophilic agents dramatically reduces dermal absorption of 2,3,7,8-TCDD compared with absorption of pure compound dissolved in solvents. In a control experiment, 26 ng of 2,3,7,8-TCDD in 50 μL methanol was administered to the skin of rats; 24 hours later the liver contained 14.8$\pm$2.6% of the dose. By comparing this value to the hepatic levels obtained after oral administration in 50% ethanol (in the same study), the amount absorbed from a dermal application can be estimated at $\sim$40% of the amount absorbed from an equivalent oral dose. This comparison assumes that hepatic levels are valid estimates of the amount absorbed from both oral and dermal routes and that absorption from methanol is equivalent to absorption from 50% ethanol. The dose-dependent distribution of 2,3,7,8-TCDD in the liver is another factor that may limit quantitative conclusions regarding bioavailability that are based solely on hepatic levels following exposure to 2,3,7,8-TCDD. As compared with dermal application in methanol, dermal application to rats of 2,3,7,8-TCDD in vaseline or polyethylene glycol reduced the percentage of the dose in hepatic tissue to 1.4 and 9.3%, respectively, but had no observable effect on the dose of 2,3,7,8-TCDD required to induce skin lesions ($\sim$1 μg/ear) in the rabbit ear assay. Application of 2,3,7,8-TCDD in a soil/water paste decreased hepatic 2,3,7,8-TCDD to $\sim$2% of the administered dose and increased the amount required to produce skin lesions to 2-3 μg in rats and rabbits, respectively. Application in an activated carbon/water paste essentially eliminated absorption, as measured by percent of dose in the liver, and increased the amount of 2,3,7,8-TCDD required to produce skin lesions to $\sim$160 μg. These results suggest that dermal

absorption of 2,3,7,8-TCDD depends on the formulation (vehicle or adsorbent) containing the toxin.

Shu et al. (1988b) investigated the dermal absorption of soil-bound 2,3,7,8-TCDD in rats. Relative dermal bioavailability was estimated by comparing the level of 2,3,7,8-TCDD in the liver of rats given soil-bound 2,3,7,8-TCDD dermally to that of rats given oral doses of 2,3,7,8-TCDD dissolved in corn oil. The level of 2,3,7,8-TCDD in livers of rats dosed orally with 2,3,7,8-TCDD in corn oil, following correction for unabsorbed 2,3,7,8-TCDD, is assumed to represent 100% bioavailability. The dermal penetration of 2,3,7,8-TCDD after 4 hours of contact with skin was ~60% of that after 24 hours of contact. After 24 hours of contact with the skin, the degree of dermal uptake from contaminated soil was ~1% of the administered dose. The authors observed that the degree of uptake does not appear to be influenced significantly by the concentration of 2,3,7,8-TCDD in soil, by the presence of crankcase oil as co-contaminants, or by environmentally versus laboratory-contaminated soil.

A major limitation of these studies is the uncertainty regarding the extrapolation of dermal absorption data on these compounds from the rat to the human. The in vitro dermal uptake of 2,3,7,8-TCDD has been investigated in hairless mouse and human skin (Gallo et al., 1992; Rahman et al., 1992). In vitro dermal uptake of 2,3,7,8-TCDD from laboratory-contaminated soil indicated that aging of soils (up to 4 weeks) and the presence of additives (2,4,5-trichlorophenol and motor oil) in the soil did not have any significant effect on dermal uptake (Gallo et al., 1992). Because most of the 2,3,7,8-TCDD remained in the stratum corneum layer of human skin, the permeation of 2,3,7,8-TCDD was significantly lower in human than in hairless mouse skin. Although there are no published quantitative in vivo data on the dermal absorption of 2,3,7,8-TCDD and related compounds in the human, data on the rhesus monkey are very limited. Brewster et al. (1988) found that 1,2,3,7,8-PeCDF was poorly absorbed in the monkey after dermal application, with <1% of the administered dose being absorbed in 6 hours. This provides further evidence for the very slow rate of dermal absorption of 2,3,7,8-TCDD and related compounds.

1.1.3. Transpulmonary Absorption

The use of incineration as a means of solid and hazardous waste management results in the emission of contaminated particles that may contain TCDD and related compounds into the environment. Thus, significant exposure to TCDD and related compounds may result from inhalation of contaminated fly ash, dust, and soil. In an attempt to address the bioavailability and potential health implications of inhaling contaminated particles, Nessel et al. (1990) examined the potential for transpulmonary absorption of TCDD in female Sprague-Dawley rats after intratracheal instillation of the compound in a corn oil vehicle or as a laboratory-prepared contaminant of gallium oxide particles. Several biomarkers of systemic absorption were measured, including the dose-dependent effects of TCDD on hepatic microsomal cytochrome P-450 content, AHH activity, and liver histopathology. Significant dose-related effects were observed at an exposure of ≥ 0.55 μg TCDD/kg. The authors found that induction was slightly higher when animals received TCDD in corn oil than when animals received TCDD-contaminated particles and was comparable to induction after oral exposure. The results from Nessel et al. (1990) indicate that systemic effects occur after pulmonary exposure to TCDD, suggesting that transpulmonary absorption of TCDD does occur.

The pulmonary bioavailability of 2,3,7,8-TCDD was also examined in female Sprague-Dawley rats following intratracheal instillation of PCDD-contaminated soil from a former 2,4,5-trichlorophenoxy-acetic acid manufacturing site (Nessel et al., 1992). A size-dependent enrichment of PCDDs and PCDFs was observed, with the smaller particles being more highly contaminated. 2,3,7,8-TCDD was enriched up to 33-fold in small respirable particles as compared with unfractionated soil. Pulmonary bioavailability of 2,3,7,8-TCDD was assessed by hepatic enzyme induction (AHH activity) and 2,3,7,8-TCDD concentration. The data indicate that the relative pulmonary bioavailability of 2,3,7,8-TCDD on respirable soil particles is 100% as compared with laboratory recontaminated gallium oxide.

The transpulmonary absorption of 2,3,7,8-TCDD was assessed in male Fischer 344 rats following intratracheal instillation of a 1 nmol/kg dose in Emulphor:ethanol:water (Diliberto et al., 1992). Transpulmonary absorption was $\sim 92\%$, suggesting that there was almost complete absorption of 2,3,7,8-TCDD by inhalation under these conditions. Similar

results were also observed for the transpulmonary absorption of 2,3,7,8-TBDD under similar exposure conditions (Diliberto et al., 1993a,b). These results suggest that the transpulmonary absorption of 2,3,7,8-TCDD and 2,3,7,8-TBDD was similar to that observed following oral exposure.

1.1.4. Parenteral Absorption

In an effort to obtain more reproducible and complete absorption of 2,3,7,8-TCDD and related compounds for pharmacokinetic studies, Abraham et al. (1989a,b) used various vehicles to investigate the absorption of 2,3,7,8-TCDD after parenteral application in rats. These investigators observed optimal results with the subcutaneous injection of 2,3,7,8-TCDD with a mixture of toluene:DMSO (1:2) as the vehicle. At 3 and 5 days after treatment, the percentages of administered dose remaining at the injection site under the skin of the back were ~10% and 2%, respectively. The vehicle did not cause adverse effects at an applied volume of 0.2 mL/kg bw. The absorption of a defined mixture of CDDs and CDFs in the rat was also examined after subcutaneous injection with toluene:DMSO (1:2) as a vehicle. Of the 97 congeners analyzed, 70 were ≥95% absorbed 7 days after exposure; 21 were 90-95% absorbed; and 1,2,3,9-TCDD, 1,2,3,6,7,9-/1,2,3,6,8,9-HxCDD, 1,2,3,4,6,7,9-HpCDD, OCDD, 1,2,4,6,8,9-HxCDF and 1,2,3,7,8,9-HxCDF were 84-89% absorbed. Greater than 90% absorption of CDDs and CDFs was also observed under these conditions in the marmoset monkey, with the exception of 1,2,3,4,7,8,9-HpCDF, OCDF, and OCDD, which had ~50-80% of the administered dose absorbed (Neubert et al., 1990; Abraham et al., 1989a). Although the absorption of CDDs and CDFs after subcutaneous administration in toluene:DMSO (1:2) is somewhat slow in rats and monkeys, absorption of most congeners was >90% within 7 days. Even for highly chlorinated insoluble congeners, such as OCDD and OCDF, subcutaneous absorption was >84% in the rat and >50% in the monkey.

Less complete and slower absorption of CDDs and CDFs was observed after subcutaneous injection of these compounds using an oil-containing vehicle (Brunner et al., 1989; Abraham et al., 1989a). Using a corn oil:acetone vehicle (24:1, v/v), Lakshmanan et al. (1986) observed that only 7% of the administered dose of 2,3,7,8-TCDD was absorbed

24 hours after subcutaneous injection and that only 35% was absorbed after intraperitoneal injection. Brunner et al. (1989) also reported that intraperitoneal administration of CDDs and CDFs revealed a delayed absorption from the abdominal cavity that varied for the different congeners. Therefore, concentrations measured in abdominal adipose tissue after intraperitoneal administration may not represent average values of adipose tissue in the whole body, particularly at early time points following exposure. This may also be true following oral exposure due to different perfusion rates of different fat depots (McKinley et al., 1993).

1.2. DISTRIBUTION

1.2.1. Distribution in Blood and Lymph

Once a compound is absorbed, its distribution is regulated initially by its binding to components in blood and its ability to diffuse through blood vessels and tissue membranes. Lakshmanan et al. (1986) investigated the absorption and distribution of 2,3,7,8-TCDD in thoracic duct-cannulated rats. Their results suggest that after gastrointestinal absorption, 2,3,7,8-TCDD is absorbed primarily by the lymphatic route and is transported predominantly by chylomicrons. Ninety percent of the 2,3,7,8-TCDD in lymph was associated with the chylomicron fraction. The plasma disappearance of 2,3,7,8-TCDD-labeled chylomicrons followed first-order decay kinetics, with 67% of the compound leaving the blood compartment very rapidly ($t_{1/2}$=0.81 minutes), whereas the remainder of the 2,3,7,8-TCDD had a $t_{1/2}$ of 30 minutes. 2,3,7,8-TCDD was then found to distribute primarily to the adipose tissue and the liver.

In vitro studies have investigated the distribution of 2,3,7,8-TCDD in human whole blood. Henderson and Patterson (1988) found ~80% of the compound associated with the lipoprotein fraction, 15% associated with protein (primarily human serum albumin), and 5% associated with cellular components. A subsequent in vivo investigation reported a similar distribution of 2,3,7,8-TCDD in the various fractions of human whole blood (Patterson et al., 1989b). Theoretical and limited experimental data also suggest that 2,3,7,8-TCDD and related compounds may be associated with plasma prealbumin (McKinney et al., 1985; Pedersen et al., 1986). The distribution of [^{3}H]-2,3,7,8-TCDD among lipoprotein fractions

from three fasting, normolipemic donors indicated a greater percentage associated with LDL (55.3±9.03% SD) than with VLDL (17.4±9.07% SD) or HDL (27.3±10.08% SD). The distribution of 2,3,7,8-TCDD among the lipoprotein fractions was similar to that reported earlier by Marinovich et al. (1983). When the binding of 2,3,7,8-TCDD was calculated per mole of lipoprotein, it was suggested that the maximal binding capacity was exerted by VLDL, followed by LDL and HDL (Marinovich et al., 1983). The results also suggest that variations in the amounts of each lipoprotein class may alter the distribution of 2,3,7,8-TCDD among lipoproteins in a given subject. Significant species differences also exist; in the case of the rat, which has markedly lower plasma lipids compared to humans, 2,3,7,8-TCDD was distributed almost equally among the lipoprotein fractions (Marinovich et al., 1983).

Congener-specific differences have been observed for the in vivo binding of the 2,3,7,8-substituted PCDDs and PCDFs to different serum fractions in human blood (Patterson et al., 1989b). Binding to the lipoproteins gradually decreased with increasing chlorine content, with about 75% of 2,3,7,8-TCDD bound to lipoproteins, while approximately 45% of OCDD was bound to this fraction. In contrast, binding to other serum proteins increased with chlorine content from approximately 20% for 2,3,7,8-TCDD to 50% for OCDD. The results indicate that the higher chlorinated PCDDs and PCDFs do not partition according to the lipid content of these blood fractions.

In addition, there is indirect evidence that suggests that the binding of 2,3,7,8-TCDD to lipoproteins may alter the pharmacokinetics and toxic potency of the compound. Marinovich et al. (1983) found that experimentally induced hyperlipidemia in rats delayed the development of overt toxicity (lethality). However, the disposition of 2,3,7,8-TCDD was not investigated under these conditions. These investigators suggest that the release of lipoprotein-bound 2,3,7,8-TCDD is related to the metabolic turnover of lipoproteins. In hyperlipidemic rats, the turnover of VLDL and LDL is delayed significantly compared to that in normolipidemic animals, and this may contribute to the plasma lipoprotein binding modifying the toxicity of 2,3,7,8-TCDD in hyperlipidemic rats.

The time- and temperature-dependent cellular uptake of lipoprotein-associated 2,3,7,8-TCDD by cultured human fibroblasts was greatest from LDL, intermediate from HDL, and

least from serum (Shireman and Wei, 1986). Decreased cellular uptake of LDL and 2,3,7,8-TCDD was observed in mutant fibroblasts, which lack the normal cell membrane receptor for LDL. This provides some evidence that specific binding of LDL and the LDL receptor pathway may account for some of the rapid early uptake of 2,3,7,8-TCDD with LDL entry. The results suggest that the entry of 2,3,7,8-TCDD into cells may not be solely by simple diffusion. However, nonspecific binding of the LDL and transfer of 2,3,7,8-TCDD from LDL to the cell membranes are probably also important, as significant time- and temperature-dependent uptake of 2,3,7,8-TCDD and LDL occurred in the mutant fibroblasts.

Thus, upon absorption, 2,3,7,8-TCDD and probably related compounds are bound to chylomicrons, lipoproteins, and other serum proteins that assist in distributing these uncharged, lipophilic compounds throughout the vascular system. These compounds then partition from blood components into cellular membranes and tissues, probably largely by passive diffusion. In addition, cellular uptake may be facilitated partly through the cell membrane LDL receptor, the hepatic receptor for albumin (Weisiger et al., 1981), and other systems.

1.2.2. Tissue Distribution

Once absorbed into blood, 2,3,7,8-TCDD and related compounds readily distribute to all organs. Tissue distribution within the first hour after exposure parallels blood levels and reflects physiological parameters such as blood flow to a given tissue and relative tissue size. For example, high initial concentrations of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDF, and 3,3′,4,4′-TCB were observed in highly perfused tissue such as the adrenal glands during the 24-hour period after a single exposure (Birnbaum et al., 1980; Olson et al., 1980; Pohjanvirta et al., 1990; Brewster and Birnbaum, 1988; Durham and Brouwer, 1990). A high percentage of the dose of 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF was also found in muscle within the first hour after intravenous exposure due to the large volume of this tissue (Birnbaum et al., 1980; Birnbaum, 1985; Brewster and Birnbaum, 1988). Nevertheless, within several hours the liver, adipose tissue, and skin become the primary sites of disposition, when expressed as percent of administered dose per gram tissue and as percent of dose per organ. Liver, adipose tissue, skin, and thyroid were the only tissues to show an increase in the concentra-

tion of 2,3,7,8-TCDD during the initial 4 days after a single intraperitoneal exposure of rats (Pohjanvirta et al., 1990). In this study, a similar general pattern of disposition was observed in Han/Wistar and Long-Evans rats, which are, respectively, most resistant and susceptible to the acute toxicity of 2,3,7,8-TCDD (Pohjanvirta et al., 1990).

Table 1-4 illustrates the tissue distribution of 2,3,7,8-TCDD in female Wistar rats 7 days after a single subcutaneous exposure (Abraham et al., 1988). This general pattern of distribution is similar to that observed in mice, rats, rhesus monkeys, hamsters, and guinea pigs, where liver and adipose tissue consistently have the highest concentrations of 2,3,7,8-TCDD (Piper et al., 1973; Fries and Marrow, 1975; Rose et al., 1976; Allen et al., 1975; Van Miller et al., 1976; Kociba et al., 1978a,b; Gasiewicz et al., 1983a; Manara et al., 1982; Olson et al., 1980; Gasiewicz and Neal, 1979; Birnbaum, 1986; Pohjanvirta et al., 1990; Abraham et al., 1988). A similar pattern of disposition also was observed for 2,3,7,8-TCDF in the guinea pig, rat, C57BL/6J and DBA/2J mouse, and rhesus monkey, with 2,3,7,8-TCDF concentrations highest in liver and adipose tissue (Decad et al., 1981b; Birnbaum et al., 1980, 1981). In summary, there do not appear to be major species or strain differences in the tissue distribution of 2,3,7,8-TCDD and 2,3,7,8-TCDF, with the liver and adipose tissue being the primary disposition sites.

The tissue distribution of the coplanar PCBs and PBBs also appears to be similar to that of 2,3,7,8-TCDD and 2,3,7,8-TCDF. Limited studies in rats and mice found that 3,3′,4,4′-TCB, 3,3′,4,4′-TBB, and 3,3′,4,4′,5,5′-HxBB distributed preferentially to adipose tissue and liver (Clarke et al., 1983, 1984; Millis et al., 1985; Wehler et al., 1989; Clevenger et al., 1989).

While the liver and adipose tissue contain the highest concentrations of 2,3,7,8-TCDD and 2,3,7,8-TCDF, there are some congener-specific differences in the relative tissue distribution of related compounds. 2,3,7,8-TBDD and 1,2,3,7,8-PeCDD disposition in the rat was very similar to that of 2,3,7,8-TCDD (Kedderis et al., 1991a; Wacker et al., 1986). The hepatic concentration of OCDD and 2,3,4,7,8-PeCDF in the rat, however, was approximately 10- to 20-fold greater than that in adipose tissue, which generally contains the second highest levels of these compounds (Birnbaum and Couture, 1988; Norback et al., 1975; Williams et al., 1972; Brewster and Birnbaum, 1987). The tissue distribution of a

Table 1-4. Tissue Distribution of [^{14}C]-2-3,7,8-TCDD in Female Wistar Rats[a,b]

Tissue	Range of 2,3,7,8-TCDD Concentrations (ng/g)
Liver	29.23–30.99
Adipose tissue	3.72–4.14
Adrenal glands	0.89–1.08
Ovaries	0.76–0.96
Thymus	0.60–1.05
Skin	0.64–0.68
Lung	0.32–0.33
Kidney	0.27–0.29
Pancreas	0.21–0.31
Spleen	0.18–0.23
Serum	0.16–0.18
Bone (with marrow)	0.16–0.16
Muscle	0.08–0.12
Brain	0.07–0.09

[a]Source: Abraham et al., 1988.
[b]Distribution was assessed 7 days after a single subcutaneous exposure (3 μg/kg bw).

defined mixture of CDDs and CDFs (28.8 μg CDDs+CDFs/kg bw containing 120 ng 2,3,7,8-TCDD/kg bw) was measured in marmoset monkeys 7 days after a single subcutaneous exposure (Abraham et al., 1990). For most of the 2,3,7,8-substituted congeners, the highest concentrations were detected in hepatic and adipose tissue, with correspondingly lower values detected in kidney, brain, lung, heart, thymus, or testes. The hepatic and adipose tissue concentrations were similar for 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, and 1,2,3,7,8-/1,2,3,4,8-PeCDF. Nonetheless, the hepatic concentrations were approximately 10-fold or more greater than those of adipose tissue for 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-/1,2,3,4,7,9-HxCDF, 1,2,3,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF, and OCDF. The lungs and thymus contained higher concentrations of all of these congeners than were detected in kidney, brain, heart, and testes. Unexpectedly, the concentrations of these HxCDDs, HpCDDs, OCDD, and HxCDFs were similar in the adipose tissue, lungs, and thymus. In the case of HpCDFs and OCDF, the concentrations were greater in the lungs than in the adipose tissue. The enhanced disposition of highly chlorinated congeners to the lungs and thymus is of interest and deserves further investigation. For example, it is possible that the high concentration in the lungs could be related to the insolubility of these compounds.

Whole-body autoradiography of mice and rats after intravenous administration of [^{14}C]-2,3,7,8-TCDD showed a selective localization of radioactivity in the liver and nasal olfactory mucosa (Appelgren et al., 1983; Gillner et al., 1987). The selective localization of 2,3,7,8-TCDD in the nasal olfactory mucosa was apparently overlooked by other distribution studies that only examined selected organs. Gillner et al. (1987) found no 2,3,7,8-TCDD-derived radioactivity in the olfactory mucosa after solvent extraction of sections, suggesting that 2,3,7,8-TCDD was not covalently bound in this tissue. In addition, Gillner et al. (1987) reported induction of mRNA coding for cytochrome P-4501A2 in the absence of P-4501A1 induction in olfactory mucosa of rats. The selective distribution of 2,3,7,8-TCDD in the liver and olfactory mucosa correlates with the tissue-specific localization of cytochrome P-24501A2, which represents a potential sequestration (binding) protein (see Section 1.2.5).

Increases in the incidence of squamous cell carcinoma of nasal turbinates and carcinoma of the liver were observed in rats after a 2-year exposure to 2,3,7,8-TCDD in rat chow (Kociba et al., 1978a); however, this effect was not observed in nasal tissues of mice or rats intubated with 2,3,7,8-TCDD. Gillner et al. (1987) suggested that 2,3,7,8-TCDD may not be an initiator in this tissue and indicated that future studies should investigate the possibility that 2,3,7,8-TCDD may act as a promoter or cocarcinogen in nasal tissue.

Evidence has also been reported that suggests that 2,3,7,8-TCDD uptake and retention by the liver is dependent on the cell type within the liver. Hakansson et al. (1989) found that at 4 days after exposure of rats to 2,3,7,8-TCDD, 60% of the dose distributed to hepatocytes and 12% was retained by stellate cells. Half-lives for 2,3,7,8-TCDD in hepatocytes and stellate cells were also calculated to be 13 and 50 days, respectively, suggesting that 2,3,7,8-TCDD is more persistent in nonparenchymal cells. Further studies are needed to understand the pharmacokinetic and pharmacodynamic significance of the cell-specific distribution of 2,3,7,8-TCDD and related compounds.

1.2.2.1. *Tissue Distribution in Humans*

Fachetti et al. (1980) reported tissue concentrations of 2,3,7,8-TCDD at levels of 1-2 ng/g in adipose tissue and pancreas, 0.1-0.2 ng/g in the liver, and $\leq$0.1 ng/g in thyroid, brain, lung, kidney, and blood in a woman who died 7 months after potential exposure to 2,3,7,8-TCDD from the Seveso accident. This pattern of 2,3,7,8-TCDD distribution, however, may not be representative for humans because the woman at the time of death had an adenocarcinoma (which was not considered related to the accident) involving the pancreas, liver, and lung.

Ryan et al. (1985a) examined the distribution of 2,3,7,8-TCDD in two humans at autopsy. They determined on a weight basis that 2,3,7,8-TCDD distributed in descending order to fat ($\sim$6 ppt) and liver ($\sim$2 ppt), with levels in muscle and kidney below detection; however, 2,3,7,8-TCDD levels compared on a per lipid basis were similar between tissues. These data should be interpreted with caution because only two subjects were examined and one of the subjects was suffering from fatty liver syndrome; therefore, the data cannot be generalized to the entire population.

Poiger and Schlatter (1986) estimated that ~90% of the body burden of 2,3,7,8-TCDD was sequestered in the fat after a volunteer ingested [^{3}H]-2,3,7,8-TCDD in corn oil at a dose of 1.14 ng/kg. During this 135-day study, elevated radioactivity was detected in the blood only during the first 2 days after treatment. The data would be consistent with the high lipid bioconcentration potential of 2,3,7,8-TCDD in humans, as calculated by Geyer et al. (1986) from daily intake assumptions, levels in human adipose tissue, and pharmacokinetic models. Geyer et al. (1986) estimated a BCF of between 104 and 206 for 2,3,7,8-TCDD in human adipose tissue.

In human adipose tissue, levels of 2,3,7,8-TCDD averaging 5-10 ppt have been reported for background populations in St. Louis, Missouri, by Graham et al. (1986); in Atlanta, Georgia, and Utah by Patterson et al. (1986); and in Canada by Ryan et al. (1985b). Sielken (1987) evaluated these data and concluded that the levels of 2,3,7,8-TCDD in human adipose are log-normally distributed and positively correlated with age. Among the observed U.S. background levels of 2,3,7,8-TCDD in human adipose tissue, more than 10% were > 12 ppt.

Patterson et al. (1987) developed a high-resolution gas chromatographic/high-resolution mass spectrometric analysis for 2,3,7,8-TCDD in human serum. The arithmetic mean of the individual human serum samples was 47.9 ppt on a whole-weight basis and 7.6 ppt on a lipid-weight basis. Paired human serum and adipose tissue levels of 2,3,7,8-TCDD have been compared by Patterson et al. (1988), Kahn et al. (1988), and Schecter et al. (1990b). All three laboratories reported a high correlation between adipose tissue and serum 2,3,7,8-TCDD levels when the samples were adjusted for total lipid content. This correlation indicates that serum 2,3,7,8-TCDD is a valid estimate of the 2,3,7,8-TCDD concentration in adipose tissue.

Congener-specific partitioning of 2,3,7,8-substituted PCDDs and PCDFs between adipose tissue and plasma lipids has also been reported in a study of 20 Massachusetts Vietnam veterans (Schecter et al., 1990b). The distribution ratio between plasma lipid and adipose tissue increased with chlorine substitution on the PCDDs and PCDFs. While 2,3,7,8-substituted TCDD, TCDF, PeCDD, PeCDF, HxCDD, and HxCDF had a plasma lipid to adipose tissue ratio of about 1.0, OCDD had a ratio of 2.0. On the other hand,

whole blood PCDDs and PCDFs seem to be found at the same concentrations as in adipose tissue on a lipid basis (Schecter, 1991).

The disposition of 2,3,7,8-substituted PCDDs and PCDFs in human liver and adipose tissue was assessed in a study of 28 people from the Munich area (Thoma et al., 1989; 1990). Table 1-5 summarizes these results, which are expressed both on a lipid and wet weight basis. The concentrations of PCDDs and PCDFs in adipose tisue and liver are not the same when calculated on a lipid basis. This is in contrast to the high correlation that was reported between adipose tissue and serum TCDD levels when expressed on a lipid-weight basis (Patterson et al., 1988; Kahn et al., 1988; Schecter et al., 1990b). Furthermore, the liver/adipose tissue ratio increased with the higher chlorinated PCDDs and PCDFs. The congener-specific hepatic disposition is also similar to that observed in rats and marmoset monkeys exposed to a complex mixture of PCDDs and PCDFs (Abraham et al., 1989b; Neubert et al., 1990). Therefore, it is important to consider congener- and tissue-specific differences in disposition of PCDDs and PCDFs when blood levels are used to estimate tissue levels or body burdens.

In a study of potentially heavily exposed Vietnam veterans, MMWR (1988) reviewed an Air Force study of Ranch Hand veterans who were either herbicide loaders or herbicide specialists in Vietnam. The mean serum 2,3,7,8-TCDD levels of 147 Ranch Hand personnel was 49 ppt in 1987, based on total lipid weight, while the mean serum level of the 49 controls was 5 ppt. In addition, 79% of the Ranch Hand personnel and 2% of the controls had 2,3,7,8-TCDD levels $\geq$10 ppt. The distribution of 2,3,7,8-TCDD levels in this phase of the Air Force health study indicates that only a small number of Ranch Hand personnel had unusually heavy 2,3,7,8-TCDD exposure. Similar results were obtained by Kahn et al. (1988) who compared 2,3,7,8-TCDD levels in blood and adipose tissue of Agent Orange-exposed Vietnam veterans and matched controls (Kahn et al., 1988). This study also examined moderately exposed Vietnam veterans who handled herbicides regularly while in Vietnam. Although this study can distinguish moderately exposed men from others, the data do not address the question of identifying persons whose exposures are relatively low and who constitute the bulk of the population, both military and civilian, that may have been exposed to greater than background levels of 2,3,7,8-TCDD.

Table 1-5. 2,3,7,8-Substituted PCDDs and PCDFs in Human Liver and Adipose Tissue

	Tissue Concentrations on a Lipid Basis (ppt)			Tissue Concentrations on a Wet Weight Basis (ppt)	
	Fat	Liver	Liver/Fat	Liver[a]	Liver/Fat
TCDD	8.0	16.4	2.05	1.1	0.14
PeCDD	16.4	20.1	1.22	1.4	0.09
HxCDD	94.7	166.8	1.76	11.7	0.12
HpCDD	106.7	1,002.4	9.39	70.2	0.66
OCDD	373.2	4,416.2	11.83	309.1	0.83
TCDF	2.5	5.5	2.20	0.4	0.15
PeCDF	35.2	173.7	4.93	12.2	0.35
HxCDF	41.5	389.5	9.38	27.3	0.66
HpCDF	14.2	218.9	15.42	15.3	1.08
OCDF	4.0	29.7	7.43	2.1	0.52

Values are the mean of 28 people from the Munich area.
[a]Estimated from the % fat in the liver ($7.02 \pm 5.33\%$, mean$\pm$SD)

Source: Thoma et al., 1990.

1.2.3. Time-Dependent Tissue Distribution

2,3,7,8-TCDD and related compounds exhibit congener-specific disposition, which depends on tissue, species, and time after a given exposure. In general, these compounds are cleared rapidly from the blood and distributed to liver, muscle, skin, adipose tissue, and other tissues within the first hour(s) after exposure. This is followed by redistribution to the liver and adipose tissue, which exhibit increasing tissue concentrations over several days after exposure. Elimination from tissues then occurs at rates that are congener, tissue, and species specific. Thus, the ratio of the concentration of 2,3,7,8-TCDD and related compounds in different tissues (i.e., liver/adipose) may not remain constant over an extended period after a single exposure. Abraham et al. (1988) examined the concentrations of 2,3,7,8-TCDD in liver and adipose tissue of female Wistar rats over a 91-day period after a single subcutaneous exposure at a dose of 300 ng/kg bw (Figure 1-1). The maximum concentration of 2,3,7,8-TCDD in the liver and adipose tissue was reached at 3 and 7 days after exposure, respectively. The liver/adipose tissue concentration ratio does not remain constant over time because the concentration of 2,3,7,8-TCDD decreases more rapidly in the liver than in the adipose tissue. For example, the liver/adipose tissue concentration ratio (for 2,3,7,8-TCDD) was 10.3 at 1 day after exposure and 0.5 at 91 days after exposure (Figure 1-1). Results from other disposition studies also indicate that the ratio of the concentration of 2,3,7,8-TCDD and related compounds in liver, adipose tissue, and other tissues does not remain constant over an extended period after a single exposure (Pohjanvirta et al., 1990; Birnbaum, 1986; Birnbaum et al., 1980; Decad et al., 1981a; Birnbaum and Couture, 1988; Olson et al., 1980; Kedderis et al., 1993a; Brewster and Birnbaum, 1987, 1988; Neubert et al., 1990). This relationship is important in attempting to correlate dose-response data with tissue concentrations of 2,3,7,8-TCDD and related compounds.

In an attempt to maintain constant 2,3,7,8-TCDD levels in tissues to study long-term effects, Krowke et al. (1989) investigated several loading-dose/maintenance-dose exposure regimens. They found that similar liver/adipose tissue concentrations ranging from 5 to 8 could be maintained in rats over a 22-week period with a loading dose of 25 μg/kg followed by weekly maintenance doses of 5 μg/kg.

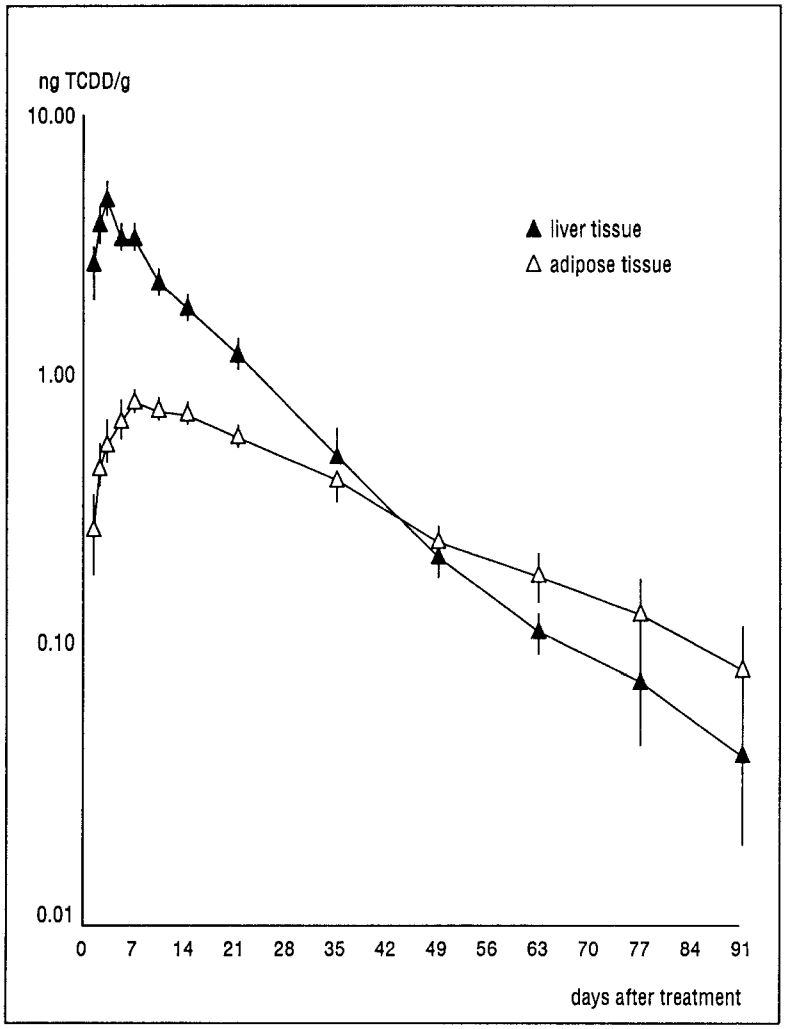

Figure 1-1. Time course of the concentration of ^{14}C-TCDD in rat liver and adipose tissue after a single subcutaneous injection of 300 ng TCDD/kg bw to female rats (M$\pm$SD).

Source: Abraham et al., 1988.

A large body of data on the tissue concentrations of 2,3,7,8-TCDD and related compounds over time after exposure can be evaluated by estimating congener-specific half-life values for a given tissue and species. Table 1-6 summarizes pharmacokinetic elimination parameters for 2,3,7,8-TCDD and related compounds from major tissue depots. Data from Abraham et al. (1988) (see Figure 1-1) were used to estimate the half-life for 2,3,7,8-TCDD in the liver and adipose tissue of rats (Table 1-6). The decrease in the 2,3,7,8-TCDD concentration in adipose tissue is a linear function in the semilogarithmic plot in Figure 1-1 (log concentration versus time), which indicates apparent first-order elimination kinetics with a half-life of 24.5 days (Table 1-6). Liver tissue exhibits a biphasic (two-component) exponential decay pattern, with a half-life of 11.5 days for the first component (days 10-49) and a half-life of 16.9 days for the second component (days 49-91) (see Figure 1-1 and Table 1-6). Results of Abraham et al. (1988) and Lakshmanan et al. (1986) indicate that in the rat, 2,3,7,8-TCDD is more persistent in the adipose tissue than in the liver. This is in contrast to the mouse, where liver and adipose tissue have similar half-lives (Birnbaum, 1986). 2,3,7,8-TCDD is exceptionally persistent in the adipose tissue of the rhesus monkey, with a half-life approximately 10- to 40-fold greater than that observed in the rat and mouse (Bowman et al., 1989). Thus, the relative persistence of 2,3,7,8-TCDD is tissue specific and exhibits marked interspecies variability.

Most of the pharmacokinetic data on the relative persistence of other congeners in Table 1-6 have been reported in rat studies, which limits interspecies comparisons. Results in the rat suggest that the distribution and elimination of 2,3,7,8-TBDD from tissue are similar to those of 2,3,7,8-TCDD. The most persistent congeners are OCDD, 2,3,4,7,8-PeCDF, and 1,2,3,6,7,8-HxCDF, which distribute almost entirely to the liver. OCDD and 2,3,4,7,8-PeCDF also exhibit similar elimination kinetics, with a relative half-life in the liver more than twofold greater than that in adipose tissue. The least persistent congeners are 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and 3,3′,4,4′-TCB. These congeners exhibit similar elimination kinetics in the rat, with half-lives in the adipose tissue greater than those in liver. The relative tissue distribution of these congeners varies, however, with 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF distributing primarily to the liver while 3,3′,4,4′-TCB distributes predominantly to the adipose tissue.

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Table 1-6. Elimination of 2,3,7,8-TCDD and Related Compounds from Major Tissue Depots

Chemical	Species (Sex)	Dose	Tissue	Half-Life (days)	Remarks	Reference
CDDs						
2,3,7,8-TCDD	Wistar rat (F)	0.3 μg/kg, s.c.	liver liver liver adipose	11.5 16.9 13.6 24.5	95% Confidence interval (time period investigated): 10.7-12.3 (10-49 days) 14.0–21.4 (49–91 days) 12.8–14.4 (10–91 days) 22.4–26.8 (14-91 days)	Abraham et al., 1988
2,3,7,8-TCDD	Wistar rat (M)	1.0 μg//kg, i.p.	liver adipose	37.1 53.2	Tissue levels were measured for 20 weeks following exposure	Lakshmanan et al., 1986
2,3,7,8-TCDD	Sprague-Dawley rat (M)	7 or 20 ppb in diet for 42 days	liver	11	85% total dose	Fries and Marrow, 1975
2,3,7,8-TCDD	Sprague-Dawley rat (F)	7 or 20 ppb in diet for 42 days	liver	13	70% of total dose	Fries and Marrow, 1975
2,3,7,8-TCDD	C57BL/6J mice (M) Ah^b/Ah^d	0.5 μg/kg, i.p.	liver adipose skin	8.5 10.3 16.0	Pool size (% of total dose): 36.8 23.6 7.6	Birnbaum, 1986
2,3,7,8-TCDD	C57BL/6J mice (M) Ah^d/Ah^d	0.5 μg/kg, i.p.	liver adipose skin	7.1 7.6 14.9	Pool size (% of total dose): 20.6 31.3 10.2	Birnbaum, 1986
2,3,7,8-TCDD	DBA/2J mice (F) Ah^b/Ah^d	0.5 μg/kg, i.p.	liver adipose skin	12.4 13.3 13.2	Pool size (% of total dose): 29.2 30.9 21.4	Birnbaum, 1986

Table 1-6 (continued)

Chemical	Species (Sex)	Dose	Tissue	Half-Life (days)	Remarks	Reference
2,3,7,8-TCDD	DBA/2J mice (F) Ah^d/Ah^d	0.5 μg/kg, i.p.	liver adipose skin	11.9 11.8 12.8	Pool size (% of total dose): 20.2 42.3 26.6	Birnbaum, 1986
2,3,7,8-TCDD	rhesus monkey (F)	25 ppt in diet	adipose	391±88	Mean±SE (n=7)	Bowman et al., 1989
OCDD	Fischer 344 rat (M)	50 μg/kg, i.v.	liver adipose skin	84 38 3 69	Pool size (% of total dose): 72.7 7.1 9.0, 1st component 0.3, 2nd component	Birnbaum and Couture, 1988
BDDs						
2,3,7,8-TBDD	Fischer 344 rat (M)	0.5 μg/kg, i.v. (0.001 μmol/kg)	liver adipose skin muscle blood	4.5 16.5 57.8 2.5 57.8 1.6 26.7 18.2	1st component 2nd component 1st component 2nd component 1st component 2nd component	Kedderis et al., 1991a
CDFs						
2,3,7,8-TCDF	Fischer 344 rat (M)	30.6 μg/kg, i.v. (0.1 μmol/kg)	liver adipose skin muscle blood	0.10 1.25 3.75 0.45 11.09 0.02 0.72 0.02 1.14	Pool size (% of total dose) 29.09 1st component 31.39 2nd component 17.85 6.84 1st component 1.22 2nd component 24.85 1st component 6.73 2nd component 1.31 1st component 0.89 2nd component	Birnbaum et al., 1980

Table 1-6 (continued)

Chemical	Species (Sex)	Dose	Tissue	Half-Life (days)	Remarks	Reference
2,3,7,8-TCDF	C57BL/6J mice (M)	30.6 μg/kg, i.v. (0.1 μmol/kg)	liver adipose skin muscle	1.9 1.6 0.15 4.0 0.015 1.1	 1st component 2nd component 1st component 2nd component	Decad et al., 1981b
2,3,7,8-TCDF	DBA/2J mice (M)	30.6 μg/kg, i.v. (0.1 μmol/kg)	liver adipose muscle	1.8 7.0 0.02 4.0	 1st component 2nd component	Brewster and Birnbaum, 1988
1,2,3,7,8-PeCDF	Fischer 344 rat (M)	34 μg/kg, i.v. (0.1 μmol/kg)	 liver adipose skin muscle adrenal blood	 1.36 25.72 12.91 1.32 14.53 0.03 6.96 0.14 2.36 0.07 12.42	Pool size (% of total dose): 42.59 1st component 1.27 2nd component 10.19 7.14 1st component 1.49 2nd component 34.81 1st component 7.42 2nd component 0.26 1st component 0.02 2nd component 5.33 1st component 1.29 2nd component	Brewster and Birnbaum, 1988
1,2,3,7,8-PeCDF	Sprague-Dawley rat (F)	4.0 μg/kg, p.o.	liver	3.3	69.8% of total dose	Van den Berg et al., 1989a,b

Table 1-6 (continued)

Chemical	Species (Sex)	Dose	Tissue	Half-Life (days)	Remarks	Reference
2,3,4,7,8-PeCDF	Fischer 344 rat (M)	34 μg/kg, i.v. (0.1 μmol/kg)	liver adipose skin muscle blood	193 69 0.62 1.23 0.04 0.51 9.84 0.04 1.32 55	Pool size (% of total dose): 67.71 10.53 3.54 1st component 1.37 2nd component 29.40 1st component 2.01 2nd component 0.78 3rd component 3.18 1st component 0.37 2nd component 0.008 3rd component	Brewster and Birnbaum, 1987
2,3,4,7,8-PeCDF	Sprague-Dawley rat (F)	5.6 μg/kg, p.o.	liver	108	78.3% of total dose	Van den Berg et al., 1989b
1,2,3,6,7,8-HxCDF	Sprague-Dawley rat (F)	6.0 μg/kg, p.o.	liver	73	63.4% of total dose	Van den Berg et al., 1989b
PCBs						
3,3′,4,4′-TCB	Sprague-Dawley rat (F)	5 mg/kg/day, p.o., for 21 days	liver adipose	0.8 2.5	21-day exposure produced steady state with 300 ng/g in liver and 8 μg/g in adipose tissue. Elimination was assessed over a 22-day postexposure period.	Clarke et al., 1984
3,3′,4,4′-TCB	ICR mice (M)	8 mg/kg, p.o., every other day for 10 doses	liver adipose serum	2.15 2.60 1.07	Steady-state tissue concentrations: 1.5 μg/g 19.2 μg/g 0.04 μg/mL	Clevenger et al., 1989

i.v. = intravenous; s.c. = subcutaneous; i.p. = intraperitoneal; p.o. = per os.

The experimental tissue distribution and elimination data in Table 1-6 were obtained after exposure to a single congener, while real-world exposure to 2,3,7,8-TCDD and related compounds occurs as a complex mixture of congeners. Recently, Neubert et al. (1990) examined the persistence of various CDDs and CDFs in hepatic and adipose tissue of male and female marmoset monkeys. Animals received a single subcutaneous exposure to a defined CDD/CDF mixture (total dose of 27.8 μg/kg bw), which contained 0.12 μg 2,3,7,8-TCDD/kg bw. Using the international toxicity equivalency (I-TE) factors (NATO, 1988; U.S. EPA, 1989), the total administered dose corresponded to 0.464 μg I-TE/kg bw. The concentrations of specific congeners in liver and adipose tissue were measured at 1, 6, 16, or 28 weeks after exposure, and elimination constants and half-lives were estimated assuming first-order kinetics (Table 1-7). Data in Table 1-7 were determined from pregnant and nonpregnant female and male marmosets (total of 12 animals) since no obvious differences in tissue concentrations were observed among these groups. All 2,3,7,8-substituted CDDs and CDFs were consistently more persistent in the adipose tissue than in the liver of marmoset monkeys. In general, the persistence in adipose tissue was from ~1.3- to 2.0-fold greater than that in liver, with the exception of 1,2,3,4,7,8-/1,2,3,4,7,9-HxCDF, HpCDFs, and OCDF, which were even more persistent in adipose tissue. For the latter congeners and OCDD, there was marked variance in half-life values, which may be due to delayed and incomplete absorption of the exceptionally persistent congeners and the relatively short (28 weeks) period of investigation. A significant species difference exists for OCDD and 2,3,4,7,8-PeCDF, which, in contrast to the marmoset monkey, was found to be more persistent in the liver of the rat, with half-lives more than twofold greater than that in adipose tissue (Birnbaum and Couture, 1988; Brewster and Birnbaum, 1987) (see Table 1-6). Further comparison of tissue elimination data in the rat (Table 1-6) and monkey (Table 1-7) indicates that 2,3,7,8-TCDD, OCDD, 2,3,7,8-TCDF, 1,2,3,6,7,8-HxCDF, and 2,3,4,7,8-PeCDF (adipose tissue only) are more persistent in the marmoset monkey than in the rat. The exception to this relationship is 2,3,4,7,8-PeCDF, which is more persistent in rat liver compared to the monkey.

The exposure of marmoset monkeys to a complex mixture of CDDs and CDFs included exposure to both 2,3,7,8- and non-2,3,7,8-substituted congeners (Neubert et al.,

Table 1-7. Elimination Constants and Half-Lives of Various 2,3,7,8-Substituted CDDs and CDFs in Hepatic and Adipose Tissue of Marmoset Monkeys[a,b]

Congener	Hepatic Tissue			Adipose Tissue		
	K_e ($weeks^{-1}$)	Half-Life (weeks)	95% Conf. Interval (weeks)	K_e ($weeks^{-1}$)	Half-Life (weeks)	95% Conf. Interval (weeks)
2,3,7,8-TCDD[c]	0.0841±0.0109	8.3	6.6–11.1	0.0658±0.0072	10.5	8.7–13.4
1,2,3,7,8-PeCDD[c]	0.0649±0.0101	10.7	8.2–15.4	0.0490±0.0057	14.2	11.5–18.3
1,2,3,4,7,8-HxCDD	0.0702±0.0059	9.9	8.4–11.8	0.0411±0.0083	16.9	12.1–27.9
1,2,3,6,7,8-HxCDD	0.0558±0.0046	12.4	10.7–14.9	0.0373±0.0073	18.6	13.4–30.2
1,2,3,7,8,9-HxCDD	0.0767±0.0078	9.0	7.5–11.3	0.0525±0.0089	13.2	9.9–19.7
1,2,3,4,6,7,8-HpCDD	0.0518±0.0081	13.4	10.2–19.3	0.0372±0.0060	18.6	14.2–27.2
OCDD	0.0089±0.0084	78	27–∞[d]	0.0122±0.0093	101	20–∞[d]
2,3,7,8-TCDF	0.8012±0.0549	<0.87[e]	-<1.00	0.4986±0.0829	1.39	1.05–2.06
1,2,3,7,8-/1,2,3,4,8-PeCDF	0.7476±0.0294	0.93	0.86–1.00	0.4735±0.0408	1.46	1.25–1.76
2,3,4,7,8-PeCDF	0.0786±0.0048	8.8	7.9–10.0	0.0563±0.0059	12.3	10.2–15.5
1,2,3,4,7,8-/1,2,3,4,7,9-HxCDF	0.0307±0.0039	23	18–30	0.0103±0.0074	68	28–∞[d]
1,2,3,6,7,8-HxCDF	0.0486±0.0037	14.3	12.4–16.7	0.0290±0.0091	24	15–62
1,2,3,7,8,9-HxCDF	0.0848±0.0057	8.2	7.2–9.4	not analyzed[f]	NA	NA
2,3,4,6,7,8-HxCDF	0.0373±0.0057	18.6	14.3–26.5	0.0182±0.0082	38	20–327

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Table 1-7 (continued)

Congener	Hepatic Tissue			Adipose Tissue		
	K_e (weeks^{-1})	Half-Life (weeks)	95% Conf. Interval (weeks)	K_e (weeks^{-1})	Half-Life (weeks)	95% Conf. Interval (weeks)
1,2,3,4,6,7,8-HpCDF	0.0186±0.0072	37	21–152	-0.0140±0.0137	∞[d]	54–∞[d]
1,2,3,4,7,8,9-HpCDF	0.0088±0.0127	79	20–∞[d]	0.0011±0.0112	660	30–∞[d]
OCDF	0.0040±0.0096	174	30–∞[d]	-0.0042±0.0148	∞[d]	28–∞[d]

[a]Source: Neubert et al., 1990.
[b]Animals were treated subcutaneously with a single dose of a defined CDD/CDF mixture, and the tissues were analyzed at different times following treatment. Half-lives were calculated from tissue concentrations of the 2,3,7,8-substituted congeners in hepatic and adipose tissue. Values are given as elimination rate constant K_e including estimated SD and half-life including 95% confidence intervals.
[c]Calculated from the time period: >6 weeks after injection.
[d]Calculated half-life is apparently infinite. Data for OCDD and OCDF are unreliable due to delayed absorption.
[e]Not detected in hepatic tissue 6 weeks after treatment; limits of detection used for calculation.
[f]Due to interference.
NA = Not applicable.

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1990). One week after exposure to this complex mixture, the non-2,3,7,8-substituted CDDs and CDFs were present in liver and adipose tissue in relatively minor quantities when compared with 2,3,7,8-substituted congeners; however, non-2,3,7,8-substituted compounds represented a considerable percent of the exposure mixture. In this study, none of the non-2,3,7,8-substituted TCDDs, PeCDDs, TCDFs, or PeCDFs could be detected in the liver. Some of the hexa- and hepta-congeners were detected in adipose tissue and liver, but after 1 week, the total amount in the liver was >5% of the administered dose only in the case of 1,2,4,6,8,9-HxCDF. Similar results were obtained in rats after exposure to a defined, complex mixture of CDDs and CDFs (Abraham et al., 1989c). Additional short-term studies in rats provide evidence that the low tissue concentrations of non-2,3,7,8-substituted congeners, measured 1 week after exposure, were the result of rapid elimination, since these congeners were detected at higher levels in the liver 13 to 14 hours after exposure (Abraham et al., 1989d). These results in monkeys and rats are compatible with data from analysis of human tissue samples and milk in which the non-2,3,7,8-substituted congeners have also not been shown to be present in significant concentrations when compared with the 2,3,7,8-substituted congeners (Schecter et al., 1985, 1986; Ryan, 1986; Rappe et al., 1986; Beck et al., 1987, 1989; Thoma et al., 1989).

A potential problem of tissue distribution and elimination studies after exposure to a complex mixture of CDDs and CDFs is the possible interaction of the mixture during the uptake and elimination of specific congeners from tissues. A similar hepatic distribution (~25% of dose) and liver/adipose tissue concentrations ratio (~2) for 2,3,7,8-TCDD were observed in rats 7 days after exposure to 2,3,7,8-TCDD (100 ng/kg bw) when the compound was administered alone or in combination with a large amount of other CDDs/CDFs (total 23,222 ng/kg bw) (Abraham et al., 1988, 1989d). This suggests that under these experimental conditions, the tissue distribution of 2,3,7,8-TCDD was not altered when the exposure included a complex mixture of CDDs/CDFs. Van den Berg et al. (1989b) studied the hepatic disposition and elimination of CDFs administered individually (see Table 1-6) and as mixtures. Co-administration of 1,2,3,7,8- and 2,3,4,7,8-PeCDF resulted in 46% of the dose of 1,2,3,7,8-PeCDF distributing to the liver, while 70% was distributed to the liver after administration of the single compound (see Table 1-6). Nevertheless, this combined

exposure did not alter the rate of elimination of 1,2,3,7,8-PeCDF from the liver. Co-administration of 2,3,4,7,8-PeCDF and 1,2,3,6,7,8-HxCDF did not alter the hepatic uptake of either congener or the hepatic elimination of 2,3,4,7,8-PeCDF but increased the hepatic half-life of 1,2,3,6,7,8-HxCDF to 156 days from the single compound exposure half-life of 73 days (see Table 1-6). However, these values must be considered rough estimates since the experimental period of 42 days was too short to calculate half-lives accurately. Although there are few investigations of potential interactions of mixtures of CDDs and CDFs on the uptake and elimination of individual congeners, the limited available data suggest that exposure to complex mixtures (see Table 1-7) may alter the tissue disposition of individual congeners.

Several studies have investigated potential pharmacokinetic interactions following exposure to two or more PCDDs, PCDFs, and PCBs (DeJongh et al., 1992, 1993a, 1993b). Interactive effects on the hepatic disposition of these compounds may in some cases explain, in part, the potentiation and antagonistic effects on CYP1A1/1A2 activities observed with combined exposure to some of these compounds. In the case of 1,2,3,7,8-PeCDD and 2,4,5,2′,4′,5′-HxCB, no pharmacokinetic bases were found to explain the antagonistic effects of the combined exposure on CYP1A1/1A2 activities in mice (DeJongh et al., 1992). In a subsequent study the hepatic disposition of 1,2,3,7,8-PeCDD in mice was increased with combined exposures to 1,2,3,6,7,8-HxCDD, 2,3,4,7,8-PeCDF, and/or 2,4,5,2′,4′,5′ HxCB (De Jongh et al., 1993b). Although there is evidence that PCDDs, PCDFs, and PCBs may influence each other's pharmacokinetics when administered in mixtures, this area needs investigation.

1.2.4. Dose-Dependent Tissue Distribution

Recent evidence suggests that the tissue distribution of 2,3,7,8-TCDD and related compounds is dose dependent. Abraham et al. (1988) investigated the distribution of 2,3,7,8-TCDD in liver and adipose tissue of rats 7 days after a single subcutaneous exposure to 2,3,7,8-TCDD at doses of 1-3,000 ng/kg bw. Greater than 97% of the administered 2,3,7,8-TCDD was absorbed at all doses, with the exception of the 3,000 ng/kg group where 84% of the dose was absorbed. Figure 1-2 illustrates the dose-dependent disposition of

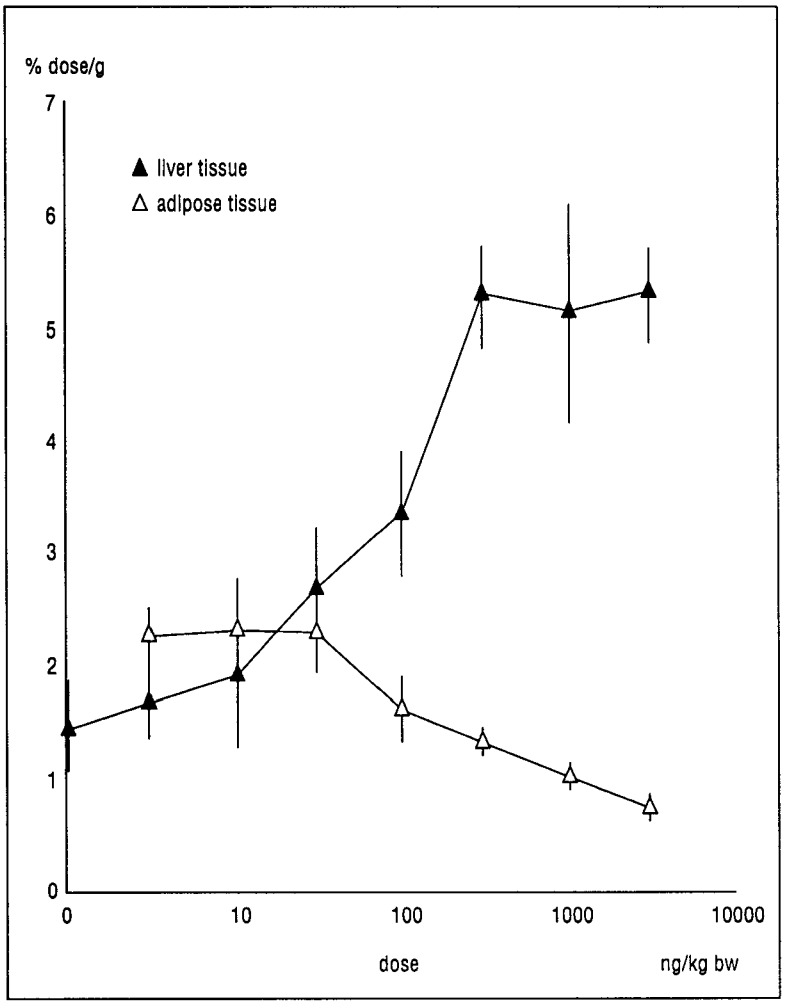

Figure 1-2. Dose dependency of the percentage of the administered dose of ^{14}C-TCDD/g of tissue recovered in liver and adipose tissue after single subcutaneous doses (values from animals treated with 3,000 ng TCDD/kg bw were corrected for 84% absorption). Concentrations were measured 7 days after the injection.

Source: Abraham et al., 1988.

2,3,7,8-TCDD in liver and adipose tissue (% dose/g) 7 days after exposure. A sharp increase in 2,3,7,8-TCDD concentration in liver was observed at exposure levels >10 ng/kg bw. Disposition in the liver increased from ~11% of the administered dose at an exposure level of 1-10 ng/kg bw to ~37% of the dose at an exposure level of 300 ng/kg bw. The increase in distribution to the liver was accompanied by a dose-related decrease in the concentration of 2,3,7,8-TCDD in the adipose tissue. As a result, the liver/adipose tissue concentration ratio for 2,3,7,8-TCDD at 7 days after exposure increased with increasing doses, starting at an exposure level of 30 ng/kg bw (Table 1-8). Thus, the tissue-specific disposition of 2,3,7,8-TCDD is regulated by a complex relationship, which includes species, time after a given exposure, and dose (see Figures 1-1 and 1-2; Tables 1-6 and 1-7).

Other studies on the tissue disposition of 2,3,7,8-TCDD and related compounds report similar dose-dependent behavior with disproportionately greater concentrations in the liver at high doses compared with low doses. Poiger et al. (1989a) observed a dose-related increase in distribution to the liver (% of dose/liver) and an increase in the liver/adipose tissue concentration ratio for 2,3,7,8-TCDD, 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDF, and 1,2,3,6,7,8-HxCDF in the rat. Kedderis et al. (1991a) also observed a dose-related increase in hepatic disposition (1.27% versus 10.05% of dose/liver) and an increase in the liver/adipose tissue concentration ratio (0.16 versus 2.59) for 2,3,7,8-TBDD at 56 days after exposure at doses of 0.001 and 0.1 μmol/kg bw, respectively. In a related study, pretreatment of mice with 2,3,7,8-TCDD (5 or 15 μg/kg) produced a dose-related, enhanced hepatic accumulation of a subsequent oral dose of 2,3,7,8-TCDD (Curtis et al., 1990). Diliberto et al. (1993b) also observed a dose-dependent tissue distribution of 2,3,7,8-TCDD in female B6C3F1 mice. In all tissues except the liver, the relative percent of the total dose of TCDD decreased while it increased in the liver with higher doses. Similarly, a dose-related increase in hepatic uptake of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin was observed after pretreatment of mice with 2,3,7,8-TCDD (Poland et al., 1989a; Leung et al., 1990b). Shen and Olson (1987) also observed an increase in the uptake of 2,3,7,8-TCDD by isolated hepatocytes from 2,3,7,8-TCDD-pretreated mice.

Chronic studies also support dose-dependent alterations in the tissue distribution of these compounds. Kociba et al. (1978a,b) found that female rats maintained on a daily

Table 1-8. 2,3,7,8-TCDD Concentrations in Liver and Adipose Tissue Following Different Doses and Calculated Concentration Ratios (Liver/Adipose Tissue)[a,b]

Dose (ng/kg)	Number	TCDD Concentration Liver (ng/g)	TCDD Concentration Adipose Tissue (ng/g)	Concentration Ratio: Liver/Adipose Tissue
1	6	0.0031±0.0009	ND	NA
3	6	0.0102±0.0020	0.0139±0.0015	0.74±0.15
10	12	0.0406±0.0121	0.0494±0.0084	0.82±0.20
30	6	0.162±0.032	0.139±0.021	1.16±0.07
100	6	0.699±0.130	0.335±0.065	2.10±0.27
300	6	3.38±0.22	0.819±0.075	4.14±0.31
1000	6	10.7±2.2	2.02±0.17	5.27±0.96
3000	5	27.9±2.4	3.66±0.31	7.65±0.64

[a]Source: Abraham et al., 1988.
[b]Concentrations were measured 7 days after injection.
ND = Not detectable; NA = not applicable.

dietary 2,3,7,8-TCDD intake of 100 ng for 2 years had an average 2,3,7,8-TCDD content of 8,100 ppt in fat and 24,000 ppt in the liver. Rats given 10 ng/kg/day had an average of 1,700 ppt 2,3,7,8-TCDD in the fat and 5,100 ppt in the liver. For both of these exposures the liver/adipose tissue concentration ratio of 2,3,7,8-TCDD was ~3. At the lowest dose level of 1 ng/kg/day, both fat and liver contained an average of 540 ppt 2,3,7,8-TCDD. Kociba et al. (1976) presented evidence that steady state had been reached by < 13 weeks of feeding of 2,3,7,8-TCDD.

Other studies do not support the dose-dependent tissue distribution of 2,3,7,8-TCDD and related compounds described above. Rose et al. (1976) reported a lack of a dose-dependent accumulation of [^{14}C]-TCDD in male and female rat liver and adipose tissue following 7, 21, and 49 days of exposure at 0.01, 0.1, or 1.0 μg/kg/day, Monday through Friday. The rates of accumulation of TCDD-derived radioactivity were similar in fat, liver and whole body; however, the concentration in the liver was about fivefold greater than that in fat. Brewster and Birnbaum (1987) also observed similar concentrations (% dose/g) of 2,3,4,7,8-PeCDF in liver, adipose tissue, and other tissues at 3 days after oral exposure at doses of 0.1, 0.5, or 1.0 μmol/kg bw. These results conflict with the above studies, which support the dose-dependent tissue distribution of these compounds. While it is not possible at this time to explain these differences, most of the available data support a dose-dependent relationship.

The dose-dependent tissue distribution of 2,3,7,8-TCDD and related compounds is a critical factor that must be considered in estimating the concentration of these compounds in human tissues after chronic low-level exposure. This is particularly important because the general human population is exposed to much smaller daily doses (possibly 0.3 pg 2,3,7,8-TCDD/kg/day) than those used in experimental disposition studies. Due at least partly to the long half-life of 2,3,7,8-TCDD in humans, however, this exposure results in concentrations of 3-18 pg/g in human adipose tissue (Leung et al., 1990c). Similar levels of 2,3,7,8-TCDD in adipose tissue (14 pg/g) were observed in rats 7 days after subcutaneous exposure to 3 ng/kg bw (see Table 1-8) (Abraham et al., 1988). Under these experimental conditions, the liver/adipose tissue 2,3,7,8-TCDD concentration was 0.74. Nonetheless, steady state was definitely not reached under these conditions, and, with increasing time after exposure, this

ratio may decrease, based on the observation that 2,3,7,8-TCDD was more persistent in adipose tissue than in liver in rats exposed to 300 ng/kg bw (see Figure 1-1 and Table 1-6) (Abraham et al., 1988). Human data on the liver/adipose tissue concentration ratio of 2,3,7,8-TCDD and related compounds are limited but suggest that the ratio may vary by at least an order of magnitude between individuals. Leung et al. (1990c) observed a geometric mean adipose tissue 2,3,7,8-TCDD concentration of 7.78 ppt in 26 individuals and a concentration in liver at about one-tenth of that in adipose tissue on a whole-weight basis. When measured on a total lipid basis, the concentrations of 2,3,7,8-TCDD in both tissues were approximately the same. Thoma et al. (1990) reported a liver/adipose tissue 2,3,7,8-TCDD concentration on a wet weight basis of 0.14, while on a lipid basis the ratio was 2.05 (Table 1-5). Considerable variability in CDD and CDF concentrations in liver and adipose tissues was also observed between individual marmoset monkeys (Neubert et al., 1990), suggesting that individual variability may also contribute to the difficulty in assigning a constant liver/adipose tissue ratio for CDDs and CDFs in humans and nonhuman primates.

1.2.5. Potential Mechanisms for the Dose-Dependent Tissue Distribution

The observation that exposure to higher doses of 2,3,7,8-TCDD and related compounds results in a disproportionately greater hepatic concentration of these compounds may be explained by a hepatic binding species that is induced by 2,3,7,8-TCDD and other agonists for the Ah receptor. The studies of Voorman and Aust (1987, 1989) and Poland et al. (1989a,b) provide evidence that this binding species is cytochrome P-4501A2.

Poland et al. (1989a,b) reported that TCDD and other Ah agonists (2,3,7,8-TCDF, β-naphthoflavone, 3,3′,4,4′,5,5′-hexabromobiphenyl) act through the Ah receptor to increase a liver binding species that increases the hepatic uptake of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin (a radiolabeled isosteric analogue of TCDD) in vivo and binding of this radioligand to liver homogenate in vitro. Twenty-four hours after the administration of a non-AHH-inducing dose ($1x10^{-10}$ mol/kg) of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin to C576BL/6J mice, the hepatic concentration of radioactivity was 1-2% of the administered dose, whereas in mice pretreated 48 hours earlier with an AHH-inducing dose of TCDD ($1x10^{-7}$ mol/kg), the hepatic accumulation of radiolabel was 25-30% of that administered. A similar, though

less dramatic, effect was observed in vitro, with liver homogenate from TCDD-treated mice binding about four times more [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin than homogenate from control mice. The administration of TCDD to C57BL/6J mice produced a dose-related stimulation of in vivo hepatic uptake of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin, increased binding of radioligand to liver homogenate, and induction of hepatic activity, with an ED_{50} ranging from 1.5 to 4.0×10^{-9} mol/kg. In congenic C57BL/6J (Ah^d/Ah^d) mice, which express the lower affinity Ah receptor, the ED_{50} values for all three responses were shifted to doses that were about 10-fold higher. The observed effects on hepatic disposition were tissue specific, with no remarkable dispositional changes being observed in kidney, lung, spleen, small intestine, or muscle. This is significant in that TCDD and other agonists for the Ah receptor induce cytochrome P-4501A1 in liver and other tissues, whereas cytochrome P-4501A2 is apparently inducible only in liver (Tuteja et al., 1985; Gillner et al. 1987). Furthermore, the changes in hepatic disposition were not species specific; similar responses were observed in guinea pigs, rats, mice, and hamsters (Poland et al., 1989a).

The following evidence reported by Poland et al. (1989b) supports the hypothesis that the TCDD-inducible hepatic binding protein is cytochrome P-4501A2: the TCDD-induced hepatic binding species was found predominantly in the microsomal fraction and was inactivated by heating at 60°C, trypsin and mercurials; the TCDD-induced hepatic binding species was specific for the liver, with a large pool size (B_{max} of 22 ± 5 nmol/g liver); and the major microsomal binding species covalently labeled with the photoaffinity ligand [^{125}I]-2-iodo-3-azido-7,8-dibromodibenzo-*p*-dioxin migrates with that immunochemically stained with polyclonal antiserum that binds to cytochrome P-4501A2.

One observation of Poland et al. (1989a,b) does not support the hypothesis that the TCDD-inducible hepatic protein is cytochrome P-4501A2. These investigators found that dietary administration of isosafrole did not stimulate hepatic uptake of [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin or the in vitro binding of this ligand to liver homogenate. Isosafrole is not an agonist for the Ah receptor, but it selectively induces cytochrome P-4501A2 (Ryan et al., 1980). Poland et al. (1989a,b) suggest that this may be attributable to the high affinity binding of an isosafrole metabolite to the protein, which might inhibit the binding of [^{125}I]-iodo-3,7,8-trichlorodibenzo-*p*-dioxin to cytochrome P-4501A2 at or near the

active site of the enzyme. This does not explain why TCDD, which also has high affinity for cytochrome P-4501A2, cannot displace some of the isosafrole metabolite from the protein, which should produce enhanced hepatic disposition of TCDD.

A more recent study used pretreatment with isosafrole to investigate the role of CYP1A2 in the hepatic disposition of 2,3,7,8-TCDD (Kedderis et al., 1993a). Although isosafrole is an inducer of CYP1A2, it also has high affinity for the protein and thus is a selective inhibitor of CYP1A2. A greater than threefold decrease in the hepatic disposition of TCDD in isosafrole-pretreated rats supports the conclusion that TCDD is bound to CYP1A2 in the liver.

Voorman and Aust (1987, 1989) support further the hypothesis that cytochrome P-4501A2 is the TCDD-inducible hepatic binding species. These investigators found that 3,3′,4,4′,5,5′-HxBB, an agonist for the Ah receptor, was associated only with cytochrome P-4501A2 through the immunoprecipitation of cytochromes P-4501A1 and 1A2, which were induced in 3,3′,4,4′,5,5′-HxBB treated rats. In addition, they found that 3,3′,4,4′,5,5′-HxBB inhibited estradiol 2-hydroxylase activity of purified cytochrome P-4501A2. A similar association of PAHs with immunoprecipitated cytochrome P-4501A2 was observed for other agonists for the Ah receptor, including 2,3,7,8-TCDD, 3,3′,4,4′-TCB, 3,3′,4,4′,5-PeCB, and 3,3′,4,4′,5,5′-HxCB. The association of 2,3,7,8-TCDD with cytochrome P-4501A2 occurred within 2 minutes, with maximum inhibition of estradiol 2-hydroxylase occurring at a concentration comparable to the concentration of the enzyme (50 nm). Cytochrome P-4501A2 was inhibited (complexed) by 2,3,7,8-TCDD with nearly 1:1 stoichiometry, and the K_i for 2,3,7,8-TCDD was calculated to be 8 nM. Therefore, 2,3,7,8-TCDD can be considered a higher binding inhibitor of cytochrome P-4501A2.

The TCDD-induced binding species was found to have an apparent equilibrium dissociation constant, K_D, for [^{125}I]-2-iodo-3,7,8-trichlorodibenzo-*p*-dioxin of 56 ± 16 nM, and a pool size, B_{max}, of 22 ± 5 nmol/g of liver in C57BL/6J mice (Poland et al., 1989b). The induced microsomal binding species has an affinity about 10^4 times less than the Ah receptor but a pool size that is $\sim 2 \times 10^3$ greater. Thus, agonists for the Ah receptor may significantly affect their disposition through a dose-related enhancement of hepatic uptake, which should correlate with induction of cytochrome P-4501A2.

The disposition and pharmacokinetics of 2,2',4,4',5,5'-HxCB and -HxBB have been investigated in several species (Tuey and Matthews, 1980; Lutz et al., 1984). These lipophilic compounds are similar to 2,3,7,8-TCDD in that they are slowly metabolized and that metabolism is required for urinary and biliary elimination; however, they do not exhibit dioxin-like activity. 2,2',4,4',5,5'-HxCB and -HxBB distribute primarily to adipose tissue, with partition coefficients (tissue/blood ratio) ranging from 300 to 500 in the mouse, rat, monkey, dog, and human. The liver is not a major site for the disposition of 2,2',4,4',5,5'-HxCB and -HxBB in contrast to 2,3,7,8-TCDD and related compounds. Partition coefficients in the liver range from 10 to 30 in these species. 2,2',4,4',5,5'-HxCB and -HxBB do not induce hepatic cytochrome P-4501A1 or 1A2 and do not exhibit dioxin-like activity. The lack of induction of hepatic cytochrome P-4501A2 may explain the lack of a dose-dependent hepatic disposition of these compounds.

Kedderis et al. (1991b) assessed the dose-response relationship for the induction of hepatic cytochrome P-4501A1 and P-4501A2 in male Fischer 344 rats exposed to 2,3,7,8-TBDD at doses as low as 0.1 nmol/kg. They reported that induction of P-4501A2 by 2,3,7,8-TBDD appeared to be a more sensitive response than P-4501A1 induction over the dose range studied. In addition, comparison of hepatic P-4501A2 levels and liver:adipose tissue concentration ratios suggested that induction of P-4501A2 alone would not directly account for the preferential hepatic accumulation of 2,3,7,8-TBDD and that additional factors must be involved. One explanation may be that at low 2,3,7,8-TBDD concentrations, endogenous substrates bind to CYP1A2, not allowing 2,3,7,8-TBDD to be sequestered by the protein (Kedderis et al., 1993b). At higher 2,3,7,8-TBDD concentrations, new protein is formed and 2,3,7,8-TBDD can compete for binding to CYP1A2, resulting in the increased hepatic disposition observed at higher exposures of 2,3,7,8-TBDD (Kedderis et al., 1991b).

Other factors may also regulate the intracellular distribution of 2,3,7,8-TCDD and related compounds. The possible role of hepatic lipoproteins as intracellular carriers in the transport of 2,3,7,8-TCDD has been assessed by in vitro and in vivo studies (Souès et al., 1989a,b). 2,3,7,8-TCDD and 2,3,7,8-TCDF were bound to lipoproteins in mouse and rat liver, which subsequently underwent rapid and pronounced degradative processing, possibly catalyzed by lipoprotein lipase, to heavier entities. The in vitro incubation of 2,3,7,8-

TCDD-lipoprotein complex with separated Ah receptor demonstrated that a passive transfer occurred. The authors suggest a carrier role for lipoproteins in the intracellular transport of 2,3,7,8-TCDD and related compounds.

1.3. METABOLISM AND EXCRETION

Although early in vivo and in vitro investigations were unable to detect the metabolism of 2,3,7,8-TCDD (Vinopal and Casida, 1973; Van Miller et al., 1976), there is evidence that a wide range of mammalian and aquatic species are capable of biotransforming 2,3,7,8-TCDD to polar metabolites (Ramsey et al., 1979, 1982; Poiger and Schlatter, 1979; Olson et al., 1980; Olson, 1986; Gasiewicz et al., 1983b; Poiger et al., 1982; Sijm et al., 1990; Kleeman et al., 1986a,b, 1988). Although metabolites of 2,3,7,8-TCDD have not been directly identified in humans, recent data regarding feces samples from humans in a self-dosing experiment suggest that humans can metabolize 2,3,7,8-TCDD (Wendling et al., 1990).

Table 1-9 summarizes data on the metabolism and excretion of 2,3,7,8-TCDD and related compounds after exposure to a single radiolabeled congener. Investigations of 2,3,7,8-TCDD in rats, mice, guinea pigs, and hamsters found that >90% of the radiolabeled material excreted in urine and bile represented polar metabolites. Similar results were also observed for other congeners (see Table 1-9), with the exception of OCDD; however, studies were often limited to the rat. OCDD is apparently not metabolized by the rat or metabolized to a very minimal extent (Birnbaum and Couture, 1988). For all of the congeners in Table 1-9, essentially all of the CDD, BDD, CDF, or PCB-derived radioactivity in liver, adipose tissue, and other tissues represented parent compound, suggesting that metabolites of these compounds were readily excreted. Thus, with the exception of OCDD, the metabolism of 2,3,7,8-TCDD and related compounds is required for urinary and biliary elimination and therefore plays a major role in regulating the rate of excretion of these compounds. In addition, direct intestinal excretion of parent compound is another route for excretion of 2,3,7,8-TCDD and related compounds that is not regulated by metabolism.

Table 1-9. Metabolism and Excretion of 2,3,7,8-TCDD and Related Compounds[a]

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
CDDs									
2,3,7,8-TCDD	Sprague-Dawley rat (M)	50 μg/kg, p.o.	NA	NA	NA	4.0	17.4±5.6[c]	NC	Piper et al., 1973
2,3,7,8-TCDD	Sprague-Dawley rat (M)	7 or 72 ppb in diet for 42 days	NA	NA	NA	NA	12	NC	Fries and Marrow, 1975
2,3,7,8-TCDD	Sprague-Dawley rat (F)	7 or 72 ppb in diet for 42 days	NA	NA	NA	NA	15	NC	Fries and Marrow, 1975
2,3,7,8,-TCDD	Sprague-Dawley rat (M, F)	1.0 μg/kg, p.o	NA	NA	NA	9.9	31±6[d]	NC	Rose et al., 1976
2,3,7,8-TCDD	Sprague-Dawley rat (M, F)	0.1 and 1.0 μg/kg/day, 5 days/week for 7 weeks	NA	NA	NA	8.5	23.7	NC	Rose et al., 1976
2,3,7,8-TCDD	Han/Wistar rat (M)	5 μg/kg, i.p.	>90	NA	~70-90	14.1	21.9	NC	Pohjanvirta et al., 1990
2,3,7,8-TCDD	Long-Evans rat (M)	5 μg/kg, i.p.	>90	NA	~20-90	12.0	20.8	NC	Pohjanvirta et al., 1990
2,3,7,8-TCDD	Sprague-Dawley (M)	500 μg/kg, i.p.	100	100	NA	NA	NA	NC	Neal et al., 1982
2,3,7,8-TCDD	C57BL/6J mice (M)	10 μg/kg, i.p.	100	100	85	2.7	11.0±1.2[d]	NC	Gasiewicz et al., 1983a

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Table 1-9 (continued)

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
2,3,7,8-TCDD	DBA/2J mice (M)	10 μg/kg, i.p.	100	100	82	1.2	24.4±1.0[d]	NC	Gasiewicz et al., 1983a
2,3,7,8-TCDD	B6D2F1J mice (M)	10 μg/kg, i.p.	100	100	86	2.5	12.6±0.8[d]	NC	Gasiewicz et al., 1983a
2,3,7,8-TCDD	C57BL/6J mice Ah^b/Ah^d (M)	500 ng/kg, i.p.	NA	NA	NA	3.1	9.42	NC	Birnbaum, 1986
2,3,7,8-TCDD	C57BL/6J mice Ah^d/Ah^d (M)	500 ng/kg, i.p.	NA	NA	NA	2.1	9.74	NC	Birnbaum, 1986
2,3,7,8-TCDD	DBA/2J Ah^b/Ah^d (F)	500 ng/kg, i.p.	NA	NA	NA	5.3	10.40	NC	Birnbaum, 1986
2,3,7,8-TCDD	DBA/2J Ah^d/Ah^d (F)	500 ng/kg, i.p.	NA	NA	NA	6.8	11.11	NC	Birnbaum, 1986
2-Iodo-3,7,8-TCDD	C57BL/6J mice (F)	[^{125}I] 0.1 nmol/kg, i.p.	NA	NA	NA	NA	14.2	whole body counting was used to estimate body burden over 30-day period	Leung et al., 1990b
2-Iodo-3,7,8-TCDD	C57BL/6J mice (F)	[^{125}I] 0.1 nmol/kg, i.p., 3 days following pretreatment with 2,3,7,8-TCDD (0.1 μmol/kg, i.p.)	NA	NA	NA	NA	8.0	whole body counting was used to estimate body burden over 30-day period	Leung et al., 1990b
2,3,7,8-TCDD	Hartley guinea pig (M)	0.5 μg/kg, i.p.	NA	NA	NA	15.7	30.2±5.8[d]	NC	Gasiewicz and Neal, 1979

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Table 1-9 (continued)

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
2,3,7,8-TCDD	Hartley guinea pig (M)	0.56 μg/kg, i.p.	100	100	19	11.2	93.7±15.5[d]	NC	Olson, 1986
2,3,7,8-TCDD	Golden Syrian hamster (M)	[^{3}H] 650 μg/kg, i.p.	NA	NA	NA	1.4	11.95±1.95[d]	NC	Olson et al., 1980; Neal et al., 1982
2,3,7,8-TCDD	Golden Syrian hamster (M)	[^{14}C] 650 μg/kg, i.p.	100	100	55-75	NA	10.82±2.35	NC	Olson et al., 1980; Neal et al., 1982
2,3,7,8-TCDD	Golden Syrian hamster (M)	[^{3}H] 650 μg/kg, p.o.	NA	NA	NA	NA	14.96±2.53	NC	Olson et al., 1980; Neal et al., 1982
2,3,7,8-TCDD	human (M)	1.14 ng/kg, p.o.	NA	NA	~50	>3.1	2120[e]	NC	Poiger and Schlatter, 1986; Wendling et al., 1990
2,3,7,8-TCDD	rainbow trout	494 ppt in diet for 13 weeks	NA	~75	NA	NA	105	elimination followed for 13 weeks following exposure	Kleeman et al., 1986b
2,3,7,8-TCDD	yellow perch	494 ppt in diet for 13 weeks	NA	~90	NA	NA	126	elimination followed for 13 weeks following exposure	Kleeman et al., 1986a
1,2,3,7,8-PeCDD	Sprague-Dawley rat (M, F)	8.42-10.06 μg/kg, p.o.	NA	100	NA	12	29.5±2.7	NC	Wacker et al., 1986

Table 1-9 (continued)

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
OCDD	Fischer 344 rat (M)	50 μg/kg, iv	<33	0	0	>65	~70	whole body $t_{1/2}$ estimated from body burden in liver, skin, and adipose tissue over 56-day period	Birnbaum and Couture, 1988
OCDD	Fischer 344 rat (M)	50 μg/kg/day, p.o., for 10 days	NA	NA	NA	NA	~173	whole body $t_{1/2}$ estimated from body burden in liver, skin, and adipose tissue over 112-day period	Birnbaum and Couture, 1988
BDDs									
2,3,7,8-TBDD	Fischer 344 rat (M)	0.001 μmol/kg, iv	NA	100	80-90	11.1	0.7 2.9 17.8	Pool size (% of dose): 11.63 1st component 2.78 2nd component 1.45 3rd component	Kedderis et al., 1991a
2,3,7,8-TBDD	Fischer 344 rat (M)	0.1 μmol/kg, iv	NA	100	80-90	9.2	0.6 17.8	Pool size (% of dose): 22.47 1st component 2.35 2nd component	Kedderis et al., 1991a
CDFs									
2,3,7,8-TCDF	Fischer 344 rat (M)	0.1 μmol/kg, iv	100	>96	99	31.4	1.8 0.3	fecal excretion urinary excretion	Birnbaum et al., 1980
2,3,7,8-TCDF	C57BL/6J mice (M)	0.1 μmol/kg, iv	100	NA	80	6.5	2.8 1.8 2.0	urine feces urine and feces	Decad et al., 1981b
2,3,7,8-TCDF	DBA/2J mice (M)	0.1 μmol/kg, iv	100	NA	80	2.8	4.9 5.4 4.0	urine feces urine and feces	Decad et al., 1981b

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Table 1-9 (continued)

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
2,3,7,8-TCDF	Hartley guinea pig (M)	0.02 μmol/kg, iv	>90	NA	<10	1.0	20	animal exhibited body weight loss	Decad et al., 1981a
2,3,7,8-TCDF	Hartley guinea pig (M)	4 μg/kg, p.o.	NA	NA	NA	NA	40	no observable toxicity	Ioannou et al., 1983
2,3,7,8-TCDF	rhesus monkey (M)	0.1 μmol/kg, iv	100	>92	>92	5.4	6.24 10.30 ~8	urine feces urine and feces	Birnbaum et al., 1981
1,2,3,7,8-PeCDF	Fischer 344 rat (M)	0.1 μmol/kg, iv	~90	100	NA	12.8	 0.92 3.32 1.26 17.32 1.12 6.30	Pool size (% of dose): feces: 57.79 1st component 6.92 2nd component urine: 2.68 1st component 0.16 2nd component feces and urine: 59.97 1st component 2.51 2nd component	Brewster and Birnbaum, 1988
2,3,4,7,8-PeCDF	Fischer 344 rat (M)	0.1 μmol/kg, iv	NA	>90	>90	>100	 1.27 63.82	Pool size (% of dose): feces 1.22 1st component 0.57 2nd component	Brewster and Birnbaum, 1987
2,3,4,7,8-PeCDF	rhesus monkey (M)	0.1 μmol/kg, iv	NA	NA	63–70	~34	38–49	$t_{1/2}$ represents minimum value; all animals lost body weight and exhibited other signs of toxicity	Brewster et al., 1988
CBs									
3,3'4,4'-TCB	CD rat (M, F)	0.6 mg/kg, iv	>90	NA	>90	42	~1.3–1.5	NC	Abdel-Hamid et al., 1981

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Table 1-9 (continued)

Chemical	Species	Dose	Chemical Nature of Excretion Products (% Metabolites)			Ratio of % of Dose Excreted (Feces/Urine)	Half-Life[b] (days)	Comment	Reference
			Urine	Bile	Feces				
3,3'4,4'-TCB	rhesus monkey (F)	0.6 mg/kg, iv	97	NA	97	7.2	~8-10	NC	Abdel-Hamid et al., 1981

[a]All studies measure the excretion of radiolabeled parent compound and metabolites following exposure to a single congener labeled with ^{3}H, ^{14}C, or ^{125}I.
[b]Half-life for excretion estimates assume first-order elimination kinetics.
[c](mean±SE).
[d](mean±SD).
[e]n=1.
i.p. = intraperitoneal; i.v. = intravenous; NA = not available; NC = no comment; p.o. = per os.

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1.3.1. Structure of Metabolites

Several metabolites of 2,3,7,8-TCDD have recently been identified. Sawahata et al. (1982) investigated the in vitro metabolism of 2,3,7,8-TCDD in isolated rat hepatocytes. The major product was deconjugated with β-glucuronidase, derivatized with diazomethane and separated into two compounds by high-performance liquid chromatography. These metabolites were subsequently identified as 1-hydroxy-2,3,7,8-TCDD and 8-hydroxy-2,3,7-trichlorodibenzo-*p*-dioxin. Poiger et al. (1982) identified six metabolites in the bile of dogs that were given a lethal dose of [^{3}H]-2,3,7,8-TCDD. The major metabolite was 1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin; however, 3,7,8-trichloro-3-hydroxydibenzo-*p*-dioxin and 1,2-dichloro-4,5-hydroxybenzene were identified as minor metabolites. The structures of the three remaining metabolites were not determined; however, two appeared to be trichlorohydroxydibenzo-*p*-dioxins and the third was apparently a chlorinated 2-hydroxydiphenyl ether. Poiger and Buser (1984) reported differences in the relative amounts of various 2,3,7,8-TCDD metabolites in dog and rat bile. Trichlorodihydroxy-dibenzo-*p*-dioxin and tetrachlorodihydroxydiphenyl ether appear to be major metabolites in rat bile. Furthermore, conjugates, presumably glucuronides, were formed in the rat but not in the dog. The investigators also observed a generally higher metabolism rate of 2,3,7,8-TCDD in the dog. This is in good agreement with the unique ability of the dog to readily metabolize persistent PCBs such as 2,4,5,2′,4′,5′-HxCB (Sipes et al., 1982).

Biliary metabolites of 2,3,7,8-TCDF have been investigated by Poiger et al. (1984); however, unequivocal structure assignment of the metabolites could not be made using gas chromatography/mass spectroscopy. With the use of synthetic standards and GC/MS, Burka et al. (1990) identified 4-hydroxy-2,3,7,8-TCDF and 3-hydroxy-2,7,8-TCDF as major biliary metabolites of 2,3,7,8-TCDF in rats. Small amounts of 3-hydroxy-2,4,7,8-TCDF and 2,2′-dihydroxy-4,4′,5,5′-TCB were also detected. 4-Hydroxy-2,3,7,8-TCDF was also the major TCDF metabolite formed by hepatic microsomes from TCDD-pretreated rats (Tai et al., 1993). This suggests that the preferred site of metabolism of 2,3,7,8-TCDF is near the furan oxygen with oxygenation at C4 predominating over C3. The authors speculate that epoxidation of the C4-C4a bond or the C3-C4 bond could lead to formation of the above metabolites. The results of Burka et al. (1990) and Sawahata et al. (1982) suggest that

oxygenation of the unsubstituted carbon nearest the bridging oxygen in both 2,3,7,8-TCDF and 2,3,7,8-TCDD is the major route of metabolism of these compounds in the rat. Furthermore, data on the rate of elimination of these compounds summarized in Tables 1-6 and 1-8 indicate that this reaction occurs at a faster rate for the furan, since the rate of urinary and biliary elimination and resulting persistence of these compounds depend on metabolism.

Data summarized in Tables 1-6 and 1-9 indicate that 1,2,3,7,8-PeCDF is metabolized and eliminated at a greater rate than 2,3,4,7,8-PeCDF. The preference for oxygenation at C4 in 2,3,7,8-TCDF offers an explanation for the observation that 2,3,4,7,8-PeCDF is metabolized at a much slower rate than 1,2,3,7,8-PeCDF because one of the preferred sites for metabolism is blocked in the 2,3,4,7,8-substituted compound. The rate of metabolism of these compounds and their resulting relative persistence in rodents correlate with analysis of human tissues from the Yusho cohort where the relative concentrations were 2,3,4,7,8-PeCDF > 1,2,3,7,8-PeCDF > 2,3,7,8-TCDF (Masuda et al., 1985).

Pluess et al. (1987) investigated the structure of 1,2,3,7,8-PeCDF metabolites in rat bile. A dihydroxy-tetra-CDF was identified as the major metabolite. The authors propose that this compound could be formed either via further oxidation of the hydroxy-tetra-CDF or possibly via hydrolytic dechlorination of a hydroxy-penta-CDF. Minor metabolites include dihydroxy-tri-CDF, hydroxy-tetra-CDF, and hydroxy-penta-CPF.

Pluess et al. (1987) also investigated the metabolites of 2,3,4,7,8-PeCDF in rat bile. A total of 10 metabolites were detected, with dihydroxy-penta-CB and hydroxy-penta-CDF representing the major metabolites. The biphenyl metabolite indicates that cleavage of the ether bridge of the furan is an important pathway for metabolism of this congener. Other less abundant metabolites of 2,3,4,7,8-PeCDF include hydroxy-tetra-CDF, dihydroxy-tri-CDF, dihydroxy-tetra-CDF, and thio-tetra-CDF. Sulfur-containing metabolites were also identified as minor metabolites of 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF in rats (Kuroki et al., 1990). These sulfur-containing metabolites probably arise from CDF-glutathione conjugates.

In another study, a dihydroxy-PeCDF was identified as the only detectable biliary metabolite of 1,2,3,6,7,8-HxCDF, while no metabolites of 1,2,3,4,6,7,8-HpCDF were detected in the bile of rats treated with this congener (Poiger et al., 1989a).

Several in vivo and in vitro studies have investigated the metabolism of 3,3′,4,4′-TCB. Rat feces were found to contain 5-hydroxy-3,3′,4,4′-TCB and 4-hydroxy-3,3′,4′,5-TCB as major metabolites (Yoshimura et al., 1987b) and 2,5-dihydroxy-3,3′,4,4′-TCB, 4,4′-dihydroxy-3,3′,5,5′-TCB, 5,6-dihydroxy-3,3′,4,4′-TCB, 4-hydroxy-3,3′,4-TCB, and 4-hydroxy-4′,5′-epoxy-3,3′,4′,5-TCB as minor metabolites (Koga et al., 1989).

Mouse feces were found to contain 5-hydroxy- and 6-hydroxy-3,3′,4,4′-TCB and 4-hydroxy-3,3′,4′,5-TCB, whereas urine contained 2-hydroxy-3,3′,4,4′-TCB in addition to these metabolites (Wehler et al., 1989). 3,3′,4,4′-TCB was the major compound present in mouse liver and a minor portion was due to 4-hydroxy-3,3′,4,4′-TCB (Wehler et al., 1989). Darnerud et al. (1986) found 2-hydroxy-3,3′,4,4′-TCB and methylsulphonyl-TCB as major metabolites in the mouse fetus. Sulfur-containing metabolites of noncoplanar, nondioxin-like PCBs have also been reported to accumulate in the bronchial mucosa and uterine luminal fluid of mice (Bergman et al., 1979; Brandt et al., 1982) and in human lung, liver, and adipose tissue (Haraguchi et al., 1986, 1989). PCB methyl sulfones are stable lipophilic metabolites formed by the mercapturic acid pathway. The toxicological significance of these metabolites of nondioxin-like PCBs remains generally unknown.

1.3.2. Toxicity of Metabolites

The discussion above indicates that metabolism of 2,3,7,8-TCDD and related compounds is required for urinary and biliary elimination and thus plays a major role in regulating the rate of excretion of these compounds. At present, metabolism is also generally considered a detoxification process.

Data on the metabolism of 2,3,7,8-TCDD suggest that reactive epoxide intermediates may be formed. Poland and Glover (1970) investigated the in vivo binding of [1,6-^{3}H]-2,3,7,8-TCDD-derived radioactivity to rat hepatic macromolecules and found maximum levels equivalent to 60 pmol of 2,3,7,8-TCDD/mol of nucleotide in RNA and 6 pmol of 2,3,7,8-TCDD/mol of nucleotide in DNA. This corresponds to one 2,3,7,8-TCDD-DNA adduct per 35 cells. These investigators suggest that it is unlikely that 2,3,7,8-TCDD-induced oncogenesis is through a mechanism of covalent binding to DNA and somatic mutation. Further studies of 2,3,7,8-TCDD and related compounds are needed to confirm

these results and assess the relationship between covalent binding and the short- and long-term toxicity of these compounds.

Weber et al. (1982a) investigated the toxicity of 2,3,7,8-TCDD metabolites by administering extracts of bile from 2,3,7,8-TCDD-treated dogs to male guinea pigs in single oral doses equivalent to 0.6, 6.0, and 60 μg/kg of parent compound. Other groups of guinea pigs were given bile extract from untreated dogs or 2,3,7,8-TCDD itself. A comparison of the mortality data at 5 weeks after dosing indicated that the acute toxicity of 2,3,7,8-TCDD to guinea pigs was at least 100 times higher than the acute toxicity of its metabolites.

Mason and Safe (1986) synthesized 2-hydroxy-3,7,8-TCDD and 2-hydroxy-1,3,7,8-TCDD, which are metabolites of 2,3,7,8-TCDD, and assessed the toxicity of these compounds in male Wistar rats. The compounds produced no significant effect on body weight gain and thymus, liver, or spleen weights after exposure to a dose of $\leq$5000 μg/kg bw. 2-Hydroxy-3,7,8-TCDD induced hepatic microsomal AHH, EROD, and 4-chlorobiphenylhydroxylase activity at an exposure of 1,000 and 5,000 μg/kg bw, whereas 2-hydroxy-1,3,7,8-TCDD was inactive as an inducer. Thus, while 2-hydroxy-3,7,8-TCDD has dioxin-like activity as an inducer of the hepatic monooxygenase system, the potency of the metabolite is more than three orders of magnitude less than that of 2,3,7,8-TCDD. Furthermore, results are consistent with the expected rapid conjugation and excretion of these 2,3,7,8-TCDD metabolites (Weber et al., 1982b).

Metabolism of coplanar PCBs and PBBs also appears to be a detoxification process. 5-Hydroxy-3,3′,4,4′-TCB and 4-hydroxy-3,3′,4′,5-TCB did not produce liver hypertrophy, induction of hepatic AHH or DT-diaphorase activities or thymus atrophy (Yoshimura et al., 1987b). Thus, monohydroxy metabolites of 3,3′,4,4′-TCB are much less toxic than the parent compound. Further evidence for metabolism as a detoxification process comes from comparison of the metabolism and toxicity of two coplanar PBBs. Millis et al. (1985) found that 3,3′,4,4′,5,5′-HxBB exhibited greater toxic potency in rats than 3,3′,4,4′-TBB, even though 3,3′,4,4′-TBB had about a 10-fold greater affinity for the Ah receptor. Although receptor binding affinities imply that 3,3′4,4′-TBB should be more toxic than 3,3′,4,4′5,5′-HxBB, it was less toxic than the HxBB because 3,3′,4,4′-TBB was metabolized at a much greater rate than 3,3′,4,4′,5,5′-HxBB. In addition to supporting metabolism as a

detoxification process, the results of Millis et al. (1985) also suggest that receptor binding and in vitro AHH induction do not accurately reflect toxicity for PAHs, which are more readily metabolized, presumably because continued occupation of the receptor is required for toxicity.

Structure-activity studies of 2,3,7,8-TCDD and related compounds support the widely accepted principle that this parent compound is the active species. The relative lack of activity of readily excreted monohydroxylated metabolites of 2,3,7,8-TCDD and 3,3'4,4'-TCB suggests that metabolism is a detoxification process necessary for the biliary and urinary excretion of these compounds. This concept has also been generally applied to 2,3,7,8-TCDD related compounds, although data are lacking on the structure and toxicity of metabolites of other CDDs, BDDs, CDFs, BDFs, PCBs, and PBBs.

It is possible that low levels of unextractable and unidentified metabolites may contribute to one or more of the toxic responses of 2,3,7,8-TCDD and related compounds. Further studies on the nature of the biotransformation products of these compounds will help address this uncertainty.

1.3.3. Autoinduction of Metabolism

Accurate rate constants for metabolism are important in developing pharmacokinetic models that describe the disposition of 2,3,7,8-TCDD and related compounds. Metabolism plays a major role in regulating the excretion and relative persistence of these compounds because metabolism is required for urinary and biliary excretion. Although the relative rate of metabolism of 2,3,7,8-TCDD and related compounds can be estimated from tissue and excretion half-life data (see Tables 1-6 and 1-9), other factors such as relative body composition, hepatic and extrahepatic binding proteins, and direct intestinal elimination of the parent compound can also regulate the excretion of 2,3,7,8-TCDD and related compounds. Therefore, in vivo disposition data (see Tables 1-6 and 1-9) provide only a limited approximation of the relative rate of metabolism of a specific congener in a given species. In vivo disposition data were also obtained at exposures that were associated with induction of cytochromes P-4501A1 and 1A2 and other potentially adverse responses that could alter metabolism and disposition. Therefore, it may not be appropriate to directly extrapolate

these data to predict the pharmacokinetics at low levels of exposure. Low-dose extrapolations can be assisted by assessments of the potential for autoinduction of metabolism that may occur at exposures associated with enzyme induction. Characterization of the dose-dependent disposition of 2,3,7,8-TCDD and related compounds is particularly important in the extrapolation of high-exposure animal data to low-exposure human data.

The excretion of metabolites of 2,3,7,8-TCDD and related compounds into bile represents a direct means for estimating the rate of metabolism because biliary elimination depends on metabolism and is the major route for excretion of these compounds. The rate of metabolism of CDFs was estimated from the relative abundance of metabolites in rat bile (Poiger et al., 1989a). The rates of biotransformation of 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF and 1,2,3,6,7,8-HxCDF were characterized as fairly high, moderate, low, and very low, respectively. Kedderis et al. (1991b, 1993a) observed 10% of the dose of [^{3}H]-2,3,7,8-TBDD excreted in bile 5 hours after intravenous administration of 1 nmol/kg to male Fischer 344 rats. All biliary radioactivity was attributable to metabolites. This rate of elimination is similar to the fecal excretion ($\sim$8% of the dose) 24 hours after intravenous administration of 1 nmol/kg [^{3}H]-2,3,7,8-TBDD (Kedderis et al., 1991a) and reflects the effect of intravenous bolus versus oral administration on distribution and elimination. The large percent dose excreted within the first few days may also be due to a rapidly excreted impurity in the radiolabeled 2,3,7,8-TBDD (Kedderis et al., 1993a). To assess the ability of 2,3,7,8-TCDD and 2,3,7,8-TBDD to induce their own metabolism (biliary elimination), rats were pretreated with 100 nmol/kg, per os, of each compound 3 days prior to intravenous injection of 1 nmol/kg of the respective [^{3}H]-congeners. Biliary excretion of the radiolabeled dose was quantitatively and qualitatively unaffected by pretreatment, despite a twofold increase in hepatic levels of [^{3}H] in the pretreated animals and significant induction of cytochrome P-4501A1 and 1A2 (Kedderis et al., 1991b). Therefore, under these conditions, autoinduction of 2,3,7,8-TCDD and 2,3,7,8-TBDD metabolism did not occur in the rat in vivo at doses that elicited enhanced hepatic uptake. Similarly, Curtis et al. (1990) observed no change, or even an apparent decrease, in gastrointestinal contents and fecal elimination of TCDD equivalents in pretreated versus naive mice 24 hours after oral administration of

[^{14}C]-2,3,7,8-TCDD despite significantly enhanced levels of 2,3,7,8-TCDD in the livers of pretreated mice.

While the above studies suggest that autoinduction of 2,3,7,8-TCDD metabolism does not occur, other results indicate that metabolism may be induced under certain conditions. Poiger and Buser (1984) observed a small yet significant increase in biliary excretion over a 72-hour period, with pretreated rats (10 μg/kg, intraperitoneal) excreting 9.7$\pm$1.9% of the radiolabeled dose of 2,3,7,8-TCDD (200-300 μg/kg, per os) compared with 7.0$\pm$0.9% excreted by naive animals. In addition to being small changes, these results were obtained using a dose of 2,3,7,8-TCDD in excess of the LD_{50} in the rat. Poiger and Schlatter (1985) examined the influence of pretreatment with phenobarbital and 2,3,7,8-TCDD on the biliary excretion of [^{3}H]-2,3,7,8-TCDD metabolites in a dog given a single oral dose of the [^{3}H]-congener (31 or 33.8 ng/kg). Without pretreatment, 24.5% of the absorbed dose was excreted in the bile within 110 hours. Phenobarbital did not alter this rate, whereas pretreatment with 2,3,7,8-TCDD (10 μg/kg) 9 days earlier resulted in a doubling of the amount of metabolites excreted in bile (47.4%). Although this observation is limited to one dog and requires further investigation, the results suggest that significant autoinduction of 2,3,7,8-TCDD metabolism and biliary excretion may occur in the dog. Nonetheless, the small increase in metabolism and biliary excretion of 2,3,7,8-TCDD in the rat observed by Poiger and Buser (1984) and the negative results of Kedderis et al. (1991b; 1993a) and Curtis et al. (1990) suggest that autoinduction of 2,3,7,8-TCDD metabolism and biliary excretion in the rat may not occur or occurs to an extent that is not biologically relevant.

Limited data suggest that autoinduction of metabolism and biliary excretion does occur for CDFs in contrast to CDDs and BDDs. Pretreatment of rats with 2,3,7,8-TCDF (1.0 μmol/kg, 3 days earlier) significantly increased the biliary excretion of a subsequent dose of [^{14}C]-2,3,7,8-TCDF (McKinley et al., 1993). The naive rats excreted 5.69$\pm$2.35% of the dose over the initial 8 hours, while the pretreated rats excreted 13.18$\pm$3.15% of the [^{14}C]-2,3,7,8-TCDF. Similarly, pretreatment of rats with 2,3,4,7,8-PeCDF (500 μg/kg, per os, 3 days earlier) resulted in a twofold increase in the biliary elimination of a subsequent dose of [^{14}C]-2,3,4,7,8-PeCDF (Brewster and Birnbaum, 1987). These results suggest that

pretreatment with 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF induces the metabolism of these congeners.

3,3′,4,4′-TCB and 3,3′,4,4′-TBB appear to be metabolized by a 3-methylcholanthrene-inducible form of hepatic cytochrome P-450 (1A1 or 1A2), which is also induced by 3,3′,4,4′-TCB (Shimada and Sawabe, 1983; Mills et al., 1985; McKinley et al., 1993). This suggests that these compounds can induce their own rate of metabolism and subsequent excretion.

Isolated hepatocytes in suspension culture have been demonstrated to provide a useful in vitro system for studying the hepatic metabolism of 2,3,7,8-TCDD under the same conditions in species that have a wide range of sensitivity to the compound (Olson et al., 1981). The in vitro rate of metabolism of 2,3,7,8-TCDD in guinea pig, rat, C57BL/6J mouse, DBA/2J mouse, and hamster hepatocytes was estimated to be 0.2, 1.2, 1.1, 0.9, and 1.2 pmol/mg protein/hour, respectively (Wroblewski and Olson, 1985, 1988; Shen and Olson, 1987). These results indicate that 2,3,7,8-TCDD is metabolized by the guinea pig liver at a rate about fivefold less than that observed for the rat, mouse, and hamster. The limited ability of the guinea pig to metabolize 2,3,7,8-TCDD can explain the limited excretion of 2,3,7,8-TCDD metabolites in feces, which represents the major route for 2,3,7,8-TCDD excretion (Olson, 1986). In addition, the limited metabolism in the guinea pig may partly explain the relatively long excretion half-life for 2,3,7,8-TCDD in the guinea pig and may contribute to the remarkable sensitivity of the guinea pig to the acute toxicity of this agent (Olson, 1986).

Isolated hepatocytes in suspension culture have been used as an in vitro system for studying the autoinduction of metabolism of 2,3,7,8-TCDD and related compounds. Wroblewski and Olson (1988) investigated the metabolism of [^{14}C]-2,3,7,8-TCDD (2.2 μM) in hepatocytes isolated from untreated 2,3,7,8-TCDD-, 3-MC-, isosafrole-, and phenobarbital-pretreated rats and hamsters. In both species, 2,3,7,8-TCDD and 3-MC pretreatments elevated the rate of 2,3,7,8-TCDD metabolism by five- to sixfold, while phenobarbital pretreatment had no effect. Isosafrole produced a 1.8- to 2.5-fold increase in metabolism. These in vitro results at a high substrate concentration (2.2 μM) indicate that 2,3,7,8-TCDD can induce its own rate of metabolism in the rat and hamster. In contrast,

2,3,7,8-TCDD was not able to induce its own rate of metabolism in guinea pig and mouse hepatocytes (Wroblewski and Olson, 1985; Shen and Olson, 1987). Together, these results indicate that 2,3,7,8-TCDD is metabolized in the liver by a 2,3,7,8-TCDD-inducible enzyme, which is expressed in the rat and hamster but not in the guinea pig and mouse. More recently, the kinetics of 2,3,7,8-TCDD metabolism were investigated in isolated rat hepatocytes incubated with [^{3}H]-2,3,7,8-TCDD at concentrations of 0.01, 0.1, and 1.0 μM (Olson et al., 1994). Lower 2,3,7,8-TCDD concentrations in the media result in concentrations in hepatocytes that are more similar to the levels in the liver after in vivo exposure. For example, the concentration of 2,3,7,8-TCDD in hepatocytes incubated at 0.01 μM is similar to hepatic levels after in vivo exposure of rats at a dose of $\sim$10 μg/kg. At 0.01 and 0.1 μM, the rate of metabolism of [^{3}H]-2,3,7,8-TCDD was similar in hepatocytes isolated from control and 2,3,7,8-TCDD-pretreated rats, whereas at 1.0 μM, [^{3}H]-2,3,7,8-TCDD metabolism was greater in hepatocytes isolated from 2,3,7,8-TCDD-pretreated rats. The results indicate that 2,3,7,8-TCDD can induce its own rate of metabolism in the rat but only at high hepatic concentrations, which are generally not attained after in vivo exposure. Therefore, in vitro studies of the hepatic metabolism of TCDD (at 0.01 and 0.1μM) are consistent with the lack of autoinduction of 2,3,7,8-TCDD metabolism and biliary excretion observed in vivo in the rat (Kedderis et al., 1991b; Curtis et al., 1990).

The metabolism of [^{3}H]-2,3,7,8-TCDF was also investigated in isolated rat hepatocytes incubated at concentrations of 0.01, 0.1, and 1.0 μM (Olson et al., 1994). At all concentrations, hepatocytes from 2,3,7,8-TCDD-pretreated rats metabolized 2,3,7,8-TCDF at a rate from 4- to 25-fold greater than that observed in hepatocytes from control rats. The results indicate that 2,3,7,8-TCDF is metabolized in rat liver by a 2,3,7,8-TCDD-inducible enzyme, possibly cytochrome P-4501A1 or 1A2. These in vitro results support the in vivo autoinduction of 2,3,7,8-TCDF metabolism and biliary elimination observed in the rat (McKinley et al., 1993).

TCDF metabolism was also investigated in rat liver, kidney, and lung microsomes in the presence and absence of selective chemical inhibitors and antibodies to CYP1A1 and CYP1A2 (Tai et al., 1993). Together, the results of this investigation indicate that CYP1A1 is the primary enzyme responsible for the metabolism of TCDF. TCDF was also

metabolized by recombinant yeast microsomes expressing human CYP1A1 and reductase. However, based on EROD activity, a marker of CYP1A1, the relative rate of TCDF metabolism was about 100-fold greater in TCDD-induced rat liver microsomes than in yeast microsomes expressing human CYP1A1 and reductase (Tai et al., 1993). Although TCDF was metabolized by rat and human CYP1A1, the results indicated that there are marked quantitative differences in metabolism that suggest that TCDF will be more persistent in humans.

There are in vivo and in vitro data suggesting that autoinduction of 2,3,7,8-TCDD and 2,3,7,8-TBDD metabolism does not occur in the rat after exposure to sublethal doses of these agents. This is in contrast to 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF where in vivo and in vitro results support the autoinduction of metabolism and biliary elimination of these compounds in the rat.

1.3.4. Excretion in Animals

Data regarding the excretion of 2,3,4,7-TCDD and related compounds after exposure to a single radiolabeled congener (see Table 1-9) support the assumption of a first-order elimination process consisting of one or more components. These studies show that 2,3,7,8-TCDD was excreted slowly from all species tested, with half-lives ranging from 11 days in the hamster to 2,120 days in humans. 2,3,7,8-TCDD is exceptionally persistent in humans relative to other animal models. Elimination data in tissues (see Tables 1-6 and 1-7) also indicate that 2,3,7,8-TCDD and related compounds are exceptionally persistent in nonhuman primates (Bowman et al., 1989; Neubert et al., 1990). These differences may also be in part related to the dose dependency of the excretion of these compounds. In general, the congener- and species-specific rates of elimination of 2,3,7,8-TCDD and related compounds from major tissue depots (see Table 1-6) are similar to the excretion data summarized in Table 1-9.

In the Syrian Golden hamster, the mammalian species least sensitive to the acute toxicity of 2,3,7,8-TCDD, excretion occurred readily through both the urine (35% of administered dose, 41% of total excreted radioactivity) and feces (50% of the administered dose, 59% of total excreted radioactivity) (Olson et al., 1980). A similar excretion pattern

was observed in mice, although there was significant strain variability (Gasiewicz et al., 1983a; Birnbaum, 1986). In all the other species, excretion occurred mainly through the feces, with relatively minor amounts of 2,3,7,8-TCDD metabolites found in the urine (Piper et al., 1973; Allen et al., 1975; Olson, 1986; Rose et al., 1976; Gasiewicz and Neal, 1979; Pohjanvirta et al., 1990). Results in Table 1-9 also indicate that fecal elimination was the primary route for the excretion of 1,2,3,7,8-PeCDD, OCDD, 2,3,7,8-TBDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, and 3,3′,4,4′-TCB. Only Piper et al. (1973) reported the excretion of metabolites in the expired air. During 21 days following administration of a single oral dose of [^{14}C]-2,3,7,8-TCDD to rats, 3.2% of the administered radioactivity (4.6% of the excreted radioactivity) was recovered in the expired air.

Studies in the rat, guinea pig, hamster, and mouse have found that essentially all of the 2,3,7,8-TCDD-derived radioactivity excreted in the urine and bile corresponds to metabolites of 2,3,7,8-TCDD (see Table 1-9). The apparent absence of 2,3,7,8-TCDD metabolites in liver and fat suggests that, once formed, the metabolites of 2,3,7,8-TCDD are excreted readily. Thus, urinary and biliary elimination of 2,3,7,8-TCDD depends on metabolism of the toxin. The more limited data for other compounds also suggest that this relationship may be true for 1,2,3,7,8-PeCDD, 2,3,7,8-TBDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, and 3,3′,4,4′-TCB (see Table 1-9).

Although urine and bile appear to be free of unmetabolized 2,3,7,8-TCDD, data indicate that 2,3,7,8-TCDD and its metabolites are excreted in the feces of guinea pigs, rats, mice and hamsters treated with [^{3}H]- or [^{14}C]-2,3,7,8-TCDD (see Table 1-9). While 15% to 35% of the 2,3,7,8-TCDD-derived radioactivity in rat, mouse, and hamster feces represents unchanged 2,3,7,8-TCDD, 81% of the radioactivity in guinea pig feces represents unmetabolized 2,3,7,8-TCDD (Olson, 1986; Neal et al., 1982; Gasiewicz et al., 1983a; Olson et al., 1980). The daily presence of unchanged 2,3,7,8-TCDD in feces and its absence in bile suggest that direct intestinal elimination may be the source for the fecal excretion of 2,3,7,8-TCDD. Data also suggest that direct intestinal elimination of parent compound contributes to the fecal excretion for 2,3,7,8-TBDD (Kedderis et al., 1991a). While direct intestinal elimination of the parent compound may occur for other congeners (see Table 1-9), this conclusion cannot be made at this time due to the lack of experimental

data. Nonetheless, the species-specific fecal excretion of 2,3,7,8-TCDF is very similar to that observed for 2,3,7,8-TCDD, with >90% of the 2,3,7,8-TCDF-derived radioactivity excreted in guinea pig feces representing parent compound (Decad et al., 1981a). In addition, the excretion of unchanged CDDs and CDFs was detected in rat feces after subcutaneous exposure to a defined mixture of congeners (Abraham et al., 1989d). Studies in lactating rats have also found that unchanged 2,3,7,8-TCDD may be excreted in the milk of lactating animals (Moore et al., 1976; Lucier et al., 1975; Nau et al., 1986). Lactation, direct intestinal elimination, and perhaps sebum may serve as routes for excretion of 2,3,7,8-TCDD that do not depend on metabolism of the toxin. These data suggest that the in vivo half-life for elimination of 2,3,7,8-TCDD and related compounds only provides an approximation of the rate of metabolism of these compounds in a given animal. The results in Table 1-9 suggest that 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and 3,3′,4,4′-TCB are metabolized and excreted more rapidly than 2,3,7,8-TCDD, 2,3,7,8-TBDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, and OCDD.

The rate of excretion of 2,3,7,8-TCDD and related compounds is species and congener specific (see Table 1-9). 2,3,7,8-TCDD is most persistent in human and nonhuman primates. In the hamster, the least sensitive species to the acute toxicity of 2,3,7,8-TCDD, the mean $t_{½}$ was 10.8 days (Olson et al., 1980), and in the guinea pig, the most sensitive species to the acute toxicity of 2,3,7,8-TCDD, the mean $t_{½}$ was 94 days (Olson, 1986). 2,3,7,8-TCDF was also most persistent in the guinea pig, with a $t_{½}$ of 20 to 40 days (Decad et al., 1981a; Ioannou et al., 1983). Furthermore, results indicate that the relatively limited ability of the guinea pig to metabolize 2,3,7,8-TCDD and -TCDF may contribute to the greater persistence and greater acute toxicity of these congeners in the guinea pig.

The tissue distribution, metabolism, and excretion of 2,3,7,8-TCDD were also investigated in Han/Wistar and Long-Evans rats, which were, respectively, more resistant (LD_{50} > 3000 μg/kg) versus more susceptible (LD_{50} ~ 10 μg/kg) to the acute toxicity of 2,3,7,8-TCDD (Pohjanvirta et al., 1990). The results suggest that the metabolism and disposition of 2,3,7,8-TCDD do not have a major role in explaining the strain differences in toxicity.

The intraspecies differences in the $t_{1/2}$ of 2,3,7,8-TCDD in three mouse strains may be due to the finding that the DBA/2J strain possesses about twofold greater adipose tissue stores than the C57BL/6J and B6D2F$_1$/J strains (Gasiewicz et al., 1983a). The sequestering of the lipophilic toxin in adipose tissue stores of the DBA/2J mouse may contribute to the greater persistence of 2,3,7,8-TCDD in this strain. Birnbaum (1986) examined the effect of genetic background on the distribution and excretion of 2,3,7,8-TCDD in two sets of congenic mouse strains in which the congenic pairs differed only at the Ah locus. The Ah locus had no effect on the tissue distribution or excretion of 2,3,7,8-TCDD. Thus, the distribution and excretion of 2,3,7,8-TCDD were primarily governed by the total genetic background rather than the allele present at the Ah locus. These findings are consistent with the in vitro results of Shen and Olson (1987), who found that the hepatic uptake and metabolism of 2,3,7,8-TCDD do not correlate with genetic differences at the murine Ah locus. However, it is important to note that all of these are relatively high-dose studies, which may not allow for detection of Ah receptor-mediated effects on disposition.

Although the dose-related tissue distribution of 2,3,7,8-TCDD and related compounds has been described recently, very limited data are available on the dose-related excretion of these compounds. Rose et al. (1976) investigated the elimination of [^{14}C]-2,3,7,8-TCDD in rats given repeated oral doses of 0.01, 0.1, or 1.0 μg/kg/day Monday through Friday for 7 weeks or a single dose of 1.0 μg/kg. In the single-dose study, no ^{14}C was excreted in the urine or expired air; in the repeated-dose study, however, 3% to 18% of the cumulative dose was excreted in the urine by 7 weeks. This study indicated that steady-state concentrations will be reached in the bodies of rats in $\sim$13 weeks. The rate constant defining the approach to steady-state concentrations was independent of the dose of 2,3,7,8-TCDD over the range studied. Relatively small changes in the excretion of 2,3,7,8-TBDD were also observed after exposures at 1 and 100 nmol/kg (Kedderis et al., 1991a). These results are consistent with the in vivo and in vitro evidence suggesting that autoinduction of 2,3,7,8-TCDD and 2,3,7,8-TBDD metabolism does not occur in the rat after exposure to sublethal doses of these compounds (Kedderis et al., 1991b; Curtis et al., 1990; Olson et al., 1994). In contrast to these compounds, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF can induce their own rate of metabolism and biliary excretion (Brewster and Birnbaum, 1987; McKinley et al., 1993;

Olson et al., 1994). Autoinduction of metabolism suggests that these compounds may exhibit dose-related excretion, with longer half-lives for elimination at lower doses, which are not associated with enzyme induction. Further data are needed to test this hypothesis.

1.3.5. Excretion in Humans

Poiger and Schlatter (1986) investigated the excretion of 2,3,7,8-TCDD in a 42-year-old man (92 kg) after ingestion of 105 ng (1.14 ng/kg) [^{3}H]-2,3,7,8-TCDD in 6 mL corn oil (see Tables 1-9 and 1-10). The half-life for elimination was estimated to be 2,120 days based on fecal excretion over a 125-day period following the single exposure. The concentration of ^{3}H-TCDD-derived radioactivity was also measured in adipose tissue in the same individual over a 6-year period following exposure. A more accurate estimate of 2,3,7,8-TCDD half-life of 9.7 years was calculated based on adipose tissue concentrations over a 6-year period (Schlatter, 1991). Table 1-10 summarizes additional half-life estimates for 2,3,7,8-TCDD and related compounds in humans, based on serum and adipose tissue concentrations at two or more time points. In another study, the half-life of 2,3,7,8-TCDD in humans was estimated to be $\sim$7 years on the basis of 2,3,7,8-TCDD levels in serum samples taken in 1982 and 1987 from 36 of the Ranch Hand personnel who had 2,3,7,8-TCDD levels $>$10 ppt in 1987 (Pirkle et al., 1989).

Wolfe et al. (1994) recently investigated the half-life of 2,3,7,8-TCDD in an expanded cohort of 337 Air Force veterans of Operation Ranch Hand that also included the 36 subjects of the earlier half-life study by Pirkle et al. (1989). Based on paired 2,3,7,8-TCDD measurements from serum collected in 1982 and in 1987, the authors reported a mean predicted half-life of 11.6 years and a median observed half-life of 11.3 years with a nonparametric 95% confidence interval of 10.0 to 14.1 years. The authors also investigated how the 2,3,7,8-TCDD half-life varied with percent body fat (PBF), relative changes in PBF from 1982 to 1987, and age. They found that the 2,3,7,8-TCDD half-life increased significantly with a high PBF, suggesting that persons with more body fat tend to eliminate 2,3,7,8-TCDD more slowly. In contrast, increasing age was associated with a shorter half-life. The redistribution of fat stores from subcutaneous to abdominal areas with aging, resulting in greater mobilization of 2,3,7,8-TCDD, could in part explain the shorter half-life

Table 1-10. Half-Life Estimates for 2,3,7,8-TCDD and Related Compound in Humans

Chemical	Exposure Incident	Number of Individuals	Sample	Time Period Between First and Last Analysis	Number of Time Points	Half-Life (years)	Reference
CDDs							
2,3,7,8-TCDD	Male volunteer	1	fecal excretion	125 days	28	5.8	Poiger and Schlatter, 1986
2,3,7,8-TCDD	Male volunteer	1	adipose tissue	6 years	5	9.7	Schlatter, 1991
2,3,7,8-TCDD	Ranch Hand Vietnam veterans	36	serum	5 years	2	7.1[a]	Pirkle et al., 1989
2,3,7,8-TCDD	Ranch Hand Vietnam veterans	337	serum	5 years	2	11.3[b]	Wolfe et al., 1994
1,2,3,6,7,8-HxCDD	technical pentachlorophenol in wood of home	1	adipose tissue	28 months	2	3.5	Gorski et al., 1984
1,2,3,4,6,7,8-HpCDD	technical pentachlorophenol in wood of home	1	adipose tissue	28 months	2	3.2	Gorski et al., 1984
OCDD	technical pentachlorophenol in wood of home	1	adipose tissue	28 months	2	5.7	Gorski et al., 1984
CDFs							
2,3,4,7,8-PeCDF	Binghamton, New York, state office building	1	adipose tissue blood combined	initial 43 months final 29 months total 6 years	4 4 7	4.7 7.2 4.5	Schecter et al., 1990a
1,2,3,4,7,8-HxCDF	Binghamton, New York, state office building	1	adipose tissue blood combined	initial 43 months final 29 months total 6 years	4 4 7	2.9 4.4 4.0	Schecter et al., 1990a
1,2,3,6,7,8-HxCDF	Binghamton, New York, state office building	1	adipose tissue blood combined	initial 43 months final 29 months total 6 years	4 4 7	3.5 4.3 4.9	Schecter et al., 1990a

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Table 1-10 (continued)

Chemical	Exposure Incident	Number of Individuals	Sample	Time Period Between First and Last Analysis	Number of Time Points	Half-Life (years)	Reference
1,2,3,4,6,7,8-HpCDF	Binghamton, New York, state office building	1	adipose tissue blood combined	initial 43 months final 29 months total 6 years	4 4 7	6.5 4.1 6.8	Schecter et al., 1990a
2,3,4,7,8-PeCDF	Yu-Cheng	4 3 2	blood	initial 2.9 years final 2.7 years total 5.6 years	2 2 3	1.3 2.9 1.7	Ryan and Masuda, 1989
1,2,3,4,7,8-HxCDF	Yu-Cheng	4 3 2	blood	initial 2.9 years final 2.7 years total 5.6 years	2 2 3	2.1 5.1 2.4	Ryan and Masuda, 1989
1,2,3,4,6,7,8-HpCDF	Yu-Cheng	4 3 2	blood	initial 2.9 years final 2.7 years total 5.6 years	2 2 3	1.6 6.1 2.4	Ryan and Masuda, 1989
2,3,4,7,8-PeCDF 1,2,3,4,7,8-HxCDF 1,2,3,4,6,7,8-HpCDF	Yu-Cheng	3	blood	9 years	5-6	2-3	Ryan and Masuda, 1991
2,3,4,7,8-PeCDF 1,2,3,4,7,8-HxCDF	Yusho	9	blood	7 years	3-5	>5	Ryan and Masuda, 1991
1,2,3,4,6,7,8-HpCDF	technical pentachlorophenol in wood of home	1	adipose tissue	28 months	2	<1.7	Gorski et al., 1984
OCDF	technical pentachlorophenol in wood of home	1	adipose tissue	28 months	2	1.8	Gorski et al., 1984
PCBs							
3,3',4,4',5-PeCB	Yu-Cheng	NA	blood	NA	NA	<1	Ryan and Masuda, 1991

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Table 1-10 (continued)

Chemical	Exposure Incident	Number of Individuals	Sample	Time Period Between First and Last Analysis	Number of Time Points	Half-Life (years)	Reference
3,3',4,4',5,5'-HxCB	Yu-Cheng	NA	blood	NA	NA	10	Ryan and Masuda, 1991

[a]95% confidence interval about the median of 5.8-9.6 years.
[b]95% confidence interval about the median of 10.0-14.1 years.
NA = Not applicable.

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observed in older veterans. An increase in PBF from 1982 to 1987 was also associated with a decrease in half-life, which can be explained by a dilution of the existing body burden of 2,3,7,8-TCDD into the increasing adipose tissue mass. Future studies will examine these relationships further with data from a third serum sample collected in 1992 from these veterans. Additional data will also help address the question of the potential biphasic elimination of 2,3,7,8-TCDD.

These studies indicate that 2,3,7,8-TCDD is exceedingly persistent in humans. Estimated half-lives for other congeners in Table 1-10 range from 0.8 to 10 years. The half-life values in Table 1-10 are rough estimates based on a small number of individuals and analysis at as few as two time points. Phillips (1989) discusses this issue. Estimates also assume a simple, single compartment, first-order elimination process.

Ryan and Masuda (1991) reported on their continuing investigation into the elimination of CDFs in humans from the Yusho and Yu-Cheng rice oil poisonings. Yu-Cheng individuals had CDF blood levels on a lipid basis of 1-50 μg/kg, wheras Yusho patients had levels of 0.1-5 μg/kg. In the Yu-Cheng individuals, half-lives for three CDFs were 2 to 3 years, while elimination from Yusho individuals was more variable and slower, with half-lives >5 years (see Table 1-10) and, in several cases, no measurable elimination occurred during the 7 years in which samples were available. The limited results suggest that clearance of these CDFs in the human is biphasic, with faster elimination at higher exposure. Schecter et al. (1990a) and Ryan and Masuda (1989) also reported longer half-life values for CDFs in humans at later time points after exposure, when concentrations are closer to the background levels of individuals with no unusual exposure.

Due to the lipophilic nature of milk, lactation can provide a relatively efficient mechanism for decreasing the body burden of 2,3,7,8-TCDD in females. As discussed by Graham et al. (1986), this elimination of 2,3,7,8-TCDD through mother's milk can result in high exposure levels in the infant. Since both milk and the fatty tissues of fish are essentially providing an oily vehicle, it would be likely that these sources would provide 2,3,7,8-TCDD in a form that is readily bioavailable.

Several investigators have quantified the levels of 2,3,7,8-TCDD in human milk samples. Many of the milk samples were pooled (Jensen, 1987). Rappe (1984) reported

levels of 1-3 ppt 2,3,7,8-TCDD in milk fat (lipid adjusted) from five volunteers in West Germany, and in a later report, Rappe et al. (1985) reported an average level of 0.6 ppt 2,3,7,8-TCDD in milk fat from four volunteers in northern Sweden. Furst et al. (1986) reported an average level of 9.7 ppt 2,3,7,8-TCDD in milk fat from three individuals in the Netherlands and < 1.0 ppt 2,3,7,8-TCDD in milk fat from two individuals in Yugoslavia. Nygren et al. (1986) reported average levels of 2,3,7,8-TCDD in human milk samples from four subjects in Sweden to be 0.6 ppt in milk fat, in five subjects from West Germany to be 1.9 ppt in milk fat, and in four subjects from Vietnam to be < 0.5 ppt in milk fat.

High levels of 2,3,7,8-TCDD have been detected in the milk of mothers exposed to high levels of 2,3,7,8-TCDD in the environment. Reggiani (1980) reported levels between 2.3 and 28.0 ppt 2,3,7,8-TCDD in whole milk from mothers in Seveso. Baughman (1975) reported levels between 40.0 and 50.0 ppt 2,3,7,8-TCDD in whole milk from mothers in South Vietnam. Schecter et al. (1987) also found high ppt levels of 2,3,7,8-TCDD in human milk samples from South Vietnam. These authors found that levels from samples taken in 1985 from South Vietnamese mothers were comparable to the level of 2,3,7,8-TCDD presently found in North American human milk samples (5 ppt).

1.4. PHYSIOLOGICALLY BASED PHARMACOKINETIC (PB-PK) MODELS

Initially, PB-PK models were developed for 2,3,7,8-TCDD in C57BL/6J and DBA/2J mice (Leung et al., 1988), rats (Leung et al., 1990b) and humans (Kissel and Robarge, 1988). PB-PK models incorporate known or estimated anatomical, physiological, and physicochemical parameters to describe quantitatively the disposition of a chemical in a given species. PB-PK models can assist in the extrapolation of high-to-low dose kinetics within a species, estimating exposures by different routes of administration, calculating effective doses and extrapolating these values across species (Scheuplein et al., 1990). Table 1-11 summarizes the pharmacokinetic parameters for 2,3,7,8-TCDD that were used in developing these PB-PK models. In many cases, these parameters were estimated from in vivo experimental data.

A five-compartment (blood, liver, fat, muscle/skin, viscera), flow-limited PB-PK model for 2,3,7,8-TCDD was developed for the Ah-responsive C57BL/6J mouse and the

Table 1-11. Pharmacokinetic Parameters for 2,3,7,8-TCDD Used in PB-PK Models

	C57BL/6J Mouse[a]	DBA/2J Mouse[a]	Sprague-Dawley Rat[b]	Human[c]	Female C57BL/6J Mice[d]	
					Naive	Pretreated
Partition Coefficient (Tissue/Blood)						
Liver	20	20	20	25	10	10
Fat	350	350	350	300	300	300
Richly perfused (kidney)	20	20	20	7-10	10	10
Slowly perfused (skin)	250	250	40	30	200	200
Slowly perfused (muscle)	250	250	40	4	3	3
Biochemical Constants						
Binding capacity to hepatic cytosolic protein (nmol/liver)	0.0042	0.0042	0.054	-	0.0042	0
Binding affinity to hepatic cytosolic protein (nM)	0.29	2	0.015	-	0.29	0.29
Binding capacity to hepatic microsomal protein (nmol/liver)	20	20	-	-	1.75	20
Noninduced binding capacity to hepatic microsomal protein (nmol/liver)	-	-	25	-	-	-
Induced binding capacity to hepatic microsomal protein (μmol/liver)	-	-	175	-	-	-
Binding affinity to hepatic microsomal protein (nM)	20	75	7	-	20	20
First-order metabolic rate constant (per hour per kg liver)	3.25	1.75	2.0	-	1.0	3.0
Absorption constant from gastrointestinal tract into liver (per hour)	0.02	0.02	0.2	-	0.04	0.15
Binding to blood	2.5	2.5	2.5	-	1.0	3.0

[a]Leung et al., 1988.
[b]Leung et al., 1990a.
[c]Kissel and Robarge, 1988.
[d]Leung et al. (1990) modeled the disposition of [^{125}I]-2-iodo-3,7,8-TCDD, an analog of 2,3,7,8-TCDD, following a single exposure (0.1 nmol/kg) in naive female C57BL/6J mice and in mice pretreated 3 days earlier with an inducing dose of 2,3,7,8-TCDD (0.1 μmol/kg).

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Ah-nonresponsive DBA/2J mouse (Leung et al., 1988). The model also included binding in the hepatic cytosol and hepatic microsomes and first-order hepatic metabolism. There was general agreement between the simulated description generated by the model and the experimental disposition data of Gasiewicz et al. (1983a). The greater accumulation of 2,3,7,8-TCDD in the liver of the C57BL/6J mouse, compared to the DBA/2J mouse, was not attributable to the twofold greater total fat content in the DBA/2J strain. The authors suggested that strain difference in hepatic disposition was due to differing affinity of 2,3,7,8-TCDD for the microsomal binding protein in the two strains and proposed using a different microsomal dissociation constant for each strain. Alternatively, the strain difference in hepatic disposition may be due to the different doses of 2,3,7,8-TCDD needed to induce cytochrome P-4501A2 in the two strains. In contrast to the high-capacity/low-affinity hepatic microsomal binding proteins, the low-capacity/high-affinity hepatic cytosolic binding protein (Ah receptor) did not play a major role in determining the overall tissue distribution pattern of 2,3,7,8-TCDD in this model.

A similar five-compartment PB-PK model was developed to describe the tissue disposition of 2,3,7,8-TCDD in the Sprague-Dawley rat (Leung et al., 1990a). This description included blood, liver (cytosolic receptor and microsomal binding protein), fat, muscle/skin, and visceral tissue compartments. The authors found generally good agreement between the PB-PK model simulated data and the experimental data for the single-dose study of Rose et al. (1976) and the 7- and 13-week multiple-dose studies of Kociba et al. (1978b). The model was not satisfactory for the 2-year feeding study of Kociba et al. (1978a), underpredicting the 2,3,7,8-TCDD concentration in the fat at the low dose (0.001 μg/kg/day) and overestimating the concentration achieved at the high dose (0.1 μg/kg/day). The model found the hepatic disposition of 2,3,7,8-TCDD to be dependent on the high-capacity/low-affinity hepatic microsomal binding protein with a dissociation constant of 7 nM and a basal and induced concentration in the liver of 25 and 175 nmol/liver, respectively. As discussed earlier, Voorman and Aust (1987, 1989) and Poland et al. (1989a,b) provided evidence that this binding species is cytochrome P-4501A2. Induction of the microsomal binding protein was necessary to account for the differences in hepatic disposition at low (0.01 μg/kg) and high (1.0 μg/kg) daily doses of 2,3,7,8-TCDD. The dose-dependent tissue distribution of

2,3,7,8-TCDD was also discussed earlier. As in the mouse PB-PK model (Leung et al., 1988), the low-capacity/high-affinity hepatic cytosolic binding protein (Ah receptor) was not a major factor in directly influencing the hepatic disposition of 2,3,7,8-TCDD. The dissociation constant of the cytosolic Ah receptor in vivo was estimated to be 15 pM by fitting enzyme induction data from McConnell et al. (1984).

A PB-PK model was also developed for female C57BL/6J mice for [^{125}I]-2-iodo-3,7,8-TCDD, an analog of 2,3,7,8-TCDD (Leung et al., 1990b). Mice were pretreated with 0.1 μmol/kg of 2,3,7,8-TCDD or the vehicle only, followed by 2-iodo-3,7,8-TCDD (0.1 nmol/kg) 3 days later. Naive mice had liver/fat 2-iodo-3,7,8-TCDD concentration ratios of 0.17–0.38, while the 2,3,7,8-TCDD pretreated mice had ratios of 2.0–6.1. This is in agreement with the dose-dependent tissue distribution of 2,3,7,8-TCDD described earlier (Abraham et al., 1988; Poiger et al., 1989a). As with 2,3,7,8-TCDD, the model found that the 2-iodo-3,7,8-TCDD concentration in the liver was most sensitive to the binding capacity of the hepatic microsomal protein. Whole-body elimination of 2-iodo-3,7,8-TCDD approximated first-order kinetics, and induction by pretreatment with an inducing dose of 2,3,7,8-TCDD almost doubled the rate of excretion ($t_{1/2}$ of 14.2 days in naive versus 8.0 days in induced mice) (see Table 1-9). The distribution in naive and pretreated mice was described by a PB-PK model in which induction (2,3,7,8-TCDD pretreatment) increased the amount of hepatic microsomal binding protein from 1.75 to 20 nmol/liver and increased the rate constant for metabolism of free 2-iodo-3,7,8-TCDD from 1 to 3 hours/kg liver. Although the more rapid elimination of 2-iodo-3,7,8-TCDD in 2,3,7,8-TCDD-pretreated mice suggests that the rate of metabolism of 2-iodo-3,7,8-TCDD was induced by pretreatment, no data were provided on the effect of this pretreatment of body weight and composition, which may in turn alter the rate of elimination. 2,3,7,8-TCDD pretreatment may also alter deiodinase activity. Furthermore, in vivo and in vitro studies suggest that the autoinduction of 2,3,7,8-TCDD metabolism may not occur under these conditions. Kedderis et al. (1991b) and Curtis et al. (1990) found no autoinduction of 2,3,7,8-TCDD metabolism and biliary excretion in the rat. In addition, Shen and Olson (1987) found that while 2,3,7,8-TCDD pretreatment of C57BL/6J mice increased the uptake of 2,3,7,8-TCDD by hepatocytes in suspension culture, pretreatment did not increase the rate of metabolism of

2,3,7,8-TCDD by hepatocytes. Therefore, this PB-PK model may not accurately describe the metabolism of 2,3,7,8-TCDD for exposures that result in varying degrees of induction of the hepatic monooxygenase system. While the dose-dependent pharmacokinetics of 2,3,7,8-TCDD may not include autoinduction of 2,3,7,8-TCDD metabolism, this may not be the case for other CDDs, BDDs, CDFs, BDFs, PCBs, and PBBs. For example, the autoinduction of metabolism has been reported for CDFs (Brewster and Birnbaum, 1987; McKinley et al., 1993; Olson et al., 1994).

Andersen et al. (1993) recently described a receptor-mediated PB-PK model for the tissue distribution and enzyme-inducing properties of 2,3,7,8-TCDD. The data used for this analysis were from two previously published studies with Wistar rats (Abraham et al., 1988; Krowke et al., 1989). The model was used to examine the tissue disposition of 2,3,7,8-TCDD and the induction of both a dioxin-binding protein (presumably cytochrome P-4501A2) and cytochrome P-4501A1.

Kohn et al. (1993) recently developed a mechanistic model of the effects of dioxin on gene expression in the rat liver (referred to as the NIEHS model). The model includes the tissue distribution of 2,3,7,8-TCDD in the rat and its effect on the concentrations of CYP1A1 and CYP1A2 and the effects of 2,3,7,8-TCDD on the Ah, estrogen, and EGF receptors over a wide 2,3,7,8-TCDD dose range. Experimental data from Tritscher et al. (1992) and Sewall et al. (1992) were incorporated into the NIEHS model. Female Sprague-Dawley rats were injected with an initiating dose of diethylnitrosamine, and after 20 days, the rats were exposed biweekly to 2,3,7,8-TCDD in corn oil by gavage at doses equivalent to 3.5-125 ng/kg/day for 30 weeks. The NIEHS model predicts a linear relationship between administered dose and the concentration in the liver over this dose range, which is in agreement with the data of Tritscher et al. (1992). The biochemical response curves for all these proteins were hyperbolic, indicating a proportional relationship between target tissue dose and protein concentration at low administered doses of 2,3,7,8-TCDD.

A fugacity-based PB-PK model for the elimination of 2,3,7,8-TCDD from humans was developed by Kissel and Robarge (1988). Transport within the body was assumed to be perfusion-limited (flow-limited). 2,3,7,8-TCDD was assumed to be uniformly distributed within each tissue or fluid phase, and tissue levels were considered to be in equilibrium with

exiting fluids (blood, bile, urine). 2,3,7,8-TCDD appears to be poorly metabolized in humans, thus reducing the necessity of modeling the fate of metabolites. 2,3,7,8-TCDD also seems to exhibit fugacity-based partitioning behavior in humans as evidenced by relatively constant lipid-based tissue distribution (Leung et al., 1990c; Ryan et al., 1987), although this is not the case in rodents (Leung et al., 1988, 1990a). With a daily human background intake of 2,3,7,8-TCDD in North America of ~50 pg/day (Travis and Hattemer-Frey, 1987), the steady-state adipose tissue concentration predicted by the model, assuming no metabolism, was 7.7 ppt. This is similar to the lipid-based blood tissue levels reported in the general population with no known unusual exposure. The model was also used to predict the elimination of 2,3,7,8-TCDD from Ranch Hand Vietnam veterans. The model simulation assumed a background exposure of 50 pg/day and no metabolism. Under these conditions, apparent half-lives of 4.4, 5.2, 5.9, 7.2, 9.1, and 20 years were estimated for individuals with adipose tissue concentrations of 100, 50, 30, 20, 15, and 10 ppt, respectively. The model predicted half-lives that are similar to the experimental value of 7.1 years, based on analysis of 2,3,7,8-TCDD in blood lipids of veterans with adipose burdens greater than 10 ppt (Pirkle et al., 1989) (see Table 1-10). The apparent half-lives derived from the model increased as the adipose tissue concentrations approached the steady-state level associated with background exposure. Ryan and Masuda (1991) also reported a similar relationship for CDFs, with experimentally derived half-lives increasing in individuals with lower body burdens of the compounds. Finally, the model was also found to approximate the elimination of 2,3,7,8-TCDD from one volunteer as reported by Poiger and Schlatter (1986). Taken together, the comparisons described above suggest that a fugacity-based PB-PK model for 2,3,7,8-TCDD in humans can provide one method for describing the elimination of 2,3,7,8-TCDD from humans.

Kedderis et al. (1993b) recently developed a PB-PK model for 2,3,7,8-TBDD in the rat. The model is based on previously developed physiologically based models for 2,3,7,8-TCDD (Leung et al., 1990a; Poland et al., 1989b) and uses published data on the disposition of a single exposure to 2,3,7,8-TBDD at a dose of 1 or 100 nmol/kg, intravenous (Kedderis et al., 1991a,b) and dermal disposition data (Jackson et al., 1993). In the model, the dose- and time-dependent accumulation in the liver was attributed to specific binding of 2,3,7,8-

TBDD with the inducible protein, CYP1A2. The model also includes diffusion-limited tissue uptake of 2,3,7,8-TBDD, transluminal excretion of parent compound via the gut into the feces, growth of tissue compartments, and a separate skin compartment. This model provides further validation of the model structure originally developed to describe important dispositional determinants for 2,3,7,8-TCDD.

A five-compartment (blood, liver, fat, skin, muscle), flow-limited physiological model was developed to describe the tissue distribution and excretion of 2,3,7,8-TCDF-derived material in rats, mice, and monkeys (King et al., 1983), based on experimental data reported earlier (Birnbaum et al., 1980, 1981; Decad et al., 1981b). Partition coefficients (tissue/blood distribution ratios) and metabolic clearances were estimated from in vivo experimental data and are summarized in Table 1-12. All pharmacokinetic parameters for 2,3,7,8-TCDF were based on in vivo data after a single intravenous exposure at a dose of 0.1 μmol/kg (30.6 μg/kg). Therefore, the model is limited in not considering the potential dose-related distribution and excretion of 2,3,7,8-TCDF. Recent studies indicate that 2,3,7,8-TCDF is able to induce its own rate of metabolism and biliary excretion at higher doses (McKinley et al., 1993; Olson et al., 1994). This model will need to be revised as additional data on the dose-related distribution and excretion of 2,3,7,8-TCDF become available.

PB-PK models are primarily limited by the availability of congener- and species-specific data that accurately describe the dose- and time-dependent disposition of 2,3,7,8-TCDD and related compounds. The pharmacokinetic parameters summarized in Tables 1-11 and 1-12 were derived from available in vivo and in vitro experimental data. As additional data become available, particularly on the dose-dependent disposition of these compounds, more accurate models can be developed. In developing a suitable model in the human, it is also important to consider that the half-life estimate of 7.1 years for 2,3,7,8-TCDD was based on two serum values taken 5 years apart, with the assumption of a single compartment, first-order elimination process (Pirkle et al., 1989). It is likely that the excretion of 2,3,7,8-TCDD in humans is more complex, involving several compartments, tissue-specific bonding proteins, and a continuous daily background exposure. Furthermore, changes in body weight

Table 1-12. Pharmacokinetic Parameters for 2,3,7,8-TCDF Used in the PB-PK Model Described by King et al. (1983)

	C57BL/6J Mouse	DBA/2J Mouse	Fischer 344 Rat	Rhesus Monkey
Partition Coefficients				
Liver	130	100	100	30
Fat	25	40	35	30
Skin	8	12	4	7
Muscle	2	4	2	2
Clearances				
Metabolism	0.07	0.06	1.0	2.25
Km (mL/minute/kg)	2.8	2.4	4.0	0.45
Metabolism excretion ratio K_k/K_L[a]	0.14	0.27	0.03	0.19

[a]Urinary clearance/biliary clearance.

and body composition should also be considered in developing PB-PK models for 2,3,7,8-TCDD and related compounds in humans.

An empirical model of dioxin (toxic equivalents) disposition in animals and humans has also been recently developed by Carrier and Brodeur (1991). The kinetic analysis begins with the observation that the tissue distribution of dioxin-like HPAH in humans and in animals is dose dependent (or, alternately, body-burden dependent). As total body burden of TCDD equivalents increases, the proportion of the body burden associated with the liver increases toward a maximum value. The data are then analyzed with an empirical, saturable binding isotherm equation:

$$\text{liver fraction}\,(f_H) = f_{max}C_{body}/(K_d + C_{body})$$

If the body burden—C_{body} (μg/kg)—is considered a surrogate for liver concentration, this equation can be loosely interpreted as the induction of binding species in the liver as dose increases. In the analyses, K_d was found to be very similar for people and experimental animals, indicating similar protein induction dynamics in various animal species. This model, however, is not physiologicallybased and the terms C_{body} and f_{max} are difficult to interpret in biological terms. In working with different isomers, f_{max} and K_d values vary somewhat, presumably due to binding affinities in the liver.

This empirical model is successful in providing a description that "fits" the observed data in various species. It still is largely a fitting exercise to a particular equation, not an examination of biology by computer modeling. In addition, there are at least two assertions that seem incorrect. First is the assumption that the limit of the hepatic fraction at very low doses is zero. It seems more likely that the limit is some finite value, determined by liver partitioning of dioxin and the binding parameters of the Ah receptor and the dioxin binding species in the liver in the linear, low-dose region. Second is the assumption that metabolism of dioxin becomes saturated with the maximum induction of liver sequestration of dioxin. There is no justification for this at present. Nevertheless, the model indicates clearly that with respect to dosimetry and induction of hepatic binding species for dioxin, people and

rodents are very similar. Furthermore, the empirical model of Carrier and Brodeur (1991) is generally consistent with the PB-PK models.

1.5. PHARMACOKINETICS IN SPECIAL POPULATIONS

1.5.1. Pregnancy and Lactation (Prenatal and Postnatal Exposure of Offspring)

The distribution and excretion of [^{14}C]-2,3,7,8-TCDD (30 μg/kg) and [^{14}C]-2,3,7,8-TCDF (800 μg/kg) were studied in pregnant C57BL/6N mice after oral exposure on gestation day 11 (Weber and Birnbaum, 1985). The distribution and excretion of 2,3,7,8-TCDD and 2,3,7,8-TCDF in pregnant mice were similar to those of males of the same strain (Gasiewicz et al., 1983a; Decad et al., 1981b) (see Tables 1-6 and 1-9), although elimination rates were higher in the pregnant mice for both congeners. For 2,3,7,8-TCDD, liver, urinary, and fecal elimination was 3.0, 3.4, and 14.4 times faster than that reported for males. For 2,3,7,8-TCDF, liver, urinary, and fecal elimination was 1.3, 1.8, and 1.8 times faster than that observed for males. Elimination data from pregnant mice were based on only three time points (gestation days 12, 13, and 14) and thus represent only rough estimates. In addition, the greater fecal excretion could have been due to incomplete absorption of 2,3,7,8-TCDD after oral exposure. Although these results need further substantiation, it is conceivable that the sex of the animal, pregnancy, and the route of exposure could have a significant impact on the pharmacokinetics of these compounds.

In a related study, Krowke (1986) compared the 2,3,7,8-TCDD concentrations in the liver of pregnant and nonpregnant NMRI mice exposed subcutaneously to 12.5 or 25 nmol/kg/day on gestation days 9-11. At 7 days after exposure to the lower dose, the hepatic 2,3,7,8-TCDD concentrations were 7 and 32 ng/g in pregnant and nonpregnant mice, respectively. At the higher exposure, 5.5 times lower concentrations of 2,3,7,8-TCDD were found in the livers of pregnant animals on gestation day 18. A similar effect on hepatic 2,3,7,8-TCDD levels was observed also in combined exposure, which contained 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, or 2,3,7,8-TCDF. The decreased hepatic levels of 2,3,7,8-TCDD in pregnant mice are consistent with the Weber and Birnbaum (1985) observation of more rapid elimination of 2,3,7,8-TCDD in pregnant mice. Further investigations are

necessary to better characterize the apparently significant effects of pregnancy on the disposition of 2,3,7,8-TCDD and related compounds.

Weber and Birnbaum (1985) also investigated the distribution of [^{14}C]-2,3,7,8-TCDD (30 μg/kg) and [^{14}C]-2,3,7,8-TCDF (800 μg/kg) to the embryos of pregnant C57BL/6N mice after oral exposure on gestation day 11. On gestation days 12, 13, and 14, the percent of the maternal dose in the embryo remained constant at 0.032-0.037%/embryo, while the concentrations in the embryo were 0.34, 0.17, and 0.15% of the dose/g embryo, respectively. Embryos had approximately 11-fold higher concentrations of 2,3,7,8-TCDD than 2,3,7,8-TCDF when exposed on a percent of total dose/g tissue basis. This may be due to the more rapid metabolism and excretion of 2,3,7,8-TCDF compared with 2,3,7,8-TCDD. Assuming that all radioactive material found in embryos was parent compound, at most 2.6 ng (8 pmol) of 2,3,7,8-TCDD and 6.4 ng (21 pmol) of 2,3,7,8-TCDF/g tissue were detected under these conditions.

The transfer of [^{14}C]-2,3,7,8-TCDD to the embryo during early gestation was assessed in NMRI mice given a dose of 25 μg/kg by intraperitoneal injection on either days 7, 8, 9, 10, 11 or 13 of gestation (Nau and Bass, 1981). The mice were sacrificed after 48 hours, and 2,3,7,8-TCDD concentrations were determined by liquid scintillation counting of solubilized tissue and by GC-ECD and GC/MS. Similar results were given by these methods, suggesting that 2,3,7,8-TCDD derived [^{14}C] in maternal and embryonic tissue was the parent compound. The maternal liver contained from 4% to 8% of the dose/g, or 40-80 ng/g. 2,3,7,8-TCDD in embryonic tissue from gestation days 11-15 ranged from 0.04% to 0.1% of the dose/g, or 0.4-1.0 ng/g. In contrast, higher levels were found earlier in gestation, with 10 ng/g embryo on gestation day 9 and 2 ng/g on day 10. The higher levels may be related to placentation, which occurs at approximately gestation days 10–11 in this mouse strain. The affinity of fetal liver for 2,3,7,8-TCDD was relatively low, as compared to maternal liver; however, 2,3,7,8-TCDD levels in fetal livers were 2 to 4 times higher than levels in other fetal organs. Nau and Bass (1981) also attempted to correlate 2,3,7,8-TCDD levels in the fetuses with the observed incidence of cleft palate. Three groups of mice were given either a single intraperitoneal exposure to 25 μg/kg 2,3,7,8-TCDD on gestation day 7 or 10 or 5 μg/kg/day, intraperitoneally, on gestation days 7-11. On gestation day 13,

2,3,7,8-TCDD concentrations in maternal tissues were very similar in the three exposure groups. At day 13, however, the embryo contained 0.038±0.011% (0.36 ng/g), 0.096±0.027% (0.92 ng/g), and 0.12±0.05% (1.1 ng/g) of the dose (mean±SD) in the 7-, 10-, and 7- to 11-day exposure groups, respectively. Cleft palate incidence on gestation day 18 was 16%, 84%, and 65% for the 7-, 10-, and 7- to 11-day exposure groups, respectively. Although further studies are needed, these results suggest that cleft palate incidence is generally related to the 2,3,7,8-TCDD concentration in the embryo. In a related study, Couture et al. (1990) found that gestation day 12 was the peak period of sensitivity for 2,3,7,8-TCDD-induced cleft palate in C57BL/6N mice; however, tissue levels were not investigated.

In the same laboratory, Abbott et al. (1989) investigated the distribution of 2,3,7,8-TCDD in the C57BL/6N mouse fetus following maternal exposure on gestation day 11 to 30 μg/kg. 2,3,7,7-TCDD was detected in the gestation day-11 embryo at 3 hours postexposure and was equally distributed between the embryonic head and body. At 72 hours postexposure, 0.035% of the total dose was in fetal tissues, and 1% of the 2,3,7,8-TCDD in the fetus (1.4-3.5 pg) was found in the palatal shelf.

Krowke (1986) also measured the concentration of 2,3,7,8-TCDD in the placenta, amniotic fluid, and fetus of NMRI mice exposed to 2.5 nmol/kg by subcutaneous injection on days 9-11 of gestation. Similar concentrations of 2,3,7,8-TCDD were observed in the placenta, amniotic fluid, and fetus (~0.5 ng/g) on day 16 of gestation. Fetal liver 2,3,7,8-TCDD concentrations were at least five times greater than in other fetal tissue. Krowke (1986) reported slightly lower 2,3,7,8-TCDD levels in the fetal head relative to other extrahepatic fetal tissue, while Weber and Birnbaum (1985) found a slightly higher 2,3,7,8-TCDD concentration in the head relative to other extrahepatic fetal tissue.

Nau et al. (1986) investigated the transfer of 2,3,7,8-TCDD via the placenta and milk in NMRI mice exposed to 25 μg/kg on day 16 of gestation. The authors confirmed the relatively low fetal tissue levels with prenatal exposure to 2,3,7,8-TCDD (Nau and Bass, 1981) and found that postnatally 2,3,7,8-TCDD was transferred efficiently to mouse neonates and offspring by lactating mothers. During the first 2 postnatal weeks, the pups were given doses of 2,3,7,8-TCDD via the milk that were, on a body-weight basis, similar to those that

had been administered prenatally to their mothers. 2,3,7,8-TCDD levels in the tissue of lactating mothers decreased within the first 3 postnatal weeks by two to three orders of magnitude to reach levels that were only ~2% of the corresponding levels in the pups that these mothers had nursed. Thus in mice, excretion into milk represents a major pathway for maternal elimination of 2,3,7,8-TCDD and for the subsequent exposure of pups.

The disposition of 2,3,7,8-TCDD in rat pups was assessed after the prenatal (via placental transfer) and postnatal (via milk) exposure from pregnant Wistar rats given a single dose of 3, 30, or 300 ng/kg, subcutaneously, on day 19 of gestation (Korte et al., 1990). Lactation resulted in the rapid elimination of 2,3,7,8-TCDD from maternal tissues, with the half-life of 2,3,7,8-TCDD in the liver of lactating rats estimated to be ~7 days. This compares to a half-life of 13.6 days in the liver of nonlactating rats (Abraham et al., 1988). At postnatal day 7, exposure via the milk resulted in pup liver 2,3,7,8-TCDD concentrations that were greater than the corresponding levels in maternal liver. In cross-fostering experiments, the concentrations of 2,3,7,8-TCDD in the liver of offspring at postnatal day 7 were 0.47, 2.59, and 4.16 ng/g in the 300 ng/kg groups exposed through the placenta only, via the milk only and through the placenta and via the milk, respectively. These results support the earlier observations that the placental transfer of 2,3,7,8-TCDD in rats and mice is relatively limited compared with the efficient transfer via maternal milk.

Van den Berg et al. (1987b) investigated the transfer of CDDs and CDFs to fetal and neonatal rats. Prenatal exposure of the fetus was assessed in pregnant Wistar rats fed a diet containing a fly ash extract from a municipal incinerator on days 10-17 of gestation. Postnatal exposure of 10-day-old pups was assessed through feeding lactating mothers the same contaminated diet for the first 10 days after delivery. Although the fly ash extract contained almost all of the 136 tetra- to octa-CDDs and -CDFs, only 17 CDD and CDF congeners were detected as major compounds in the tissue of fetuses, pups, and dams. All of the congeners were 2,3,7,8-substituted with the exception of 2,3,4,6,7-PeCDF. 2,3,7,8-TCDD had the highest retention (0.0092% of the dose/g) in the fetus, while 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and hepta- and octa-CDDs and -CDFs were not detected in the fetus. In the liver of offspring, the highest retention was found for 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and the three 2,3,7,8-substituted HxCDDs (0.74-1.13% dose/g). The 2,3,7,8-substituted

penta- and hexachlorinated congeners showed the highest retention in the livers of dams (2.05-5.17% of dose/g liver), while 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, and 2,3,4,6,7-PeCDF had the lowest retention. A linear relationship was found between the retention of CDDs and CDFs in the livers of pregnant and lactating rats. Furthermore, a linear relationship was found between the retention of CDDs and CDFs in the livers of the lactating rats and livers of the offspring.

In a related study, Hagenmaier et al. (1990) investigated the transfer of CDDs and CDFs through the placenta and via milk in a marmoset monkey. A defined mixture of CDDs and CDFs was given as a single subcutaneous injection to a pregnant marmoset monkey at the end of the organogenesis period (week 10 of gestation, 11 weeks prior to delivery). Transfer of CDDs and CDFs through the placenta was investigated in a newborn 1 day after birth, and transfer through the placenta and via milk was assessed in an infant of the same litter after a lactation period of 33 days. Tissue concentrations of the offspring were compared with those of the mother at the end of the lactation period and with data from other adult marmosets obtained at this time of maximum absorption (1 week after injection) and 6 weeks after injection. Deposition of CDDs and CDFs into the newborn liver was very low, suggesting very little transplacental transport and hepatic accumulation of these compounds. 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD were found at the highest concentration in the liver of the newborn ($\sim$0.15% of dose/g). For all other congeners, the concentrations in the liver of the newborn were $<$10% of the corresponding concentrations in adults. In contrast to liver, concentrations of 2,3,7,8-substituted congeners in the adipose tissue of the newborn were at least 33% of the levels in adults, and in the case of OCDD and OCDF, levels were threefold higher in the newborn than in the adult. The adipose tissue/liver concentration ratios for 2,3,7,8-substituted congeners in the newborn ranged from 2.2 for 1,2,3,4,6,7,8-HpCDF to 10.9 for 2,3,7,8-TCDF. Furthermore, the concentration of these congeners in the newborn was highest in the adipose tissue, followed by the skin and liver. This is in contrast to the relative distribution in the adult where the liver generally contains the highest levels of these congeners. The results indicate that hepatic concentrations in the fetus may not be representative of the rate of placental transfer of CDDs and CDFs. In the marmoset monkey, substantial placental transfer into fetal adipose tissue can be observed for

most of the 2,3,7,8-substituted congeners during the fetal period. As expected from rodent studies, the transfer of CDDs and CDFs via mothers' milk was considerable, resulting in hepatic concentrations of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, and 1,2,3,6,7,8-HxCDD in the suckled infant (postnatal day 33) higher than those in the dam. The hepatic concentration of 2,3,7,8-TCDD in the 33-day-old infant was ~0.9% of the dose/g tissue. Transfer of hepta- and octa-CDDs and CDFs to the suckled infant was rather low, only ~10% of the levels in the dam. When total exposure of the mother and offspring at the end of the 33-day nursing period was assessed in terms of I-TE factors (U.S. EPA, 1989), the liver of the mother contained 2,494 pg I-TE/g, while the offspring liver contained 2,022 pg I-TE/g. This approach is necessary to assess total exposure due to the congener-specific transfer via lactation.

The pre- and postnatal transfer of 2,3,7,8-TCDD to the offspring of rhesus monkeys was investigated by Bowman et al. (1989). Animals were fed a diet containing 2,3,7,8-TCDD at concentrations of 5 or 25 ppt for ~4 years and were on a 2,3,7,8-TCDD-free diet for ~18 months prior to parturition. Maternal 2,3,7,8-TCDD levels (mean±SE) in adipose tissue were 49±11 (n=7) and 173±81 (n=3) ppt in the 5 and 25 ppt groups, respectively. Corresponding levels in the adipose tissue of offspring at weaning (4 months) were 187±58 and 847±298 ppt in the 5 and 25 ppt groups, respectively. From these data, a 2,3,7,8-TCDD BCF of 4.29 was estimated from mother to nursing infant. This value is similar to that observed for 2,3,7,8-TCDD in the marmoset monkey (Hagenmaier et al., 1990). The milk of the rhesus monkeys in the 25 ppt group contained from 4 to 14 ppt of 2,3,7,8-TCDD, which corresponds to 150-500 ppt on a lipid basis. The authors calculated that the three mothers in the 25 ppt group excreted from 17% to 44% of their 2,3,7,8-TCDD body burden by lactation. They also concluded that the results are generally consistent with overall triglyceride movement as mediating the excretion of 2,3,7,8-TCDD in milk.

In a subsequent study, Bowman et al. (1990) reported the relative persistence of 2,3,7,8-TCDD in the offspring of rhesus monkeys that were exposed earlier to 5 or 25 ppt of 2,3,7,8-TCDD in the diet. The concentration of 2,3,7,8-TCDD in adipose tissue was measured in offspring at ~4-5, 12, and 24 months of age. The decrease of 2,3,7,8-TCDD levels in adipose tissue of seven young monkeys departed somewhat from first-order, single-

compartment kinetics, but with the limited data and an assumption of first-order kinetics, a half-life of 121 days was estimated. When the data were adjusted within each animal for body weight gain and for average fat content at each age, the adjusted data apparently followed first-order, single-compartment kinetics, with a half-life of ~181 days. Thus, young monkeys apparently eliminate 2,3,7,8-TCDD from adipose tissue at a faster rate than adult rhesus monkeys, which had individual half-lives ranging from 180 to 550 days (Bowman et al., 1989).

Furst et al. (1989) examined the levels of CDDs and CDFs in human milk and the dependence of these levels on the period of lactation. The mean concentrations of CDDs in human milk (on a fat basis) ranged from 195 ppt for OCDD to 2.9 ppt for 2,3,7,8-TCDD, with the levels of the other congeners decreasing with decreasing chlorination. This is in contrast to the generally lower levels of CDFs in human milk, which range from 25.1 ppt for 2,3,4,7,8-PeCDF to 0.7 ppt for 1,2,3,7,8-PeCDF. An evaluation of the CDD and CDF levels in relation to the number of breast-fed children found that the concentrations in milk generally decreased with the greater number of children. The CDD and CDF levels in milk from mothers nursing their second child are on average 20-30% lower than those for mothers breast-feeding their first child. CDD and CDF levels were also analyzed in one mother over a period of 1 year after delivery of her second baby to assess the effect of duration of lactation. After breast-feeding for 1 year, the mother had CDD and CDF levels that were 30-50% of the starting concentration. Levels in milk fat (ppt) at 1, 5, and 52 weeks after delivery were 251, 132, and 119 for OCDD; 7.9, 5.9, and 1.4 for 2,3,7,8-TCDD; and 33.1, 24.5, and 10 for 2,3,4,7,8-PeCDF, respectively. The results suggest a more rapid mobilization of CDDs and CDFs and excretion into human milk during the first few weeks postpartum. Although further studies are necessary, the limited data suggest that there are time-dependent, isomer-specific differences in the excretion of CDDs and CDFs in human milk.

Although data are more limited for the coplanar PCBs, 3,3′,4,4′-TCB, 3,3′,4,4′,5-PeCB, and 3,3′,4,4′,5,5′-HxCB have been detected in human milk from Swedish mothers, at concentrations of 16-32, 72-184, and 46-129 ppt on a fat basis, respectively (Noren et al., 1990). Therefore, lactation appears to be an effective means for the excretion of coplanar

PCBs from mothers and a major source of postnatal exposure of nursing infants. Since 3,3',4,4',5-PeCB and other coplanar PCBs are present in human milk at concentrations up to 60-fold higher than 2,3,7,8-TCDD, it is important to consider the relative toxic potency of these dioxin-like compounds and their potential health impact on nursing infants.

1.5.2. Aging

The influence of aging on the intestinal absorption of 2,3,7,8-TCDD was studied in 13-week-, 13-month-, and 26-month-old (senescent) male Fischer 344 rats (Hebert and Birnbaum, 1987). Absorption was measured by an in situ intestinal recirculation perfusion procedure. When absorption was calculated in terms of ng 2,3,7,8-TCDD absorbed/g mucosal dry weight/hour, the decrease between the senescent rats and the two younger age groups, from 544 ng/g/hour (young) to 351 ng/g/hour (senescent), was not statistically significant ($p < 0.05$). The results indicate that, as with other molecules that depend on diffusion for their absorption, aging does not affect the intestinal absorption of 2,3,7,8-TCDD.

Banks et al. (1990) studied the effect of age on the dermal absorption and disposition of 2,3,7,8-TCDD and 2,3,4,7,8-PeCDF in male Fischer 344 rats. When rats were administered the same dose per body weight, dermal absorption of 2,3,7,8-TCDD, at 3 days after exposure, decreased from $17.7 \pm 2.7\%$ (mean$\pm$SD) to $5.6 \pm 2.5\%$ of the administered dose in 10- and 36-week-old rats, respectively. Dermal absorption in the 96-week-old rats was similar to that of the 36-week-old rats. Dermal absorption of 2,3,4,7,8-PeCDF also decreased from $22.2 \pm 0.2\%$ to $14.7 \pm 3.8\%$ of the administered dose in 10- and 36-week-old rats, respectively. Dermal absorption of both compounds was also decreased in older rats given the same total dose per surface area. Older animals may have decreased blood flow in the upper dermis, which will decrease the clearance of these compounds from the application site. Potential age-related changes in the intercellular stratum corneum lipids may also play a role in the decreased dermal absorption observed in older animals. Changes in the percentage of the administered dose detected in various depots reflected age-related changes in dermal absorption, while age-related changes in the tissue distribution of the absorbed dose reflected changes in the total mass of these tissues at various ages. Overall elimination of the

absorbed dose was not affected by age. Although this investigation was conducted using a lipophilic solvent system and an animal model with skin that is more permeable than human skin, the results suggest that systemic bioavailability after dermal exposure to 2,3,7,8-TCDD or 2,3,4,7,8-PeCDF may be reduced in older age groups.

In a similar study, absorption, tissue distribution, and elimination were examined 72 hours after dermal application of a lower dose of 200 pmol (111 pmol/cm^2) 2,3,7,8-TCDD to weanling (3-week-old), juvenile (5-week-old), pubescent (8-week-old), young adult (10-week-old), and middle-aged (36-week-old) rats (Anderson et al., 1993). Dermal absorption using 2acetone as vehicle was greatest in 3-week-old rats (129 pmol; 64% of the administered dose), decreasing to ~80 pmol (40%) in 5-, 8-, and 10-week-old rats and to 45 pmol (22%) in 36-week-old rats. The results indicate that 2,3,7,8-TCDD is absorbed to a greater degree through skin of very young animals and that a significant decrease in potential for systemic exposure may occur during maturation and again during aging.

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2. MECHANISM(S) OF ACTION*

2.1. INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is the prototype for a class of halogenated aromatic hydrocarbons that produce similar patterns of toxicity and appear to have a common mechanism of action although they differ in potency (Poland and Knutson, 1982; Safe, 1986). Because it is the most potent, TCDD has been studied much more extensively than other structurally related compounds (Figure 2-1). TCDD achieved notoriety in the 1970s, when it was discovered to be a contaminant in the herbicide Agent Orange and was shown to produce birth defects in rodents. Subsequently, dioxin has continued to generate concern because of its widespread distribution, its persistence as an environmental contaminant, its accumulation within the food chain, and its toxic potency. In animals, TCDD elicits a wide range of biological effects, including alterations in metabolic pathways, immunological changes, teratogenic effects, and neoplasia (Poland and Knutson, 1982; Safe, 1986). In humans, the dioxin can produce the skin condition known as chloracne; the possibility that it also produces cancer and birth defects is a particular public health concern. Many individuals have been exposed to TCDD, primarily from dietary sources, although occupational and accidental exposures have also occurred. TCDD is a poor substrate for detoxification systems, such as the microsomal cytochrome P450 enzymes, which oxygenate other lipophilic compounds during their metabolic processing to inactive derivatives. Because of its relative resistance to metabolism, TCDD tends to persist in the body, and its half-life in humans is of the order of 7 to 10 years (Pirkle et al., 1989). Therefore, dioxin tends to accumulate in human tissues over time, raising the concern that repeated exposures, even to "low" concentrations, may evoke adverse health effects. Epidemiological studies have not produced a well-defined estimate of the health risk that dioxin poses to humans (Bailar, 1991), and there has been hope that knowledge of the mechanism of dioxin action may shed additional light on this issue (Gallo et al., 1991).

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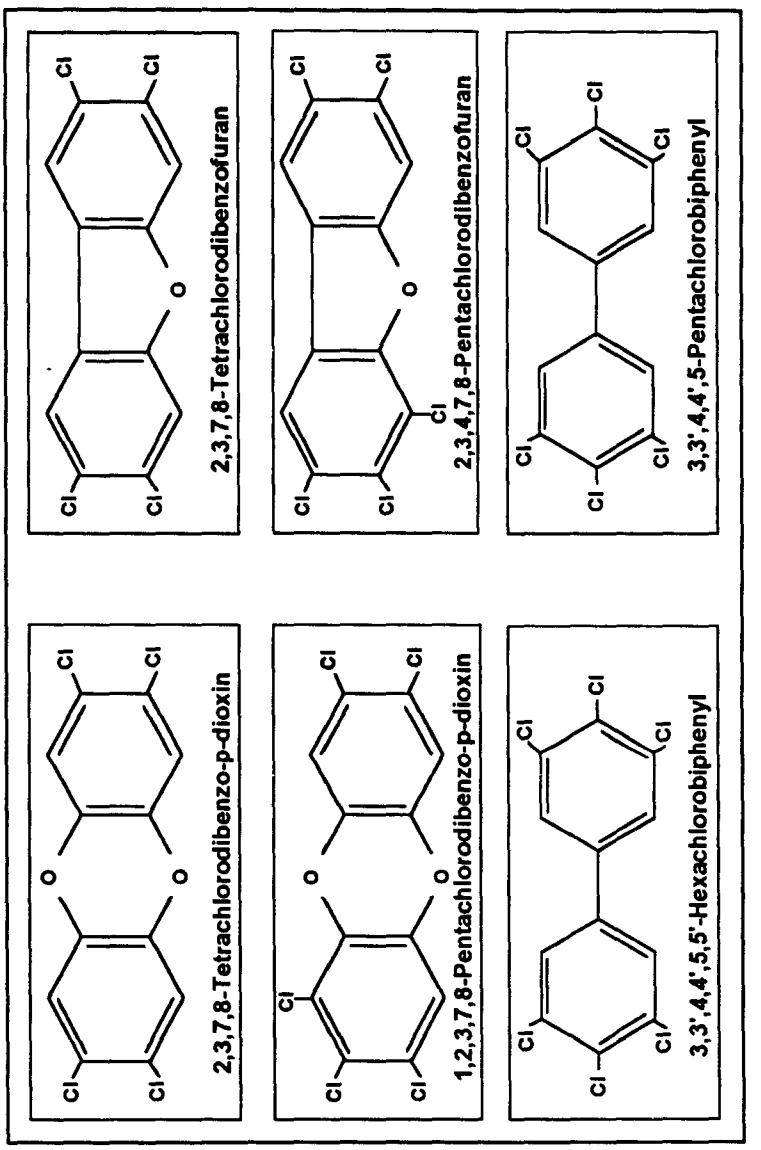

Figure 2-1. Chemical structure of dioxin and similar compounds.

mechanism of dioxin action may shed additional light on this issue (Gallo et al., 1991). Mechanistic studies can reveal the biochemical pathways and types of biological events that contribute to dioxin's adverse effects. For example, TCDD acts via an intracellular protein (the Ah receptor), which is a ligand-dependent transcription factor that functions in partnership with a second protein (known as Arnt); therefore, from a mechanistic standpoint, TCDD's adverse effects appear likely to reflect sustained alterations in gene expression. Mechanistic studies also indicate that several proteins contribute to TCDD's gene regulatory effects and that the response to TCDD probably involves a relatively complex interplay between multiple genetic and environmental factors. Such mechanistic information imposes constraints on the possible models that can plausibly account for TCDD's biological effects and, therefore, on the assumptions used during the risk assessment process. Mechanistic knowledge of dioxin action may also be useful in other ways. For example, knowledge of genetic polymorphisms that influence TCDD responsiveness may allow the identification of individuals at particular risk from exposure to dioxin. In addition, mechanistic knowledge of the biochemical pathways that are altered by TCDD may identify novel targets for the development of drugs that can antagonize dioxin's adverse effects.

As described below, biochemical and genetic analyses of the mechanism by which dioxin induces CYP1A1 gene transcription have revealed the outline of a novel regulatory system whereby a chemical signal can alter the expression of specific mammalian genes. Future studies of dioxin action have potential to provide additional new insights into mechanisms of mammalian gene regulation that are of relatively broad interest. Additional perspectives on dioxin action can be found in several recent reviews (Nebert et al., 1991; Silbergeld and Gasiewicz, 1989; Skene et al., 1989; Couture et al., 1990; Landers and Bunce, 1991; Johnson, 1992; Poellinger et al., 1992; Birnbaum, 1993).

2.2. THE "RECEPTOR" CONCEPT

The idea that a drug, hormone, neurotransmitter, or other chemical produces a physiological response by interacting with a specific cellular target molecule, i.e., a "receptor," evolved from several observations. First, many chemicals elicit responses that are restricted to specific tissues. This type of observation implied that the responsive tissue

(i.e., the adrenal cortex) contained a "receptive" component whose presence was required for the physiologic effect (i.e., cortisol secretion). Second, many chemicals are quite potent. For example, nanomolar concentrations of numerous hormones and growth factors elicit biological effects. This type of observation suggested that the target cell contained a site(s) to which the chemical could bind with high affinity. Third, stereoisomers of some chemicals (i.e., catecholamines, opioids) differ by orders of magnitude in potency. This type of observation indicated that the molecular shape of the chemical strongly influenced its biological activity; this, in turn, implied that the binding site on or in the target cell also had a specific, three-dimensional configuration. Together, these types of observations predicted that the biological responses to some chemicals involve stereospecific, high-affinity binding of the chemical to specific receptor sites located on or in the target cell.

The availability of compounds of high specific radioactivity permitted quantitative analyses of their binding to tissue components in vitro. To qualify as a potential "receptor," a binding site for a given chemical must satisfy several criteria: (1) the binding site must be saturable (i.e., there should be a limited number of binding sites per cell); (2) the binding should be reversible; (3) the binding affinity measured in vitro should be consistent with the potency of the chemical observed in vivo; (4) if the biological response exhibits stereospecificity, so should the in vitro binding, (5) for a series of structurally related chemicals, the rank order for binding affinity should correlate with the rank order for biological potency; and (6) tissues that respond to the chemical should contain binding sites with the appropriate properties.

The binding of a chemical ("ligand") to its cognate receptor is assumed to obey the law of mass action, that is, it is a bimolecular, reversible interaction. The concentration of the liganded, or occupied, receptor [RL] is a function of both the ligand concentration [L] and the receptor concentration [R]:

$$[L] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RL]$$

It follows that the fractional occupancy of the receptor $\frac{[RL]}{[R_{tot}]}$ is a function of the ligand concentration [L] and a constant K_D, which is a measure of the binding affinity of the ligand for the receptor:

$$\frac{[RL]}{[R_{tot}]} = \frac{[L]}{K_D + [L]}$$

Therefore, the relationship between receptor occupancy and ligand concentration is hyperbolic. At low ligand concentrations (where $[L] << K_D$), a small increase in [L] produces an approximately linear increase in fractional receptor occupancy. At high ligand concentration (where $[L] >> K_D$), the fractional occupancy of the receptor is already very close to 1, and a small increase in [L] produces no further meaningful increase in receptor occupancy, because the binding sites are essentially saturated.

Ligand binding constitutes only one aspect of the receptor concept. By definition, a receptor mediates a response, and the functional consequences of the ligand-receptor binding represent an essential aspect of the receptor concept. Receptor theory attempts to quantitatively relate ligand binding to biological response. The classical "occupancy" model of Clark (1933) postulated (1) that the magnitude of the biological response is directly proportional to the fraction of the occupied receptors, and (2) that the response is maximal when all receptors are occupied. However, analyses of numerous receptor-mediated effects indicate that the relationship between receptor occupancy and biological effect is not as straightforward as Clark envisioned. In certain cases, no response occurs even when there is some receptor occupancy, a threshold phenomenon that reflects the biological "inertia" of the response (Ariens et al., 1960). In other cases, a maximal response occurs well before all receptors are occupied, a phenomenon that reflects receptor "reserve" (Stephenson, 1956). Therefore, one cannot simply assume that the relationship between fractional receptor occupancy and biological response is linear. Furthermore, for a ligand (such as TCDD) that elicits multiple receptor-mediated effects, one cannot assume that the binding-response relationship for one, simple effect (such as enzyme induction) will necessarily be identical to the binding-response relationship for a different, more complex effect (such as cancer).

2.3. THE Ah (DIOXIN) RECEPTOR

The unusual potency of TCDD in eliciting its toxic effects suggested the possible existence of a receptor for the dioxin. Poland and coworkers, using radiolabeled TCDD as a ligand, demonstrated that the cytosolic fraction of C57BL/6 mouse liver contained a protein that bound the dioxin saturably (i.e., ~ 105 binding sites per cell), reversibly, and with high affinity (i.e., in the nanomolar range, consistent with TCDD's biological potency in vivo). Competition binding studies with congeners of TCDD revealed that ligands with the highest binding affinity were planar and contained halogen atoms in at least three of the four lateral positions; thus, ligand binding exhibited stereospecificity. In addition, the pattern of ligand binding in vitro resembled a rectangular hyperbola and, therefore, appeared to obey the law of mass action. Together, these findings indicated that the intracellular TCDD-binding protein had the ligand-binding properties expected for a "dioxin receptor." The protein is designated as the "Ah receptor" because it binds and mediates the response to other aromatic hydrocarbons (such as 3-methylcholanthrene) in addition to TCDD (Poland and Knutson, 1982).

The Ah receptor evolved prior to the introduction of halogenated aromatic hydrocarbons into the environment (Czuczwa et al., 1984). Therefore, some other compound(s) must represent the "natural" ligand(s) for the receptor (Poellinger et al., 1992). Naturally occurring high-affinity ligands for the receptor exist in the environment, particularly in plants (Gillner et al., 1985, 1989; Rannung et al., 1987; Bjeldanes et al., 1991). Thus, the Ah receptor might have evolved as part of a substrate-inducible system designed to metabolize dietary lipophilic substances, and TCDD may mimic the binding of such substances to the receptor. In some tissues, TCDD produces changes in the proliferative/differentiated phenotype of cells (Knutson and Poland, 1980). Thus, an additional possibility is that TCDD mimics an endogenous Ah receptor ligand that regulates such tissue-specific phenotypes.

Inbred mouse strains differ quantitatively in their responsiveness to TCDD and other aromatic hydrocarbons. For example, TCDD elicits its effects at about tenfold lower concentrations in the more responsive mouse strains (typified by C57BL/6) than in the less responsive strains (typified by DBA/2). The polymorphism is genetic in origin, and in

crossbreeding studies, the more responsive phenotype segregates as an autosomal dominant trait. Numerous responses to TCDD (e.g., enzyme induction, thymic involution, cleft palate formation, hepatic porphyria) exhibit a segregation pattern identical to that for the binding of TCDD to the Ah receptor. Thus, the genetic locus (designed Ah) that governs the receptor polymorphism also governs the biological responses to TCDD. These genetic findings implicate the Ah receptor in the mechanism of dioxin action (Poland and Knutson, 1982; Nebert et al., 1991).

The Ah receptor has been difficult to purify in substantial quantity, and insufficient knowledge of its structural and functional properties has represented a major impasse in our understanding of dioxin action. Biochemical studies of crude or partially purified receptor preparations indicate that the receptor is a soluble (as opposed to membrane-bound) intracellular protein, which, upon binding TCDD, acquires a high affinity for DNA. Studies of structure-activity relationships (SAR) reveal that, within groups of structurally related compounds, a ligand's receptor-binding affinity correlates with its potency in eliciting a biological response(s). Such SAR studies constitute biochemical evidence that implicates the Ah receptor in the mechanism of dioxin action. SAR analyses are useful for assessing the possible participation of the Ah receptor in experimental systems where genetic polymorphisms in the receptor are not available. SAR studies implicate the Ah receptor in a broad spectrum of biochemical, morphologic, immunologic, neoplastic, and reproductive effects that TCDD and related compounds elicit (Poland and Knutson, 1982; Safe, 1986).

The recent cloning and expression of receptor cDNA should generate important new insights into receptor structure and function in the near future. Receptor cDNA cloning followed the development of an [^{125}I]-labeled photoaffinity ligand for the Ah receptor, which provided a method for covalently tagging the receptor protein and permitted the application of denaturing procedures to receptor purification (Poland et al., 1986). Using two-dimensional polyacrylamide gel electrophoresis as a purification technique, Bradfield et al. isolated a small amount of receptor protein and determined the amino acid sequence of its N-terminal domain (Bradfield et al., 1991). The amino acid sequence was used to generate antipeptide antibodies (Poland et al., 1991) and to devise synthetic oligodeoxyribonucleotides, which were used to clone receptor cDNA by library screening (Burbach et al., 1992; Ema et

al., 1992). The deduced amino acid sequence of the C57BL/6 mouse Ah receptor reveals that it has a molecular mass of about 89 kDa with the following features: (1) a basic helix-loop-helix (bHLH) domain, located toward the NH_2 end of the receptor; by analogy with other bHLH systems, the basic region is likely to involve DNA binding, while the helix-loop-helix domain is likely to dimerize with other proteins; (2) a region designated as "PAS," which exhibits sequence homology with Per (a *Drosophila* circadian rhythm protein), Arnt (another protein that contributes to dioxin responsiveness, described below), and Sim (a regulatory protein that participates in *Drosophila* central nervous system [CNS] development); photoaffinity labeling studies suggest that the PAS domain may comprise part of the ligand-binding domain of the receptor; and (3) a glutamine-rich region, which resembles certain "activation domains" present in some other transcription factors; by analogy, this region could interact with coactivator proteins that are yet to be characterized. Thus, like many proteins, the Ah receptor appears to be composed of several different domains, which contribute in different ways to receptor function. It is notable that the Ah receptor does not, by itself, bind strongly to DNA; acquisition of DNA-binding capability appears to require that the receptor interact with another factor (such as the Arnt protein). Thus, the active form of the receptor is heteromeric. The Ah receptor exhibits no obvious structural similarities (such as "zinc finger" DNA-binding domains) to the steroid/thyroid/retinoid family of receptors; therefore, the Ah receptor appears to represent a novel type of ligand-activated transcription factor, distinct from those described previously. Immunohistochemical studies, using antireceptor antibodies, reveal that, in intact mouse hepatoma cells, the unliganded receptor resides in the cytoplasm; exposure of cells to TCDD leads to the accumulation of the receptor within the nucleus. Such observations suggest that the receptor protein may have a domain that controls its movement to the nucleus; however, this remains to be determined.

Human cells contain an intracellular protein whose ligand-binding and hydrodynamic properties resemble those of the Ah receptor in animals (see Cook and Greenlee,1989; Harris et al., 1989; Lorenzen and Okey, 1991; Roberts et al., 1990; Harper et al., 1991 and references therein, for examples). The human receptor has not been studied extensively, and it is unknown if the properties of the human protein differ substantially from those of the Ah

receptor in animals. Cloning, expression, and functional studies of the human receptor should increase our knowledge of its properties in the near future.

2.4. THE Arnt PROTEIN

Biochemical and hydrodynamic findings suggest that more than one protein participates in the response to TCDD. For example, protein-DNA cross-linking studies imply that the liganded Ah receptor binds to DNA as a heteromeric complex, in partnership with an additional protein(s) (Elferink et al., 1990). In addition, sedimentation experiments (Prokipak and Okey, 1988; Henry et al., 1989), as well as protein-protein cross-linking studies (Gasiewicz et al., 1991; Perdew, 1992), imply that the liganded receptor associates with other proteins in mediating the response to TCDD.

Genetic analyses of mouse hepatoma cells that exhibit diminished responsiveness to TCDD provide evidence that several genes contribute to dioxin action. Chemical selection (resistance to benzo[a]pyrene) and/or physical selection (fluorescence-activated cell sorting) can be used to isolate variant cells that respond poorly to TCDD (Hankinson, 1979; Miller and Whitlock, 1981). The variant phenotypes are stable, and the defects in TCDD responsiveness appear to be mutational in origin (Hankinson, 1981). One type of variant cell exhibits a defect in TCDD binding; it appears to contain an altered Ah receptor. In a second type of variant, TCDD binding is normal; however, the liganded receptors are unable to bind to DNA and fail to accumulate in the nucleus. These variants are defective in a protein termed Arnt (see below). Studies using cell fusion reveal that both variant phenotypes are recessive and that the variants fall into different complementation groups. The latter observation indicates that more than one gene contributes to dioxin responsiveness (Hankinson, 1983; Miller et al., 1983). The data are consistent with the idea that TCDD responsiveness requires both a ligand-binding protein (i.e., the Ah receptor) and a second protein, which mediates the binding of the liganded receptor to DNA.

Hankinson and coworkers used molecular genetic techniques to identify a human cDNA that complements the defect in the second type of variant cell described above. They designated the corresponding protein as Arnt, which stands for Ah receptor nuclear translocator, because of its perceived role in translocating the liganded receptor from the

cytoplasm to the nucleus (Hoffman et al., 1991; Reyes et al., 1992). The human Arnt cDNA encodes a protein of about 86 kDa, which has the following features: (1) like the Ah receptor, it contains a bHLH domain, which presumably contributes both to DNA binding and to protein-protein interactions; (2) like the Ah receptor, the Arnt protein contains domains homologous to Per and Sim. In addition, the Arnt protein does not bind TCDD and it does not bind to DNA in the absence of the liganded Ah receptor protein (Whitelaw et al., 1993). Immunohistochemical studies, using anti-Arnt antibodies, reveal that the Arnt protein resides in the nucleus in uninduced mouse hepatoma cells and that exposure of cells to TCDD produces no change in its intracellular distribution. Thus, Arnt appears to be a nuclear protein. Furthermore, the nuclear accumulation of the Ah receptor occurs in Arnt-defective cells. Together, these findings argue against a primary role for the Arnt protein in the translocation of the receptor from cytoplasm to nucleus per se. Instead, they suggest that Arnt interacts with the liganded Ah receptor to form a heteromeric, DNA-binding protein complex that can activate gene transcription. Experiments in vitro support this idea. For example, immunoprecipitation experiments reveal that the liganded Ah receptor and the Arnt protein can interact in solution. Neither the liganded receptor nor the Arnt protein exhibits substantial DNA-binding activity in the absence of the other; the presence of both proteins is required to generate a specific DNA-binding species and to activate the expression of a reporter gene. Deletion of the bHLH domain of Arnt abrogates its functional interaction with the liganded Ah receptor (Whitelaw et al., 1993). Together, these findings imply that the transcriptionally active component of the dioxin-responsive system is a protein heteromer consisting of (at least) the liganded Ah receptor and Arnt.

Both the Ah receptor and the Arnt protein belong to the bHLH class of transcription factors, which function as heterodimers and contribute to the control of numerous genes (Kadesch, 1993). The dimerization of bHLH proteins constitutes a potential mechanism for generating regulatory diversity. For example, different heterodimers may exhibit different stabilities, may have different DNA-binding affinities, or may recognize different DNA sequences. Its bHLH structure raises the possibility that the Ah receptor might also form heterodimeric complexes with proteins other than Arnt, generating regulatory molecules with potentially novel properties. By analogy with other bHLH systems, both the absolute amount

of each partner and their relative ratios could influence the extent and type of response to TCDD. Thus, diversity in heteromer formation might contribute to the diversity of responses that typifies dioxin action. Furthermore, some HLH proteins (typified by Id), which lack a basic region, may act as dominant negative regulators of transcription by dimerizing with bHLH proteins and inhibiting their binding to DNA (Kadesch, 1993). There is evidence for a dominant negative regulator in the TCDD-responsive system; however, it is not known whether the dominant negative phenotype is due to an HLH protein that interacts with either the Ah receptor or Arnt (Watson and Hankinson, 1988; Watson et al., 1992). In any case, the bHLH structure of the Ah receptor and the Arnt protein suggests that some of the diversity in TCDD's biological effects might reflect differential gene regulation by a combinatory mechanism involving the formation of different protein heterodimers.

Studies of the Per gene product in vitro by immunoprecipitation suggest that the PAS domain is involved in protein heterodimerization (Huang et al., 1993). By analogy, the PAS domains of the Ah receptor and the Arnt protein may also participate in the formation of specific protein-protein interactions between the protein components of the dioxin-responsive system.

2.5. OTHER PROTEINS THAT PARTICIPATE IN THE RESPONSE TO DIOXIN

Attempts to purify the unliganded Ah receptor under nondenaturing conditions revealed that it tends to associate with other proteins in vitro, particularly the 90-kDa heat shock protein (hsp90). The hsp90 protein is an abundant factor that can interact with numerous other proteins and that may have multiple functions (Welch, 1992). Immunoprecipitation studies and immunosedimentation experiments using anti-hsp90 antibodies reveal that the unliganded Ah receptor associates with hsp90 in vitro (Denis et al., 1988; Perdew, 1988). In view of previous findings implicating hsp90 in glucocorticoid receptor function (Picard et al., 1990), the association between the Ah receptor and hsp90 in vitro may be more than fortuitous. Experiments in vitro suggest that the hsp90-receptor interaction could have more than one effect; hsp90 might maintain the unliganded receptor in a configuration that facilitates ligand binding, and it might prevent the inappropriate binding of the unliganded receptor to DNA (Pongratz et al., 1992). However, it is unknown whether

hsp90 interacts with the Ah receptor in the intact cell and whether the hsp90 protein influences the response to dioxin in vivo. Studies of receptor function in hsp90-defective cells might be instructive in this regard.

Several lines of evidence suggest that phosphorylation/dephosphorylation of the Ah receptor and the Arnt protein may contribute to the function of the dioxin-responsive system. First, treatment of nuclear extracts with potato acid phosphatase inhibits the binding of the liganded receptor heteromer to its DNA recognition sequence in vitro (Pongratz et al., 1991). Second, the downregulation of protein kinase C (PKC) and the chemical inhibition of PKC are associated with a reduction in the DNA-binding capability of the receptor heteromer in vitro (Okino et al., 1992; Carrier et al., 1992; Berghard et al., 1993). Third, immunoprecipitation experiments using antireceptor antibodies reveal that the receptor can undergo phosphorylation in vivo (Berghard et al., 1993). Additional experiments in vitro suggest that phosphorylation of the Arnt protein is required for its heterodimerization with the Ah receptor; however, phosphorylation of the receptor is not required for heterodimer formation (Berghard et al., 1993). Together, these findings suggest that PKC and other protein kinases might influence both heterodimerization and the binding of the receptor-Arnt heteromer to DNA. It is known that many mammalian transcription factors undergo cycles of phosphorylation and dephosphorylation; however, in most cases, the physiological significance of the modification is unknown (Hunter and Karin, 1992). Additional research is necessary to delineate fully the role of protein phosphorylation in the response to TCDD.

It appears likely that additional proteins, which remain to be identified and characterized, also contribute to the dioxin-responsive system. For example, a protein phosphatase(s) must participate in the presumed cycles of phosphorylation/dephosphorylation that the Ah receptor and Arnt protein undergo. In addition, the superinduction of CYP1A1 transcription by cycloheximide suggests that an inhibitory gene regulatory protein may modulate the response to dioxin (Israel et al., 1985; Lusska et al., 1992). Chemical cross-linking studies suggest that the Ah receptor contacts multiple proteins as it transduces the TCDD signal to the nucleus (Gasiewicz et al., 1991; Perdew, 1992). Genetic analyses of cells that respond poorly to TCDD indicate the existence of an additional complementation group, whose precise biochemical defect remains to be identified; this observation implies the

existence of another protein (in addition to the Ah receptor and Arnt) that contributes to dioxin responsiveness (Karenlampi et al., 1988).

2.6. ACTIVATION OF GENE TRANSCRIPTION BY DIOXIN

2.6.1. In Vitro Studies

Much of our current understanding of the mechanism of dioxin action is based on analyses of the induction of microsomal aryl hydrocarbon hydroxylase (AHH) activity by TCDD. Hydroxylase activity reflects the action of the cytochrome P450 1A1 enzyme, which catalyzes the oxygenation of polycyclic aromatic substrates, as the initial step in their metabolic processing to water-soluble derivatives (Conney, 1982). TCDD induces AHH activity in many tissues; in particular, the relatively strong induction response in cultured cells has facilitated the application of molecular genetic techniques to the analysis of the induction mechanism (Whitlock, 1990). Table 2-1 and Figure 2-2 summarize some of the molecular events involved in the induction process.

In mouse hepatoma cells, nuclear transcription experiments reveal that TCDD induces hydroxylase activity by stimulating the transcription of the corresponding CYP1A1 gene. The response to TCDD occurs within a few minutes and is direct, in that it does not require ongoing protein synthesis. Thus, the regulatory components required for the activation of CYP1A1 transcription are present constitutively within the cell. TCDD fails to activate CYP1A1 transcription in Ah receptor-defective cells and in Arnt-defective cells; therefore, the response requires both the Ah receptor and the Arnt protein.

The observations that TCDD activates transcription and that the liganded Ah receptor binds to DNA led to the discovery of a dioxin-responsive regulatory DNA domain, upstream of the CYP1A1 gene. Recombinant DNA methods were used to construct chimeric genes, in which potential regulatory DNA domains from the CYP1A1 gene were ligated to a heterologous "reporter" gene. After transfection of the recombinant genes into mouse hepatoma cells, TCDD was observed to activate the expression of the reporter gene. Additional transfection experiments defined the size of the dioxin-responsive domain and revealed that it had the properties of a transcriptional enhancer (Jones et al., 1986; Neuhold

Table 2-1. Events in the Activation of *CYP1A1* Gene Transcription by Dioxin

- Diffusion into the cell
- Binding to the Ah receptor protein
- Conversion of liganded receptor to the DNA-binding form
  - Dissociation from hsp90?
  - Active translocation from cytoplasm to nucleus?
  - Association with Arnt protein?
- Binding of liganded receptor heteromer to enhancer DNA
- Enhancer activation
  - Altered DNA configuration?
  - Histone modification?
  - Recruitment of additional proteins?
- Nucleosome disruption
- Increased accessibility of transcriptional promoter
- Binding of transcription factors to promoter
- Enhanced mRNA and protein synthesis
- Primary biological response(s)
- Cascade of compensatory changes?
- Secondary biological responses?

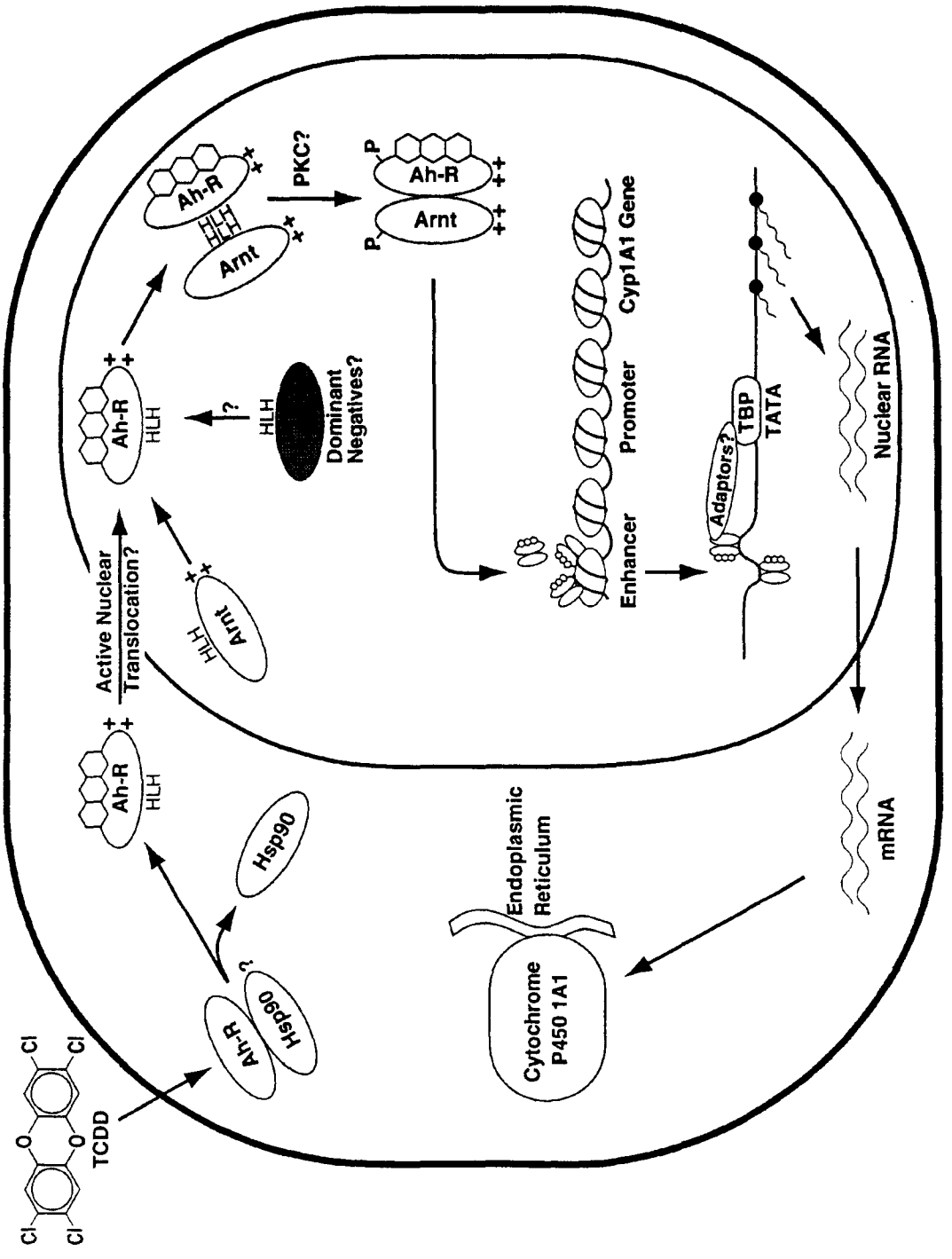

Figure 2-2. Mechanism of induction of CYP1A1 gene transcription by TCDD.

et al., 1986; Fujisawa-Sehara et al., 1987; Fisher et al., 1990). Furthermore, the recombinant gene responded poorly when transfected into Ah receptor-defective cells or Arnt-defective cells. Thus, both the receptor protein and the Arnt protein are required for enhancer function. Analyses of stable transfectants revealed that the dioxin-responsive enhancer can function in a chromosomal location distinct from that of the CYP1A1 gene (Fisher et al., 1989). Therefore, in principle, an analogous enhancer element could mediate the transcriptional response of other genes to TCDD.

In addition, the DNA upstream of the CYP1A1 gene contains a second control element (a transcriptional promoter), which functions to ensure that transcription is initiated at the correct site. The promoter binds proteins that are expressed constitutively by the cell; however, the promoter contains no binding sites for the liganded receptor heteromer. Transfection experiments indicate that neither the enhancer nor the promoter functions in the absence of the other (Jones and Whitlock, 1990). These findings raise the issue as to the mechanism by which the enhancer and promoter, which are separated by hundreds of nucleotides, function in concert. TCDD-induced alterations in the chromatin structure of the CYP1A1 gene appear to play an important role in this process, as described later.

The observations that enhancer function requires both the receptor and Arnt proteins and that the liganded, heteromeric form of the receptor exhibits an increased affinity for DNA suggested that the activation of CYP1A1 transcription involves the binding of the receptor heteromer to the enhancer. Analyses of protein-DNA interactions in vitro by gel retardation, using enhancer DNA and nuclear extracts from uninduced and TCDD-induced cells, revealed the existence of an inducible, receptor-dependent, and Arnt-dependent protein-DNA interaction, whose characteristics were those expected for the binding of the receptor heteromer to DNA (Denison et al., 1989; Hapgood et al., 1989; Saatcioglu et al., 1990a, b). The receptor heteromer recognizes a specific core nucleotide sequence:

5′T-GCGTG 3′
3′A-CGCAC 5′

which is present in multiple copies within the enhancer. Studies with an [^{125}I]-labeled dioxin indicate that the receptor heteromer binds in a 1:1 ratio to its DNA recognition sequence (Denison et al., 1989). Methylation protection and interference experiments in vitro reveal that the receptor heteromer lies within the major DNA groove and contacts the four guanines of the recognition sequence (Saatcioglu et al., 1990a, b; Shen and Whitlock, 1989; Neuhold et al., 1989). Transfection analyses of the six in vivo binding sites for the receptor heteromer as well as several mutated sites synthesized in vitro reveal that base-pairs adjacent to the core recognition sequence contribute to enhancer function. Studies of protein-DNA interactions reveal that there is no strict relationship between the affinity of the receptor heteromer for DNA and the extent of enhancer activation (Shen and Whitlock, 1992; Neuhold et al., 1989). These latter observations suggest that the protein-DNA interaction per se does not suffice to activate transcription and that an additional event (such as DNA bending--see below) is necessary. Mutational analyses reveal that the four base-pair sequence:

5′CGTG 3′
3′GCAC 5′

is required for the receptor heteromer to bind to DNA in vitro (Shen and Whitlock, 1992; Lusska et al., 1993). This sequence is also part of the recognition motif for other bHLH proteins (Kadesch, 1993). This latter observation suggests that the Ah receptor and Arnt proteins may have DNA recognition properties similar to those of some other bHLH proteins.

The DNA recognition sequence for the receptor heteromer contains two CpG dinucleotides. Studies in other systems reveal that cytosine methylation at CpG is associated with decreased gene expression, often in tissue-specific fashion. Cytosine methylation of the CpG dinucleotides within the recognition sequence diminishes both the binding of the receptor heteromer to the enhancer (as measured by gel retardation) and the function of the enhancer (as measured by transfection). Therefore, given that the TCDD-responsive receptor/enhancer system can regulate the transcription of genes other than CYP1A1,

methylation of the enhancer constitutes a mechanism for controlling the expression of such genes in tissue-specific fashion (Shen and Whitlock, 1989).

Gel retardation analyses also reveal that the binding of the receptor heteromer to its recognition sequence bends the DNA in vitro. The site of the bend is at, or very near, the site of the protein-DNA interaction (Elferink and Whitlock, 1990). These findings suggest that the binding of the receptor heteromer to chromatin might also alter the configuration of the enhancer in vivo.

2.6.2. In Vivo Studies

Studies that use transfection and in vitro techniques for analyzing protein-DNA interactions provide important clues about the components of the TCDD-responsive system and their function. However, such experimental approaches necessitate removing the DNA regulatory elements from their native context within the chromosome and, therefore, have the potential to generate misleading results. For example, in the intact cell, nuclear DNA is complexed with histones and other chromosomal proteins, and the structure of the nucleoprotein complex (chromatin) makes important contributions to the control of gene transcription (Grunstein, 1990; Felsenfeld, 1992; Kornberg and Lorch, 1992). Transfection experiments and studies of protein-DNA interactions in vitro do not adequately control for this variable. For this reason, a ligation-mediated polymerase chain reaction technique was employed to analyze the protein-DNA interactions at the dioxin-responsive enhancer in intact cells (Wu and Whitlock, 1993). These experiments reveal that the inactive (i.e., uninduced) enhancer binds few, if any, proteins within the major DNA groove. This finding implies that the inactive enhancer is relatively inaccessible to DNA-binding proteins in vivo. In addition, from a mechanistic standpoint, the absence of protein-enhancer interactions in uninduced cells argues against the idea that a specific repressor protein maintains the enhancer in an inactive configuration. Exposure of cells to TCDD leads to the rapid binding of six receptor heteromers, and few other proteins, to the enhancer. Therefore, the liganded receptor heteromer appears to activate transcription by a mechanism that does not require other enhancer-binding proteins (Wu and Whitlock, 1993).

The proteins that bind to the CYP1A1 promoter are expressed constitutively by the cell, and TCDD has no effect on their interactions with promoter DNA in vitro, as measured by DNase footprinting (Jones and Whitlock, 1990). However, in intact cells, these proteins fail to bind to the inactive (i.e., uninduced) promoter. Thus, the promoter, like the enhancer, is inaccessible in uninduced cells. Exposure of cells to TCDD induces a rapid change at the promoter, such that it becomes accessible to the constitutively expressed proteins. The change represents a primary effect of TCDD, because it is insensitive to actinomycin D at a concentration that inhibits transcription by greater than 95%. In addition, it is receptor dependent and Arnt dependent, because it does not occur in the respective variant cells. These observations indicate that the effect of the receptor-enhancer interaction is to increase the accessibility of the downstream promoter (Durrin and Whitlock, 1989; Wu and Whitlock, 1992).

Studies of the chromatin structure of the CYP1A1 gene reveal that, in the transcriptionally inactive state, the enhancer/promoter region assumes a nucleosomal structure and that nucleosomes are specifically positioned at the promoter (Morgan and Whitlock, 1992). Its organization into nucleosomes plausibly accounts for the inaccessibility of the enhancer/promoter region to DNA-binding proteins in uninduced cells. Exposure to TCDD produces a rapid and actinomycin D-insensitive loss of the positioned nucleosomes at the promoter; this change in chromatin structure mechanistically accounts for the TCDD-induced increase in promoter accessibility and increased CYP1A1 transcription in vivo (Morgan and Whitlock, 1992).

The mechanism by which the binding of liganded receptor heteromers to the enhancer alters chromatin structure is unknown. One possibility is that the DNA-bound receptor complex affects the histones (for example, by activating a histone acetylase), thereby weakening histone-DNA interactions and destabilizing nucleosomes. A second possibility is that the receptor-enhancer interaction alters the DNA structure of the enhancer/promoter region, stabilizing it in a non-nucleosomal configuration. This idea is consistent with the observation that the receptor heteromer bends the DNA in vitro (Elferink and Whitlock, 1990).

On the enhancer, the six binding sites for the receptor heteromer are arranged in an irregular pattern. The absence of regular spacing between the sites suggests that enhancer activation does not require protein-protein interactions between adjacent DNA-bound receptor heteromers. Instead, the irregular spacing of binding sites may reflect constraints imposed by chromatin structure because the receptor heteromer must bind to nucleosomes. For example, as the DNA helix wraps around the histone core of the nucleosome, the major groove (which contains the binding sites for the receptor heteromer) is periodically accessible and inaccessible. Therefore, increasing the number of binding sites at irregular intervals increases the probability that at least one site will be accessible even when the DNA is nucleosomal. In addition, the receptor heteromer contacts a relatively short (six base-pair) DNA segment, increasing the probability that the entire binding site will be accessible in the nucleosome. Thus, the multiplicity, irregular distribution, and small size of the binding sites may have evolved as a mechanism for overcoming the steric constraint imposed by the nucleosomal organization of the inactive enhancer in vivo.

2.7. FUTURE RESEARCH

The cloning of cDNAs encoding the Ah receptor and Arnt proteins and the development of antireceptor and anti-Arnt antibodies open the way to additional mechanistic studies of dioxin action. It is now practical to analyze the structure and function of these proteins using mutagenesis techniques, to analyze the structure and regulation of the corresponding genes, to determine whether different forms of the receptor and Arnt exist, and to directly analyze the role of posttranslational modifications on receptor and Arnt function. In vitro transcription can be used to study the functional components of the dioxin-responsive pathway (Wen et al., 1990). However, it will be appropriate to develop a chromatin-based system for such studies in the future.

The observation that the Ah receptor and Arnt proteins heterodimerize via HLH motifs raises the likelihood that they may also dimerize with other partners (perhaps in tissue-specific fashion) to generate different combinations of proteins that may have novel regulatory properties. For example, the partial homology between the Ah receptor, Arnt, and Sim suggests that different dimerization partners might contribute to dioxin-induced

alterations in differentiation pathways in some cells. The types of protein complexes formed will presumably depend on both the relative amount of each potential partner and the stability of each type of heterodimer. This combinational mechanism for regulating the response to dioxin may also permit the system to functionally interact with other signal transduction pathways, increasing the potential diversity of dioxin-induced responses still further (see, for example, Pimental et al., 1993). These appear to be interesting areas for future research. The information generated in future experiments is likely to provide novel insights into the mechanism of dioxin action, in particular, and into the regulation of mammalian gene transcription (especially by bHLH proteins), in general. If such studies reveal new proteins (and the corresponding genes) that influence rate-limiting steps in the response to dioxin, the findings might also prove useful from a risk assessment standpoint.

The teratogenic and tumor-promoting effects of TCDD, as well as its effects on keratinocyte differentiation, suggest that the Ah receptor and other components of the dioxin-responsive system may contribute to important developmental and proliferative pathways in some cell types. The study of transgenic animals, in which both alleles for the Ah receptor or the Arnt protein have been inactivated by homologous recombination, might provide new insights into such pathways, assuming that the transgenic animals are viable. In addition, such animals might be useful in toxicological studies to assess whether the product of the inactivated gene participates in adverse responses to particular chemicals.

Chromatin structure and nucleosome positioning have important effects on mammalian gene expression (Grunstein, 1990; Felsenfeld, 1992; Kornberg and Lorch, 1992). The TCDD-responsive CYP1A1 gene is an interesting model system for analyzing the mechanism by which a protein complex such as the liganded receptor heteromer can trigger the chromatin structural changes that increase DNA accessibility. Studies of the triggering mechanism may provide novel insights into the function of the dioxin-responsive system, in particular, and BHLH transcriptional regulatory proteins, in general.

Some dioxin-responsive genes (e.g., cytochrome P450 1A2, glutathione S-transferase Ya) exhibit a substantial constitutive ("basal") transcription, and TCDD increases expression still further. The constitutive expression implies that the promoter for such genes must be maintained in an accessible configuration even in the absence of dioxin; therefore, TCDD

may induce the transcription of such genes by a mechanism that does not involve major changes in the chromatin structure of the regulated gene. Thus, the liganded receptor heteromer may be able to increase gene transcription by both chromatin-dependent and chromatin-independent mechanisms. This may be an interesting issue for future research.

The Ah receptor presumably participates in every biological response to TCDD (Poland and Knutson, 1982; Safe, 1986; Birnbaum, 1993). Thus, it is likely that TCDD activates transcription of other genes via a receptor- and enhancer-dependent mechanism analogous to that described for the CYP1A1 gene. For example, TCDD induces the expression of genes encoding cytochrome P450 1A2, glutathione S-transferase Ya subunit, aldehyde dehydrogenase, and quinone reductase; in some cases, induction is known to occur at the transcriptional level, to be Ah receptor dependent and Arnt dependent, and to involve a DNA recognition sequence analogous to that found upstream of the CYP1A1 gene (Pimental et al, 1993; Quattrochi and Tukey, 1989; Rushmore and Pickett, 1993; Dunn et al., 1988; Takimoto et al., 1992; Favreau and Pickett, 1991). Other observations reveal that, in human keratinocytes, TCDD activates the transcription of the plasminogen activator inhibitor-2 and interleukin-10 genes, as well as additional genes that remain to be identified (Sutter et al., 1991). The mechanism by which dioxin activates keratinocyte gene expression is unknown. For dioxin-responsive genes other than CYP1A1, and especially for those genes that respond in tissue-specific fashion, the presence of the receptor/enhancer system may not be sufficient for dioxin action, and other tissue-specific, regulatory components may play a dominant role in governing the response to TCDD. For example, estrogens (presumably via estrogen receptors) influence TCDD-induced neoplasia in rat liver (Lucier et al., 1991). Also, in vitro experiments suggest the possibility that, in some cell types, a repressor protein might inhibit the response to TCDD by competing for the receptor binding site(s) on DNA (Gradin et al., 1993). Thus, future research may reveal the existence of additional stimulatory or inhibitory gene regulatory components that can modulate the activity of the dioxin-responsive receptor/enhancer system.

The potential teratogenic and neoplastic effects of TCDD have raised particular concerns about the health effects of dioxin in humans. These responses may reflect complicated cascades of biochemical changes that are difficult to analyze mechanistically;

therefore, a major challenge for the future will be to establish experimental systems in which such complex phenomena are amenable to study at the molecular level. However, the identification of a dioxin-transformable human keratinocyte cell line and the development of methods for analyzing dioxin's teratogenic effects in vitro provide reasons to be optimistic about future progress in these areas (Yang et al., 1992; Abbott et al., 1989).

2.8. MECHANISTIC INFORMATION AND RISK ASSESSMENT

A substantial body of evidence in experimental animals indicates that the Ah receptor mediates the biological effects of TCDD. Although studies of human tissue are much less extensive, it appears reasonable to assume that dioxin's effects in humans are also receptor mediated. The receptor-based mechanism predicts that, except in cases where the concentration of TCDD is already very high (i.e., $[TCDD] >> K_D$), an incremental exposure to TCDD will lead to some increase in the fraction of Ah receptor that is occupied. However, it is not valid to assume that the increased receptor occupancy will necessarily elicit a proportional increase in all biological response(s). Dose-response relationships (which may vary among responses) must be considered when using mathematical models to estimate the risk associated with exposure to TCDD.

Given TCDD's widespread distribution, its persistence, and its accumulation within the food chain, it is likely that most humans are exposed to some level of dioxin; thus, the population at potential risk is large and genetically heterogeneous. By analogy with the findings in inbred mice, polymorphisms in the Ah receptor probably exist in humans. Therefore, a concentration of TCDD that elicits a response in one individual may not do so in another. For example, studies of humans exposed to dioxin following an industrial accident at Seveso, Italy, fail to reveal a simple and direct relationship between blood TCDD levels and the development of chloracne (Mocarelli et al., 1991). These differences in responsiveness to TCDD may reflect genetic variation either in the Ah receptor or in some other component of the dioxin-responsive pathway. Therefore, analyses of polymorphisms in the Ah receptor and Arnt genes in humans with well-documented exposure to dioxin have the potential to identify genotypes that confer particular risk. Such molecular genetic

information may be useful in the future for accurately predicting the health risk that dioxin poses to humans.

Complicated responses (such as cancer) probably involve multiple events and multiple genes. For example, a homozygous recessive mutation at the hr (hairless) locus is required for TCDD's action as a tumor promoter in mouse skin (Poland et al., 1982). Thus, the hr locus influences the susceptibility of a particular tissue (skin) to a specific effect (tumor promotion) of dioxin. An analogous situation may obtain other effects of TCDD in other tissues. For example, TCDD may produce porphyria cutanea tarda only in individuals with inherited uroporphyrinogen decarboxylase deficiency (Doss et al., 1984). Such findings suggest that, for some adverse effects of TCDD, the population at risk may be limited to individuals with a particular genetic predisposition.

Other factors can influence an organism's susceptibility to TCDD. For example, female rats are more prone to TCDD-induced liver neoplasms than are males; this phenomenon is related to the hormonal status of the animals (Lucier et al., 1991). In addition, hydrocortisone and TCDD synergize in producing cleft palate in mice. Retinoic acid and TCDD produce a similar synergistic teratogenic effect (Couture et al., 1990). Therefore, in some cases, TCDD acts in combination with hormones or other chemicals to produce adverse effects. Such phenomena might also occur in humans; if so, the difficulty of risk assessment is increased, given the diversity among humans in hormonal status, lifestyle (e.g., smoking, diet), and chemical exposure.

Dioxin's action as a tumor promoter presumably reflects its ability to alter cell proliferation. There are several plausible mechanisms by which this could occur. First, TCDD might activate a gene(s) that is directly involved in tissue proliferation. Second, TCDD-induced changes in hormone metabolism may lead to tissue proliferation secondary to increased secretion of a trophic hormone. Third, TCDD-induced changes in receptors for growth factors or hormones may alter the sensitivity of a tissue to proliferative stimuli. Fourth, TCDD-induced toxicity may lead to cell death, followed by regenerative proliferation. The mechanisms might differ among tissues, might exhibit different sensitivities to TCDD, and might be modulated by different genetic and environmental factors. If so, the differences would increase the difficulty of risk assessment.

Under some circumstances, exposure to TCDD elicits beneficial effects. For example, TCDD protects against the carcinogenic effects of polycyclic aromatic hydrocarbons in mouse skin; this may reflect the induction of detoxifying enzymes (Cohen et al., 1979; DiGiovanni et al., 1980). In other situations, TCDD-induced changes in estrogen metabolism may alter the growth of hormone-dependent tumor cells, producing a potential anticarcinogenic effect (Spink et al., 1990; Gierthy et al., 1993). These (and perhaps other) potentially beneficial effects of TCDD further complicate the risk assessment process for dioxin.

Given the diversity of TCDD's biological effects and the likelihood that many effects reflect a complicated interplay between genetic and environmental factors, it may be overly simplistic to use mechanistic information derived largely from the relatively simple responses described in this chapter as the basis for developing a quantitative approach to dioxin risk assessment in humans. While this approach represents a start toward incorporating mechanistic information into risk assessment, future biologically based dose-response models will require a better understanding not only of the TCDD-induced biochemical alterations that produce disease but also of the relationships between genetic and environmental factors that influence an individual's susceptibility to TCDD. Molecular toxicology has great potential to provide new insights into such issues in the future.

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3. ACUTE, SUBCHRONIC, AND CHRONIC TOXICITY

3.1. SCOPE AND LIMITATIONS

The acute, subchronic, and chronic toxicology of the chlorinated dioxins, dibenzofurans, biphenyls, and related compounds has been reviewed extensively in recent years [CDDs and CDFs, WHO/IPCS (1989), U.S. EPA (1984, 1985); PCBs and PCTs, WHO/IPCS (1991); U.S. EPA (1990); PCBs, U.S. EPA (1990); and BDDs and BDFs, U.S. EPA (1991)]. This chapter is intended to summarize our knowledge of the toxicology of TCDD in the main, but includes references to other dioxinlike compounds when relevant data are available. In this chapter, we do not reference all published material but rather have selected various data that are considered to be of importance to risk assessment. The chapter covers experimental animal data. Immunotoxicity, reproductive/developmental toxicity, carcinogenicity, toxicity to humans, and epidemiology are all dealt with in separate chapters. Ecotoxicology also is not covered in this chapter.

3.2. ACUTE TOXICITY

The range of doses of TCDD that are lethal to animals varies extensively with both species and strain, as well as with sex, age, and the route of administration within a single strain (Table 3-1). Typically there is a delayed toxicity, with the time to death after exposure usually being several weeks. However, deaths within the first week after exposure have been observed in guinea pigs (Schwetz et al., 1973), rabbits (Schwetz et al., 1973), and Golden Syrian hamsters (Olson et al., 1980). A more than 8,000-fold difference exists between the dose of TCDD reported to cause 50 percent lethality in male Hartley guinea pigs, the most sensitive species tested (Schwetz et al., 1973), and the corresponding dose in male Syrian Golden hamsters (Henck et al., 1981). Another animal with extremely high sensitivity is the mink (*Mustela vision*); for the male, an LD_{50} value of only 4.2 μg/kg has been calculated (Hochstein et al., 1988).

Among traditional experimental animals, the rat seems to be the second most sensitive species, although there is a >300-fold variability in LD_{50} values among different strains. The Han/Wistar (Kuopio) strain of rat has been shown to be particularly resistant to TCDD

Table 3-1. Acute Lethality of TCDD to Various Species and Substrains

Species/Strain/Sex	Route	LD_{50} (μg/kg)	Time of Death (days post-exposure)	Follow-up (days)	Body Weight Loss[a] (%)	References
Guinea pig/Hartley (male)	*per os*	0.6-2.1	5-34	NR 30	50	McConnell et al., 1978b; Schwetz et al., 1973
Mink/NR (male)	*per os*	4.2	7-17	28	31	Hochstein et al., 1988
Chicken/NR (NR)	*per os*	<25	12-21	NR	NR	Greig et al., 1973
Monkey/rhesus (female)	*per os*	~70	14-34	42-47	13-38	McConnell et al., 1978a
Rat/L-E (male)	intraperitoneal	~10	15-23	48-49	39	Tuomisto and Pohjanvirta, 1987
Rat/Sherman, Spartan (male) (female)	*per os*	22 13-43	9-27	NR	NR	Schwetz et al., 1973
Rat/Sprague-Dawley (male) (female) (weanling male)	intraperitoneal	60 25 25	NR	20	NR	Beatty et al., 1978
Rat/Fischer Harlan (male)	*per os*	340	28[b]	30	43	Walden and Schiller, 1985
Rat/H/W (male)	intraperitoneal	>3000	23-34	39-48	40-53	Pohjanvirta and Tuomisto, 1987; Pohjanvirta et al., 1988
Mouse/B6 (male) D2A/2J (male) B6D2F1 (male)	*per os*	182 2570 296	24[b] 21[b] 25[b]	30	25 33 34	Chapman and Schiller, 1985
Mouse/B6 D2 B6D2F1	intraperitoneal	132 620 300	NR	NR	NR	Neal et al., 1982
Rabbit/New Zealand White (male and female)	*per os* dermal	115 275	6-39 12-22	NR 22	NR NR	Schwetz et al., 1973

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Table 3-1 (continued)

Species/Strain/Sex	Route	LD_{50} (μg/kg)	Time of Death (days post-exposure)	Follow-up (days)	Body Weight Loss[a] (%)	References
Rabbit/New Zealand White (male and female)	intraperitoneal	~50	7-10	10-20	11	Brewster et al., 1988
Hamster/Golden Syrian (male and female)	*per os*	1157-5051	2-47	50	NR	Henck et al., 1981
Hamster/Golden Syrian (male and female)	intraperitoneal	>3000	14-32	55	1[c]	Olson et al., 1980

[a]Of succumbed animals
[b]Mean time to death
[c]Data from five animals
NR = Not reported

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exposure (Pohjanvirta and Tuomisto, 1987). Among the five rats per dose group (0, 1,500, 2,000, 2,500 or 3,000 μg TCDD/kg bw), only one animal died within the 39-40 days observation period. Also, the DBA/2 male mouse has been shown to have a high resistance to TCDD toxicity (Chapman and Schiller, 1985).

Data on sex differences in sensitivity to the lethal effects of TCDD are conflicting. Acute toxicity data that address the effect of age at the point of exposure to TCDD are scarce, and comparisons are hampered by either the absence or the inadequacy of the information on the age and/or body weight of the tested animals. Additionally, as demonstrated with other chemicals, the acute toxicity may vary several fold, depending on the vehicle used or the presence of other substances that affect uptake.

The differences in sensitivity toward TCDD among various strains of mice have been claimed to depend on a genetic variability in the Ah locus (see Chapter 2). In two strains of male C57B/6J mice that differ only at the Ah locus, Birnbaum et al. (1990) found LD_{50} values of 159 and 3,351 μg/kg for the wild-type mice ($Ah^{b/b}$) and the congenic mice ($Ah^{d/d}$), respectively. The mean time to death was 22 days and was independent of dose and genotype. Signs of toxicity were similar in the two strains, and it was concluded that the spectrum of toxicity is independent of the allele at the Ah locus. However, the relative dose needed to bring about various acute responses is ~8-24 times greater in congenic mice homozygous for the "d" allele than in the wild-type mice carrying two copies of the "b" gene.

In contrast, the two strains of rats, Long-Evans and Han/Wistar (Kuopio), studied by Pohjanvirta et al. (1988) had intraperitoneal LD_{50} values of 10 and >3,000 μg TCDD/kg, respectively, although no differences with regard to the amount or the affinity of available Ah receptor could be found.

Geyer et al. (1990) utilized both their own and other data to determine a correlation between total body fat content and acute toxicity in various species and strains of laboratory mammals. They found a correlation of 0.834 and suggested that the reason for this correlation was that an increased total body fat content may enhance the capacity to remove TCDD from the systemic circulation. This factor may be important, but it almost certainly does not explain all the interspecies differences. Data from studies of the Han/Wistar

(Kuopio) rats, which are extremely resistant to TCDD-induced lethality (Pohjanvirta and Tuomisto, 1987), were not included.

In chickens, acute toxicity is characterized by clinical signs such as dyspnea, reduced body weight gain, stunted growth, subcutaneous edema, pallor, and sudden death (chick edema disease). The disease first gained attention in 1957, but the causal agents were not identified as CDDs until much later (Firestone, 1973). Chick edema occurred in birds given oral doses of 1 or 10 μg TCDD/kg/day or of 10 and 100 μg hexaCDD/kg/day, but it was not observed in chicks maintained on a diet containing 0.1 or 0.5 percent OCDD (Schwetz et al., 1973).

3.2.1. Signs and Symptoms of Toxicity

TCDD affects a variety of organ systems in different species. It should be noted that much of the comparative data base is derived from high-dose effects. The liver is the organ primarily affected in rodents and rabbits, while in guinea pigs, atrophy of the thymus and lymphatic tissues seems to be the most sensitive marker of toxicity (WHO/IPCS, 1989; U.S. EPA, 1984, 1985). It is not possible to specify a single organ whose dysfunction accounts for the lethality. Dermal effects are prominent signs of toxicity in subhuman primates, and changes in epithelial tissues dominate both cutaneously and internally. This is most apparent in nonhuman primates in which the TCDD-induced cutaneous lesions closely mimic the chloracne and hyperkeratosis observed in humans. The histopathological alterations observed in epithelial tissues include hyperplastic and/or metaplastic alterations, as well as hypoplastic responses. The toxic responses of various species to TCDD are summarized in Table 3-2 (WHO/IPCS, 1989).

Loss of body weight (wasting syndrome) is a characteristic sign observed in most animals given a lethal dose of TCDD. The weight loss usually manifests itself within a few days after exposure and results in a substantial reduction of the adipose (Peterson et al., 1984) and muscle tissue (Max and Silbergeld, 1987) observed at autopsy. With sublethal doses of TCDD, a dose-dependent decrease in body weight gain occurs.

The greatest species-specific differences in toxicity concern pathological alterations in the liver. Administering lethal doses to guinea pigs does not result in liver damage

Table 3-2. Toxic Response Following Exposure to 2,3,7,8-TCDD: Species Differences[a]

Response	Monkey	Guinea Pig	Cow[b]	Rat	Mouse	Rabbit[b]	Chicken[b]	Hamster
HYPERPLASIA AND/OR METAPLASIA								
Gastric mucosa	++	0	+	0	0			0
Intestinal mucosa	+							++
Urinary tract	++	++	++	0	0			
Bile duct and/or gall bladder	++	0	+		++			0
Lung: focal alveolar				++				
Skin	++	0	+[c]	0	0	++		0
HYPOPLASIA, ATROPHY OR NECROSIS								
Thymus	+	+	+	+	+		+	+
Bone marrow	+	+			±		+	
Testicle	+	+		+	+			
OTHER RESPONSES								
Liver lesions	+	±	++	+	++	+	+	±
Porphyria	0	0		+	++		+	0
Edema	+	0		0	+		++	+

[a]Sources: Allen and Lalich, 1962; Allen et al., 1977; Henck et al., 1981; Kimmig and Schultz, 1957; Kociba et al., 1978, 1979; McConnell, 1980; McConnell et al., 1978a,b; Moore et al., 1979; Norback and Allen, 1973; Olson et al., 1980; Schwetz et al., 1973; Turner and Collins, 1983; Vos et al., 1973; Vos and Beems, 1971; Vos and Koeman, 1970.

[b]Responses followed exposure to 2,3,7,8-TCDD or structurally related chlorinated hydrocarbons.

[c]Skin lesions in cattle have been observed, but they differ from the skin lesions observed in other species.

0 = lesion not observed, + = lesion observed (number of "+" denotes severity), ± = lesion observed to a very limited extent, blank = no evidence reported in literature.

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comparable to the liver lesions observed in rabbits and rats or to the liver changes observed in mice (McConnell et al., 1978b; Moore et al., 1979; Turner and Collins, 1983). In the hamster, manifest liver lesions do not occur even after fatal doses of TCDD; however, the ED_{50} for increased hepatic weight is only ~15 ug/kg (Gasiewicz et al., 1986). Liver-related enzyme activities in serum are elevated in those animal species where liver damage is a prominent sign of TCDD toxicity. In those animal species where hepatotoxicity is not as apparent, such as monkeys and guinea pigs, these enzyme activities are nearly normal.

Thymic atrophy has also been found in all animal species given lethal doses of TCDD. Treatment of animals with TCDD inhibits bone marrow hematopoiesis in mice, both in vivo and in vitro, by directly altering the colony growth efficiency of stem cells (Chastain and Pazdernik, 1985; Luster et al., 1980, 1985).

Among other signs and symptoms that have been demonstrated in various species, the following should be noted: hepatic porphyria, hemorrhages in various organs, testicular atrophy, reduced prostate weight, reduced uterine weight, increased thyroid weight, lesions of the adrenal glands, inhibited bone marrow hematopoiesis, decreased serum albumin, and increased serum triglycerides and free fatty acids. The details of all underlying studies for these observations have been extensively reviewed (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

Effects on heart muscle have also been observed in guinea pigs and rats (Brewster et al., 1987; Kelling et al., 1987; Canga et al., 1988). Five days after a single dose of TCDD (10 μg/kg intraperitoneally) was administered, a significantly decreased beta-adrenergic responsiveness was observed in the right ventricular papillary muscle of the guinea pig (Canga et al., 1988). In the TCDD-treated animals, a decrease in the positive inotropic effects of isoproterenol at 0.03-0.3 μM, but not at 0.1-10 nM, was also demonstrated. Additionally, the responsiveness to low-frequency stimulation and increases in extracellular calcium was enhanced in these animals. Based on these findings, the authors suggest that the heart may be a major target for TCDD toxicity.

In the monkey, several additional symptoms have been registered, such as periorbital edema, conjunctivitis, and thickening of the meibomian glands, followed by loss of the eyelashes, facial hair, and nails (McConnell et al., 1978a). These are symptoms similar to

those observed in cases of human intoxication (e.g., occupational exposure, the Seveso incident, and the Yusho and Yu-Cheng toxic oil intoxications (the latter involving exposure to PCBs and CDFs; see Chapter 1).

3.2.2. Studies In Vitro

Over 30 cell types, including primary cultures and cells from established and transformed cell lines derived from various tissues of at least six animal species, have been examined for their general cellular responses to TCDD (Beatty et al., 1975; Knutson and Poland, 1980a; Niwa et al., 1975; Yang et al., 1983a). The effects studied were changes in viability, growth rate, and morphology. Overall, there have been few or no effects documented.

However, other in vitro studies using more specific endpoints of toxicity have clearly indicated effects of TCDD at comparatively low concentrations. Thus, several studies have shown that TCDD affects cultured epidermal keratinocytes through interactions with differentiation mechanisms and that this effect may be regulated by the modulation of epidermal growth factor (EGF) binding to the cells (Hudson et al., 1986). Additionally, in epithelial cells of human origin, TCDD has been shown to alter differentiation (Hudson et al., 1985), while aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin O-deethylase (EROD) activity have been induced in vitro (see Section 3.5.4).

Wiebel et al. (1991) have identified a cell line (H4IIEC3-derived 5L hepatoma cells) that responds with decreased proliferation at low TCDD concentrations. Thus, half-maximum inhibition of proliferation occurs at a concentration of 0.1-0.3 nM, and the onset of the effect is fairly rapid, manifesting itself as early as 4-8 hours after treatment. Further studies have also demonstrated that insensitive variants of this cell line were deficient in cytochrome P-4501A1 activity and lacked measurable amounts of the Ah receptor (Göttlicher et al., 1990). In addition, 3,3',4,4'-TCB inhibited proliferation in the sensitive cell line, albeit at higher concentrations.

3.2.3. Appraisal

The numerous studies of acute toxicity in various species have demonstrated dramatic species- and strain-specific differences in sensitivity. However, the spectrum of symptoms is generally the same, although species differences exist.

Lethality is typically delayed by several weeks, and there is a pronounced wasting syndrome in almost all laboratory animals. Studies in congenic mice differing in their Ah responsiveness indicate that the sensitivity to acute toxicity of TCDD segregates with the Ah locus. Furthermore, studies of other CDDs, CDFs, and coplanar PCBs demonstrate that the potency for inducing lethality correlates with their ability to bind to the Ah receptor. In contrast, studies in various other species, as well as in various strains of rats, have demonstrated a wide range of sensitivities regardless of rather comparable levels of the Ah receptor.

3.3. SUBCHRONIC TOXICITY

The available studies on the subchronic toxicity of TCDD have been reviewed by the U.S. EPA (1984, 1985) and WHO/IPCS (1989). Overall, the signs and symptoms observed are in agreement with those observed after administration of single doses.

The study of Kociba et al. (1976) is of special interest as it has been used for comparisons of the relative toxicities of other CDDs and CDFs (Plüess et al., 1988 a and b). Adult male and female SD rats, in groups of 12, were given 0, 0.001, 0.01, 0.1, and 1.0 μg TCDD/kg bw by gavage 5 days/week for 13 weeks. At the end of the treatment period, five rats of each sex were sacrificed for histopathological examination. The remaining animals were observed for postexposure effects. The highest dose caused five deaths among the females, three during the treatment period and two after, while two deaths occurred in males in the posttreatment period. The rats given 0.01 μg TCDD/kg did not differ from the controls except for a slight increase in the mean liver-to-body weight ratio.

A 13-week dietary study in SD rats given 1,2,3,4,8-PeCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, or 1,2,3,6,7,8-HxCDF demonstrated that both the subchronic toxicity and depletion of hepatic vitamin A followed the rank order of the ability of the compounds to bind to the Ah receptor (to cause induction of AHH) (Plüess et al., 1988a and b; Håkansson

et al., 1990). However, the direct comparisons of the effects are hampered by the differences in toxicokinetic behavior of the compounds. Slightly different relationships with regard to toxicity were obtained in a tumor promotion study, where an initial loading dose (subcutaneous) of 2,3,4,7,8-PeCDF was given, followed by repeated lower doses (subcutaneous) in order to obtain a steady-state concentration (Wærn et al., 1991a). However, both of these studies support the assumption that most signs and symptoms obtained may be mediated through the Ah receptor.

In another study, groups of eight female SD rats were exposed to 16 weekly oral doses of 0, 0.01, 0.1, 1.0, and 10.0 μg TCDD/kg bw in a study primarily aimed at investigating TCDD-induced porphyria (Goldstein et al., 1982). The no-effect dose for porphyria was 0.01 μg/kg/week.

Only two studies of limited value have been performed in mice (Harris et al., 1973; Vos et al., 1973). Four weekly oral doses of 0.2, 1, 5, or 25 μg TCDD/kg bw were given to male C57Bl/6 mice in corn oil. No effects were noted at 1 μg, which corresponds to ~0.1 μg/kg bw/day.

In male and female Hartley guinea pigs, a 90-day feeding study of TCDD was performed by DeCaprio et al. (1986) where the surviving animals were subjected to extensive pathologic, hematologic, and serum chemical analyses. The diets contained 0, 2, 10, 76, or 430 ng TCDD/kg^{bw}. The two lowest doses, 2 and 10 ng/kg of diet, produced no dose-related alterations. Based on this study a no-observed-effect level of 0.6 ng TCDD/kg bw/day in guinea pigs was estimated. At the highest dose, severe body weight losses and mortality were observed. No dose-related mortality occurred at 76 ng/kg.

A cumulative dose of 0.2 μg TCDD/kg bw, which was divided into nine oral doses 3 times/week during days 20-40 of gestation, produced no clinical signs of toxicity in pregnant rhesus monkeys (*Macaca mulatta*) (McNulty, 1984). However, signs of toxicity such as body weight loss, epidermal changes, and anemia did occur in those monkeys who had received cumulative doses of 1.0 and 5.0 μg TCDD/kg bw over the same time period.

3.3.1. Appraisal

Utilizing the above data, subchronic NOAELs for rats, mice, and guinea pigs are estimated to be 0.01 μg, 0.1 μg, and 0.6 ng TCDD/kg bw/day, respectively. However, these studies cannot be directly compared with each other. Furthermore, none of the studies utilized initial loading doses, and due to the long half-life of TCDD, steady states may not have been reached in the animals except at the very end of the study periods. Distribution between tissues in the animals depends on both time of exposure and dose level (see Chapter 1), which further complicates any comparisons.

In spite of this, the limited data available seem to indicate that signs and symptoms of subchronic toxicity follow the same rank order as Ah receptor-mediated effects, such as induction of AHH.

3.4. CHRONIC TOXICITY

The results of chronic toxicity studies performed on laboratory animals exposed to TCDD are summarized in Table 3-3. Details have been reviewed by the U.S. EPA (1984, 1985) and WHO/IPCS (1989).

The most important study in rats is the chronic toxicity study of Kociba et al. (1978, 1979). Groups of 50 male and 50 female SD rats were fed diets providing daily doses of 0.001, 0.01, and 0.1 μg TCDD/kg bw for 2 years. Control rats, 86 males and 86 females, received diets containing the vehicle alone. Increased mortality was observed in females given 0.1 μg/kg/day, while increased mortality was not observed in male rats at this dose or in animals receiving doses of 0.01 or 0.001 μg/kg/day. From month 6 to the end of the study, the mean body weights of males and females decreased at the highest dose and, to a lesser degree, in females given 0.01 μg/kg/day. During the middle of the study, lower-than-normal body weights were also occasionally recorded in the low-dose group, although during the last quarter of the study, the body weights were comparable with those of the controls.

Increased urinary coproporphyrin and uroporphyrin were noted in females, but not in males, given TCDD at a dose rate of 0.01 and 0.1 μg/kg/day. Analyses of blood serum collected at terminal necropsy revealed increased enzyme activities related to impaired liver function in female rats given 0.1 μg TCDD/kg/day. Necropsy examination of the rats

Table 3-3. Studies on Chronic Exposure (Except for Studies on Cancer) to TCDD in Laboratory Animals

Species/Strain	Sex and No. per Group	Doses Tested	Treatment Schedule	Parameters Monitored	References
Rats/Sprague-Dawley	M/10	0, 1, 5, 50, 500, 1000, 5000, 50,000, 500,000, 1,000,000 ppt	continuous in diet for 65 weeks	survival	Van Miller et al., 1977
Rats/Sprague-Dawley	M, F/10	0.001, 0.01, 0.1 μg/kg/day	continuous in diet for 2 years	extensive histopathology, hematology and clinical chemistry	Kociba et al., 1978, 1979
Mice/Swiss	M/38-44	0, 0.007, 0.7, 7.0 μg/kg/week	gavage weekly for 1 year	histopathology	Toth et al., 1979
Mice/B6C3F1	M/50, F/50 M/75, F/75	0.01, 0.05, 0.5 μg/kg/week (males) 0.04, 0.2, 2.0 μg/kg/week (females) 0.0	gavage biweekly for 2 years	extensive histopathology	NTP, 1980
Monkey/*Macaca mulatta*	F/8	500 ppt	continuous in the diet for 9 months	extensive histopathology, hematology and clinical chemistry	Allen et al., 1977

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surviving TCDD exposure until the end of the study revealed that effects in the liver constituted the most consistent alteration in both males and females. Histopathological examination revealed multiple degenerative, inflammatory, and necrotic changes in the liver that were more extensive in females. Multinucleated hepatocytes and bile-duct hyperplasia were also noted. Liver damage was dose related, and no effect was observable at the low-dose rate. The NOAEL was estimated to be 0.001 μg/kg/day. At the end of the study, the fat and liver concentration of TCDD at this dose was 540 ppt.

In male Swiss mice, weekly oral doses of 0, 0.007, 0.7, and 7.0 μg TCDD/kg bw for 1 year resulted in amyloidosis and dermatitis (Toth et al., 1979). The incidence of these lesions was 0 of 38, 5 of 44, 10 of 44, and 17 of 43 in the control-, low-, medium-, and high-dose groups, respectively. The LOAEL in this study was estimated to be 0.001 μg/kg/day.

In the NTP (1980) gavage study in B6C3F1 male and female mice, no adverse effects were seen at the lowest dose tested (i.e., 0.01 and 0.04 μg/kg bw/week for males and females, respectively, corresponding to ~1.4 and 6 ng/kg bw/day).

The limited studies (9-20 months) available in rhesus monkeys (Allen et al., 1977; Barsotti et al., 1979; Schantz et al., 1978) revealed signs and symptoms similar to those recorded in more short-term studies. Adverse effects were noted down to the lowest dose tested (i.e., ~2-3 ng/kg bw/day for 20 months) (Schantz et al., 1978).

3.4.1. Appraisal

From the different long-term studies on TCDD, it can be estimated that the NOAEL for the rat is 1 ng/kg bw/day, corresponding to a fat and liver concentration (NOEL) of 540 ppt. For the male Swiss mouse, effects (dermatitis and amyloidosis in 5 of 44 animals) were noted at the lowest dose tested (i.e., the LOEL would be 1 ng/kg bw/day). However, in B6C3F1 mice, NOELs of 1.4 and 6 ng/kg/day were obtained for males and females, respectively. The studies in the rhesus monkey cannot be used for such a determination. Adverse effects were observed at the lowest dose tested, ~2-3 ng/kg body weight.

3.5. SPECIFIC EFFECTS

3.5.1. Wasting Syndrome

TCDD at high doses (lethal or near lethal) causes a starvationlike or wasting syndrome in several animal species. In young animals or following a sublethal dose to adults, this response is manifested as a cessation of weight gain. Animals exposed to near lethal or higher doses characteristically lose weight rapidly. Numerous studies utilizing pair-feeding, total parenteral nutrition, and everted intestinal sacs have been performed to elucidate the mechanisms behind the wasting syndrome (U.S. EPA, 1984, 1985; WHO/IPCS, 1989), but no single explanation has been obtained so far. No generalized impairment of intestinal absorption seems to occur.

Peterson et al. (1984) have suggested a model for the TCDD-induced wasting syndrome that is based on the assumption that body weight in rats is regulated to an internal standard or hypothalamically programmed set-point. Thus, body weight at a given age is constantly being compared to this set-point value, and if differences occur, feed consumption is adjusted. When TCDD lowers this set point, reduction in food consumption results as the rat attempts to reduce its weight to a new lower level. This hypothesis has been tested in several experiments under carefully controlled feeding conditions. Repeated studies have demonstrated that reduction of feed intake due to increased food spillage is sufficient to account for the loss of body weight in TCDD-treated SD rats. Additionally, TCDD-treated rats maintain and defend their reduced weight level with the same precision that *ad libitum*-fed control rats defend their normal weight level (Seefeld and Peterson, 1983, 1984; Seefeld et al., 1984a, b); the percentage of the daily feed intake that is absorbed by the gastrointestinal tract of TCDD-treated and control rats is similar (Potter et al., 1986; Seefeld and Peterson, 1984). Hypophagia was the major cause of adipose and lean tissue loss in male Fischer 344 rats, C57Bl/6 mice, and albino guinea pigs when exposed to a calculated LD_{80} dose of TCDD. Body weight loss followed a similar time-course in TCDD-treated and pair-fed control animals of all three species (Kelling et al., 1985).

Thus, body weight loss appears to contribute to lethality in a species- and strain-dependent fashion, but weight loss appears to play a greater role in causing death in SD rats and guinea pigs than it does in Fischer 344 rats and C57Bl/6 mice. Loss of body weight and

loss of appetite are also prominent signs of thyroid dysfunction. However, some data indicate that the effect of TCDD on thyroid hormones cannot explain the TCDD-induced decrease in body weight gain.

TCDD-induced wasting is always accompanied by the loss of adipose tissue. The rate of fat storage is determined by Lipoprotein lipase (LPL), which controls the serum level of triglycerides. Brewster and Matsumura (1984) found that the LPL activity was decreased in guinea pigs to 20 percent of the value of *ad libitum*-fed controls after 1 day, and this effect persisted throughout the study (10 days). Thus, the authors suggest that TCDD irreversibly reduces adipose LPL activity, thus making the animals less capable of adapting to nutritional changes and needs.

In a series of studies on Wistar rats, Lakshman et al. (1988, 1989, 1991) have demonstrated that single intraperitoneal injections of TCDD (from 1 μg/kg) caused a dose-dependent inhibition of fatty acid synthesis in the liver and the adipose tissue. The adipose tissue was found to be more sensitive than the liver. Furthermore, they found an increased mobilization of depot fat into the plasma compartment accompanied by an increase in plasma free fatty acid concentrations.

In vitro studies in isolated heart mitochondria have indicated that a TCDD concentration of 1.5 nmol/mg mitochondrial protein affects oxygen activation associated with cell respiration. Superoxide radicals and H_2O_2 were indicated to be involved in the development of the observed effects (Nohl et al., 1989).

Loss of muscle tissue accompanied by a decreased glucocorticoid receptor-binding capacity and an increased glutamine synthetase activity have been observed in male Fischer 344N rats given a single oral TCDD dose of 100 μg/kg (Max and Silbergeld, 1987).

Another biochemical effect associated with TCDD-induced wasting syndrome is the decrease in hepatic vitamin A storage in TCDD-exposed animals (Thunberg et al., 1979; Håkansson et al., 1989b, 1991). Vitamin A is necessary for growth, and vitamin A deficiency will result in depressed body weight gain as well as in reduced food intake. However, in contrast to TCDD-treated animals, the vitamin A-deficient animals continue to eat and grow, though body weight gain is less than normal (Hayes, 1971).

That decreased feed intake could be a result of a direct TCDD effect on the brain was initially indicated by Pohjanvirta et al. (1989), but this has been contradicted by later studies (Stahl and Rozman, 1990). The administration of TCDD at 50 μg/kg intraperitoneally to male SD rats caused a significant decrease in the serum concentration of prolactin detectable after 4 hours, compared to pair-fed vehicle controls and noninjected controls (Jones et al., 1987). The rapid onset of this effect suggests that it may be mediated by a pathway other than through interaction with the Ah receptor. Further studies have demonstrated that the effect of TCDD was reversed by pimozide, a dopamine receptor antagonist, and that the rate constant of dopamine depletion after α-methyl-*p*-tyrosine, as well as the turnover rate, were significantly elevated. This suggests a hypothalamic site of action of TCDD in their experiments (Russell et al., 1988).

Changes in intermediary metabolism have been demonstrated in TCDD-treated experimental animals. Conflicting data on effects on serum glucose and hepatic glucogen levels have been reported earlier (WHO/IPCS, 1989). Several recent studies have suggested that the ultimate cause of death in some mammalian species may be caused by a progressive hypoglycemia (Ebner et al., 1988; Gorski and Rozman, 1987; Gorski et al., 1990). However, in the guinea pig, serum glucose levels were not affected by treatment of the animals with TCDD (Gasiewicz and Neal, 1979). Slight reductions in serum glucose levels were noted in both Long-Evans and Han/Wistar rats (Pohjanvirta et al., 1989). Rozman et al. (1990) have suggested that the subchronic and chronic toxicities of TCDD are related to the inhibition of key enzymes of gluconeogenesis. They demonstrated that the induction of appetite suppression was preceded by the inhibition of PEPCK, which caused a reduction in gluconeogenesis. This was followed by a progressive increase in plasma tryptophan levels that was suggested to cause a serotonin-mediated reduction of the feed intake. In SD rats, TCDD in doses of 25 and 125 μg caused a rapid decrease (50 percent) in phosphoenolpyruvate carboxykinase (PEPCK) activity 2 days after dosing, followed by a dose-dependent decrease in glucose-6-phosphatase activity 4 or 8 days after exposure. Both appetite suppression and reduced PEPCK activity occurred in the same dose range (Weber et al., 1991). TCDD-induced impairments of carbohydrate synthesis have also been suggested by studies in chick embryos (Lentnek et al., 1991).

Numerous studies have measured serum levels of free fatty acids, cholesterol, and triglycerides in various species after TCDD treatment (WHO/IPCS, 1989), but no pronounced qualitative differences have been observed between species or strains of mice.

The wasting syndrome thus seems to be a generalized effect, elicited in all species and strains, but at various dosages (single or repeated administration). Specific studies have not been performed to elucidate if this syndrome is elicited through the interaction of TCDD with the Ah receptor. However, strong support for an Ah receptor-mediated mechanism comes from studies with other CDDs and CDFs. The binding affinities of various CDDs and CDFs to the Ah receptor as well as those of related PCBs have been shown to strongly correlate with their potency of induction of the wasting syndrome in both rats and guinea pigs (Safe, 1990).

3.5.2. Hepatotoxicity

TCDD induces hyperplasia and hypertrophy of parenchymal cells and, thus, hepatomegaly in all species investigated, even at sublethal doses. There is, however, considerable variation in the extent and severity of this lesion among the species tested. Other liver lesions are more species specific. Lethality following the administration of TCDD cannot be explained by these liver lesions alone, although they may be a contributing factor, at least in the rat and rabbit. The morphological changes in the liver are accompanied by impaired liver function, which is characterized by liver enzyme leakage, increased microsomal monooxygenase activities, porphyria, impaired plasma membrane function, hyperlipidemia, and increased regenerative DNA synthesis (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

The hepatotoxic reaction in various strains of rats given lethal doses of TCDD is characterized by degenerative and necrotic changes, with the appearance of mononuclear cell infiltration, multinucleated giant hepatocytes, increased numbers of mitotic figures and pleomorphism of cord cells, an increase in the hepatic smooth endoplasmatic reticulum, and parenchymal cell necrosis. The histological findings are accompanied by hyperbilirubinemia, hypercholesterolemia, hyperproteinemia, and increased SGOT and SGPT activities, further indicating damaged liver function (WHO/IPCS, 1989). These lesions may be severe enough

to be a contributing factor in death. The lesions observed after sublethal doses are qualitatively almost identical to those observed after lethal doses.

Earlier studies in mice have found similar effects. Recently, Shen et al. (1991) reported a comparative study on the hepatotoxicity of TCDD in Ah-responsive and Ah-nonresponsive mice (C57BL/6J and DBA/2J, respectively). C57BL/6J mice given a single dose of 3 μg/kg TCDD developed mild to moderate hepatic lipid accumulation but no inflammation or necrosis. Severe fatty change, mild inflammation, and necrosis occurred at 150 μg/kg. DBA/2J mice given 30 μg/kg developed hepatocellular necrosis and inflammation but no fatty change. Lipid accumulation was only slight after 600 μg/kg. The authors concluded that the Ah locus may be involved in determining the steatotic effects of TCDD.

The guinea pig shows less severe morphological alteration in the liver than other species. Likewise, the hamster exhibits little or no liver damage even after a fatal dose, but liver lesions have been observed after prolonged periods following the administration of nonlethal doses.

Several parameters relating to disturbed hepatic plasma membranae function have been studied (U.S. EPA, 1984, 1985; WHO/IPCS, 1989). ATPase activities were depressed and protein kinase C activity was increased in rats, but not in guinea pigs, treated with TCDD (Bombick et al., 1985). TCDD also induced a decrease in the binding of EGF. The relative doses of TCDD needed to suppress EGF binding to 50 percent of the control level were 1, 14, and 32 μg/kg for the guinea pig, the SD rat, and the Syrian Golden hamster, respectively (Madhukar et al., 1984). A single intraperitoneal dose of 115 μg TCDD/kg bw decreased the EGF binding by 93.1, 97.8, and 46.0 percent in C57Bl/6, CBA, and AKR mice, respectively, 10 days after treatment (Madhukar et al., 1984).

Further studies on the interaction of TCDD with EGF have been performed in congenic mice of the strain C57BL/6J (Lin et al., 1991a, b). The ED_{50} for the TCDD-induced decrease in maximum binding capacity of the EGF receptor was 10 times higher in the Ah-nonresponsive mice, compared to the Ah-responsive animals. This study supports the hypothesis that the effects of TCDD on EGF receptor ligand binding may be mediated by the Ah receptor.

The effects of TCDD on biliary excretion of various compounds have also been studied. Of special interest are studies on the excretion of ouabain, a model compound for neutral nonmetabolized substrates such as estradiol, progesterone, and cortisol, which was depressed in a dose-related manner by a single, oral dose of TCDD in rats (Yang et al., 1977, 1983b). The available data suggest that the hepatic membrane transport of ouabain may be selectively impaired by TCDD. Peterson et al. (1979a, b) have indicated that changes in ATPase activities are not responsible for the reduced ouabain excretion.

TCDD administration stimulates the accumulation of porphyrins in the liver and an increase in urinary porphyrin excretion. Indeed, during manifest porphyria, accumulation of porphyrins occurs not only in the liver but also in the kidney and spleen of rats (Goldstein et al., 1982).

Contradictory results on species variations have been published. It seems clear that porphyria can be produced in both mice and rats but the condition is always the result of subchronic or chronic administration. Exposure to single doses has not been demonstrated to produce porphyria. The mechanism underlying the induction of porphyria is not elucidated. Cantoni et al. (1981) exposed rats orally to 0.01, 0.1, and 1 μg TCDD/kg bw/week for 45 weeks and increased coproporphyrin levels were observed at all dose levels. A marked porphyric state appeared only at the highest dose tested, after 8 months of exposure.

TCDD is a potent inducer of rodent and murine ALA-synthetase, the initial and rate-limiting enzyme involved in heme synthesis. However, increased ALA activity was not found in mice exposed to 25 μg TCDD/kg bw/week for 11 weeks, despite porphyria being evident (Jones and Sweeney, 1980). Thus, the induction of ALA-synthetase does not seem to be a necessary event in TCDD-induced porphyria. A more likely suggestion is that decreased hepatic porphyrinogen decarboxylase is the primary event in porphyria induced by halogenated aromatics (Elder et al., 1976, 1978). TCDD depresses this enzyme activity in vivo in the liver of mice (Cantoni et al., 1984a, b; Elder and Sheppard, 1982; Jones and Sweeney, 1980), but not in vitro (Cantoni et al., 1984b).

A comparative study of TCDD-induced porphyria has not been conducted in responsive and nonresponsive mice. However, in a study on Ah-responsive (Ah^b) and Ah-nonresponsive (Ah^d) C57BL/6J female mice, the urinary excretion of porphyrins was

examined after treatment of the animals with hexachlorobenzene for ≤ 17 weeks (Hahn et al., 1988). After 15 weeks of treatment with 200 ppm hexachlorobenzene in the diet, the excretion of porphyrins was 200 times higher in the Ah^b mice, compared with controls. In contrast, the Ah^d mice only showed a sixfold increase. Induction of P-450c(1A1) was observed only in Ah^b mice, while induction of P-450d(1A2) was observed in both strains, but to a lesser degree in the Ah^d mice.

3.5.3. Epidermal Effects

Chloracne and associated dermatological changes are widespread responses to TCDD in humans. However, this type of toxicity is expressed only in a limited number of animal species (i.e., rabbits, monkeys, and hairless mice).

In the rabbit ear bioassay, a total dose of 80 ng TCDD gave a chloracnegenic response, while no response was obtained when the total dose applied to the ear was 8 ng (Jones and Krizek, 1962; Schwetz et al., 1973). The application of TCDD in various vehicles has been demonstrated to markedly decrease this response (Poiger and Schlatter, 1980). The hairless mouse is a less sensitive model for chloracnegenic response than is the rabbit ear bioassay (Knutson and Poland, 1982; Puhvel et al., 1982). However, following repeated applications of ~ 0.1 μg TCDD over several weeks, an acnegenic response was noted in the hairless mouse strains, SkH:HR1 and HRS/J. An acnegenic response was also caused by repeated applications of 2 mg of 3,4,3′,4′-TCB (Puhvel et al., 1982). Female HRS/J hairless mice have also been used to test the dermal toxicity and skin tumor-promoting activity of TCDD, PeCDF, and HxCDF (Hebert et al., 1990a). All of the tested compounds induced coarse, thickened skin with occasional desquamation; these effects were more severe after the application of PeCDF and HxCDF.

Keratinocytes, the principal cell type in the epidermis, have been utilized as an in vitro model for studies of TCDD-induced hyperkeratosis both in human- and animal-derived cell cultures. The response to TCDD is analogous to the hyperkeratinization observed in vivo.

A TCDD-induced keratinization response in vitro was first demonstrated in a keratinocyte cell line derived from a mouse teratoma (XB cells). The keratinization was

dose-related (Knutson and Poland, 1980b). Late-passage XB cells (termed XBF cells) lost their ability to respond by keratinization after TCDD treatment. Both XB cells (keratinization assay) and XBF cells (flat-cell assay) have proven to be useful in in vitro bioassays to determine the dioxinlike activities of both environmental samples and pure isomers (Gierthy and Crane, 1985a, b; Gierthy et al., 1984).

Several continuous lines of human keratinocytes, derived from neonatal foreskin or squamous cell carcinomas, have been shown to respond to TCDD in nM concentrations with a variety of signs indicating alterations in the normal differentiation process (WHO/IPCS, 1989). The responses include decreased DNA synthesis, decreased number of proliferating basal cells, decreased binding of EGF, and an increase in the state of differentiation (Osborne and Greenlee, 1985; Hudson et al., 1986). The responses were also obtained with TCDF, but not with 2,4-diCDD (Osborne and Greenlee, 1985). TCDD has also been shown to inhibit high-density growth arrest in human squamous carcinoma cell lines, and, indeed, the minimum concentration for increases in cell proliferation was 0.1 nM in the most sensitive cell line (SCC-15G). In studies on the same cell lines, a modulating effect of the transforming growth factor beta could not be demonstrated (Hebert et al., 1990 b, c).

3.5.4. Enzyme Induction

TCDD has repeatedly been found to increase the activities of various enzymes. While observations of enzyme inhibition have also been made, enzyme induction has been one of the most extensively studied biochemical responses produced by TCDD. The mixed-function oxidase (MFO) system is the most thoroughly investigated, and AHH and EROD (as markers for CYP1A1 induction) are the most frequently assayed enzyme activities. The induction of MFO activities might potentiate the toxicity of other foreign compounds that require metabolic transformation by the MFO system before they can exert their toxic effects. Furthermore, increased MFO activities might adversely affect important metabolic conversions of endogenous compounds. TCDD has also been reported to affect a variety of other enzymes (e.g., UDPGT and GST) that are components of multifunctional enzyme systems involved in the conjugation, biotransformation, and detoxification of a wide variety of endogenous and exogenous compounds.

Several investigators have studied the relative potency of various halogenated dioxins, dibenzofurans, and biphenyls to induce AHH and/or EROD activities (Safe, 1990). An apparent structure-activity relationship was found between the location of the halogen atoms on the dibenzo-*p*-dioxin molecule and the ability to induce AHH activity both in vivo and in vitro. Isomers with halogens at the four lateral ring positions produced a greater biological response than those with halogens at three lateral ring positions, while two lateral halogen atoms seemed to be insufficient to produce a biological response. Numerous studies have indicated that there is very strong agreement between the Ah-binding affinity of various CDDs, CDFs, and related PCBs and their potency to induce AHH, both in vivo and in vitro (Safe, 1990). Structure-activity studies have also demonstrated clear correlation between the toxicity and induction potency of a series of CDDs, CDFs, and coplanar PCBs (Poland and Glover, 1973; Safe, 1990).

On a molecular basis, TCDD is the most potent MFO-inducing compound known, and MFO induction seems to be the most sensitive biochemical response produced. Measurements of the induction of AHH or EROD (mediated through CYP1A1) are considered to be very sensitive markers of the TCDD-induced enzyme induction. According to Kitchin and Woods (1979), induction in the rat takes place at doses as low as 0.002 μg TCDD/kg bw. The NOEL for a single administration to rats seems to be 1 ng/kg, while a single dose of 3 ng/kg causes a detectable induction of AHH or EROD (Kitchin and Woods, 1979; Abraham et al., 1988).

Enzyme induction has also been observed in the offspring of various species after prenatal and postnatal (milk) exposure to TCDD (Lucier et al., 1975; Korte et al., 1990; Wærn et al., 1991b).

The effect of TCDD on enzyme activities has been most frequently investigated in the rat (WHO/IPCS, 1989). In the liver, TCDD has been shown to increase both the contents of cytochrome P-450lA1 and cytochrome P-450lA2, as well as other microsomal enzyme activities involved in the oxidative transformation and conjugation of xenobiotics (e.g., aniline hydroxylase, AHH, biphenyl hydroxylase, ECOD, EROD, and UDPGT) (U.S. EPA, 1984, 1985; WHO/IPCS 1989).

TCDD also affects some other hepatic enzymes not related to the MFO system, including aldehyde dehydrogenase, ß-aminolevulinic acid synthetase, DT-diaphorase, transglutaminase, ornithine decarboxylase, transaminases (ALT and AST), plasma membrane ATPases, porphyrinogen carboxylase, prostaglandin synthetase, enzymes involved in testosterone metabolism, and RNA polymerase (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

Studies in different species also have revealed that enzyme induction due to TCDD exposure is both a species- and strain-specific phenomenon. Pohjanvirta et al. (1988) studied enzyme induction in the Long-Evans and Han/Wistar (Kuopio) rat strains (LD_{50}, ~ 10 and $>3{,}000$ μg/kg, respectively). Differences in the inducibility of EROD, ECOD, or ethylmorphine N-demethylase were not found, nor were there any differences with regard to the amount of available Ah receptor or the amount of cytochrome P-450 in the hepatic microsomal fractions. Similarly, differences regarding possible induction of UDPGT were absent (Pohjanvirta et al., 1990).

Enzyme induction studies on mice have been performed mainly with strains that are genetically different at the Ah locus, thus making them responsive or nonresponsive to the induction of hepatic cytochrome P-4501A1-related enzyme activities. Qualitatively and in general, the same responses can be obtained in both strains, but there may be more than one order of magnitude difference with regard to the doses required to elicit a response. TCDD is thus 10-fold more potent in inducing hepatic cytochrome P-450lA1 and the related AHH activity in C57BL/6J mice (Ah-responsive) than in DBA/2 mice (Ah-nonresponsive) (Poland and Knutson, 1982; Nebert, 1989).

In contrast, although the guinea pig is the most sensitive species to the toxic effects of TCDD, it does not respond to the administration of TCDD with liver toxicity or with extensive enzyme induction. Indeed, even at lethal doses, the induction of MFO is only very slight (Beatty and Neal, 1977; Håkansson et al., 1992).

The data on enzyme induction in rabbits are rather limited and also somewhat conflicting with regard to increases in the amount of cytochrome P-450 (Hook et al., 1975; Liem et al., 1980).

Similarly, hepatic enzyme induction has been only partially studied in Syrian Golden hamsters. When hamsters were given a lethal dose of TCDD, increased hepatic GST and

glutathione reductase activities were found. The ED_{50} values for the induction of hepatic ECOD and reduced NADP:menadione oxidoreductase activities and cytochrome P-450 content in male Syrian Golden hamsters were 1.0, 2.0, and 0.5 μg TCDD/kg bw, respectively (i.e., extremely low doses, compared to doses that produce tissue damage and lethality in this species) (Gasiewicz et al., 1986).

In a comparative study of EROD induction in guinea pigs, rats, C57BL/6 and DBA/2 mice, and Syrian Golden hamsters, the animals were given single doses that were intended to be equitoxic (i.e., 1, 40, 100, 400, and 400 μg TCDD/kg, respectively) compared with the acute toxicity for the respective species and strain. EROD induction was noted in all species except for the hamster. During the observation period (112 days), the EROD induction dropped to more or less normal values in all rats and mice, while the induction (albeit low compared with the other species) was sustained for the whole period in the guinea pig (Håkansson et al., 1992).

The N-demethylation of caffeine has been applied as a noninvasive method for studying enzyme induction in vivo. Studies on the marmoset monkey (*Callithrix jacchus*) utilizing ^{14}C-labeled caffeine and measuring $^{14}CO_2$ exhalation by a breath test has indicated a NOEL of 1 ng/kg and a LOEL of 3 ng/kg (Kruger et al., 1990). Although the authors state that the N-demethylation of caffeine probably was P-450lA1 dependent, studies by Butler et al. (1989) indicate that this reaction is dependent on cytochrome P-450lA2.

In the chick embryo, both AHH and δ-aminolevulinic acid synthetase have been reported to be extremely sensitive to the inductive effects of TCDD and related compounds (Poland and Glover, 1973; Brunström and Andersson, 1988; Brunström, 1990).

Although TCDD is relatively nontoxic in cell cultures, it is a very potent inducer of AHH or EROD activities in in vitro systems, including lymphocytes and primary hepatocytes, as well as established and transformed cell lines.

The ED_{50} values for AHH induction by TCDD have been determined in 11 established cell lines and in fetal primary cultures from five animal species and cultured human lymphocytes. The values ranged from 0.04 ng/mL medium in C57Bl/6 mouse fetal cultures and 0.08 ng/mL in the rat hepatoma H-4-II-E cell line to >66 ng/mL in the HTC rat hepatoma cell line (Niwa et al., 1975). Several cultured human cells or cell lines

including lymphocytes (Atlas et al., 1976), squamous cell carcinoma lines (Hudson et al., 1983), breast carcinoma cell lines (Jaiswal et al., 1985), and lymphoblastoid cells (Nagayama et al., 1985) have been shown to be inducible for AHH activity by TCDD.

TCDD was demonstrated to be the most potent AHH inducer of 24 chlorinated dibenzo-*p*-dioxin analogues (Bradlaw et al., 1980) in a rat hepatoma cell culture (H-4-II-E) that is extremely sensitive to AHH induction. The EC_{50} values for AHH and EROD induction in the same cell system varied over 7 orders of magnitude for 14 different CDDs, the most potent being TCDD and the least potent being 2,3,6-triCDD (Mason et al., 1986).

3.5.5. Appraisal

Based on data from Kitchin and Woods (1979), Abraham et al. (1988), and Kruger et al. (1990), Neubert (1991) has calculated NOEL values for enzyme induction in both rats and marmoset monkeys to a single dose of 1 ng/kg bw. At this dose, the tissue concentrations for both species were found to be 4 ppt for adipose tissue and 3 ppt for the liver. It is interesting to note that the wide range of sensitivities toward the acute toxicity of TCDD is also reflected in a wide range of sensitivities for enzyme induction both in vivo and in vitro. However, it is evident that the guinea pig is fairly insensitive to enzyme induction, while the hamster is highly sensitive in this respect.

Finally, it is evident that the structure-activity relationships revealed from in vitro testing correlate fairly well with in vivo studies within a given species or strain.

3.5.6. Endocrine Effects

Alterations in endocrine regulation have been suggested from human exposure to TCDD that resulted in hirsutism and chloracne. Chronic exposure to TCDD causes impaired reproduction in experimental animals, possibly by interfering with the estrus cycle in combination with some steroidlike activities of TCDD. This has prompted studies on the interaction of TCDD with steroid hormones and their receptors.

Increased systemic levels of glucocorticoids may mimic some of the symptoms of TCDD toxicity (e.g., involution of lymphoid tissues, edema, and mobilization of fatty acids from adipose tissues). Thus, TCDD has been suggested to increase glucocorticoid activity

through indirect effects on glucocorticoid receptors. Poland et al. (1976) demonstrated that cortisol and synthetic glucocorticoids did not bind to the TCDD receptor.

Conflicting data have been reported on TCDD-induced levels of glucocorticoids. However, significant changes to the liver cytosolic glucocorticoid receptor were induced by TCDD at doses 10,000-fold lower in adrenalectomized SD rats, compared with control rats (Sunahara et al., 1989). The data furthermore indicate that it is the binding properties of the receptor that are affected rather than the amount of receptor protein. Studies in congenic strains of Ah-responsive and Ah-nonresponsive C57BL/6J female mice (Goldstein et al., 1990; Lin et al., 1991a, b) have also demonstrated that TCDD decreased the maximum binding capacity of the hepatic glucocorticoid receptor in both strains of mice by ~30 percent. Differences in dose-response curves between the different strains could not be observed. These data suggest that this effect may be mediated by a pathway different from that mediated by the Ah receptor.

Steroids are endogenous substrates for the hepatic MFO system. TCDD, which influences the activity of this enzyme system, may thus alter steroid metabolism in vivo and, consequently, also the magnitude of steroid-mediated functions.

Early studies also reported contradictory data on changes in steroid levels. However, Umbreit and Gallo (1988) suggest that estrogen receptor modulation and the animal's physiological response to this modulation can explain some of the toxicity observed in TCDD-treated animals. The susceptibility of different species to TCDD correlates, to some extent, with their steroid glucuronidation capacity. Thus, hamsters have low steroid UDPGT activity while guinea pigs have a corresponding high activity. Another example is given by comparing the SD and Gunn rat, the latter being defective in producing some UDPGTs. The homozygous Gunn rat is 3-10 times more resistant to the effects of TCDD than is the SD rat (Thunberg, 1984; Thunberg and Håkansson, 1983). However, the results of TCDD exposure in various species and strains are complex. [In order to counteract the TCDD-induced modulation of the estrogen receptor, the effects observed will be dependent on the ability of the organism to synthesize and excrete estrogens.] Interactions of TCDD and related compounds with estrogen have recently been reviewed by Safe et al. (1991).

The importance of estrogens as modulators of TCDD-induced toxicity has also been demonstrated by Lucier et al. (1991), who found that the tumor-promoting effects of TCDD could be effectively prevented by removing the ovaries from female rats before exposure to TCDD. This finding agrees well with the results obtained from the long-term bioassays that demonstrated liver tumors only in female rats (Kociba et al., 1978; NTP, 1982).

In studies on congenic strains of Ah-responsive and Ah-nonresponsive C57BL/6J female mice, a statistically significant difference in the responsiveness of the hepatic estrogen receptor was found, thus indicating that the Ah receptor regulates the effects of TCDD on the binding of estrogen to the hepatic estrogen receptor (Goldstein et al., 1990; Lin et al., 1991a, b).

TCDD-induced changes in levels or activities of testosterone or its metabolites have been reported from several studies (Keys et al., 1985; Mittler et al., 1984; Moore and Peterson, 1985; Neal et al., 1979). A single oral dose of 50 μg TCDD/kg bw increased the plasma corticosterone level in SD rats 7 and 14 days postexposure (Neal et al., 1979). However, a single oral dose of 25 μg TCDD/kg bw has also been shown to decrease the plasma corticosterone in SD rats 14 and 21 days postexposure. It is essential to note that Neal et al. (1979) also observed a slight decrease in serum corticosterone during days 1-4 posttreatment.

Mittler et al. (1984) demonstrated a decreased activity of testicular 16-α-testosterone hydroxylase, 6-ß-hydroxytestosterone, and 7-α-hydroxytestosterone in young SD rats 90 hours postexposure to single intraperitoneal doses of 0.2, 1, or 5 μg TCDD/kg bw.

A single dose of 0.06 μmol TCDD/kg bw decreased levels of 3α-, 6α-, and 16ß-hydroxytestosterone; an increase of 7α-hydroxytestosterone have also been observed in young male Wistar rats (Keys et al., 1985). Moore et al. (1985) also noted decreases in serum testosterone and dihydrotestosterone levels in 15 μg TCDD/kg bw-dosed male SD rats. The data do not, however, allow for any conclusions with regard to the possible relationship to receptor-mediated toxicity. TCDD induces several enzymes related to testosterone metabolism, which suggests that the changes observed may be secondary to the induction of various enzymes. Serum testosterone and dihydrotestosterone were found to be dose-dependently depressed by TCDD treatment in male SD rats, when compared with pair-fed

and *ad libitum*-fed controls. The ED_{50} for this effect was ~15 μg/kg (Moore et al., 1985). It was further shown that testosterone synthesis was decreased in the animals due to depressed production of pregnenolone by the testis (Kleeman et al., 1990). In the same strain of rats, a single oral dose of TCDD of 100 μg/kg was found to cause a 55 percent decrease in testicular cytochrome $P\text{-}450_{scc}$ activity but also to cause the inhibition of the mobilization of cholesterol to cytochrome $P\text{-}450_{scc}$. The authors concluded that the latter effect probably was responsible for the inhibition of testicular steroidogenesis (Moore et al., 1991). Maternal exposure to TCDD has been shown to affect the male reproductive system at low doses (i.e., the lowest dose tested was 64 ng/kg) (Mably et al., 1991, 1992a, b, c) (see Chapter 5).

In ovo exposure of white Leghorn chickens to TCDD in the dose range of 1-10,000 pmol/egg increased the cardiac release of prostaglandins (Quilley and Rifkind, 1986). Studies on chick embryos have indicated that the induction of cytochrome P-450 by TCDD results in a major increase in the NADPH-dependent metabolism of arachidonic acid (Rifkind et al., 1990). These effects are thus clearly related to receptor-mediated enzyme induction.

Rather conflicting data have been published regarding TCDD-induced effects on thyroid hormones (WHO/IPCS, 1989). The available data on serum T4, T3, and TSH levels are not sufficient to state whether or not TCDD-treated rats are functionally hypothyroidic, euthyroidic, or hyperthyroidic.

However, Brouwer (1987) has demonstrated that a dioxinlike PCB (i.e., 3,4,3′,4′-TCB, through a rapidly produced metabolite, 5-OH-TCB) binds to TTR. This binding causes interactions with the physiological functions of TTR and thyroid hormone transport is severely affected. This finding may explain some of the characteristic toxicological lesions found after PCB exposure.

3.5.7. Vitamin A Storage

Decreased hepatic vitamin A storage has been reported in animals exposed to various chlorinated aromatic compounds. Because minute quantities are needed to produce its effect and its persistence in nature TCDD is unique in its ability to reduce the vitamin A content of the liver. A single oral dose of 10 μg TCDD/kg bw decreased both the total amount and the

concentration of vitamin A in the liver of adult male SD rats (Thunberg et al., 1979). The decrease was evident 4 days after dosing and progressed with time. After 8 weeks, the treated animals had a total liver vitamin A content corresponding to 33 percent of that of controls. Decreased dietary intake of vitamin A could not account for this difference. A significant increase in the UDPGT activity was observed, suggesting an increased excretion of glucuronide-conjugated vitamin A. However, no correlation between the UDPGT activity and the hepatic vitamin A reduction was seen when homozygous Gunn rats lacking inducible UDPGT (Aitio et al., 1979) and heterozygous Gunn rats with inducible UDPGT were treated with a single, oral dose of 10 μg TCDD/kg bw (Thunberg and Håkansson, 1983).

In a study combining pair-feed restriction and a single TCDD treatment, it was found that the decreases in liver reserves of vitamin A were not related to a decreased intake of vitamin A via the diet (Håkansson et al., 1989a).

Puhvel et al. (1991) reported a comparative study in which congenic haired (+/+) and hairless (hr/hr) HRS/J mice were fed a vitamin A-deficient diet and treated topically with TCDD. The sensitivity to TCDD-induced cutaneous changes was essentially 100 times higher in hairless mice than in haired mice (0.01 and 1.0 μg 3 times/week for 3 and 2 weeks, respectively). In the haired phenotype, the effects of vitamin A depletion by itself were not seen by cutaneous histology, nor were any changes in cutaneous morphology attributable to TCDD observed. In the hairless mice, however, vitamin A deficiency increased the keratinization of dermal epithelial cysts and increased the sensitivity of these cysts to TCDD-induced keratinization. Analysis of vitamin A demonstrated that TCDD-exposure did not affect cutaneous levels of the vitamin but did significantly lower levels of vitamin A in the liver. TCDD-induced body weight loss and atrophy of the thymus glands were not affected by the vitamin A status in either strain.

In a study on tumor promotion by TCDD, utilizing the induction of enzyme-altered hepatic foci in the liver and performed on female SD rats, Flodström et al. (1991) found that vitamin A deficiency by itself enhanced foci development. The effect of TCDD treatment was also markedly enhanced, as were other TCDD-induced toxicities including thymus atrophy.

Several studies have been performed to elucidate the mechanism of TCDD-vitamin A interaction. Håkansson et al. (1989c) and Håkansson and Hanberg (1989) have demonstrated that TCDD specifically inhibits the storage of vitamin A in liver stellate cells. Brouwer et al. (1989) demonstrated that a single dose of TCDD (10 μg/kg) to female SD rats reduced vitamin A in the liver, lung, intestines, and adrenal glands, while increasing its concentration in serum, kidneys, and urine. They also found a 150 percent increase in the free fraction of serum retinol binding protein. Taken together, all of these data in the rat indicate that TCDD induces an increased mobilization of vitamin A from hepatic and extrahepatic storage sites into the serum, accompanied by an enhanced elimination of the vitamin via the kidney into the urine.

In a comparative study of TCDD toxicity in male SD rats and Hartley guinea pigs (Håkansson et al., 1989b), the animals were given single intraperitoneal doses of 40 and 0.5 μg/kg bw, respectively (i.e., comparable fractions of their respective LD_{50}). In these species there were similar reductions in hepatic vitamin A, while serum and renal vitamin A concentrations were increased in the rat but unaffected in the guinea pig. Hepatic EROD activity was markedly increased in the rat but unchanged in the guinea pig. Furthermore, although rats seemed to recover from the wasting, thymic atrophy, and liver enlargement, and resumed their ability to store vitamin A in the liver at 4-8 weeks after exposure, no such trends for wasting and vitamin A storage were observed in guinea pigs, even 16 weeks after exposure. A complementary study also included C57BL/6 mice, DBA/2 mice, and Syrian Golden hamsters (Håkansson et al., 1991). The effects on TCDD-induced decrease of vitamin A in the liver and the lung correlated reasonably well with other toxic symptoms observed in the animals. On the other hand, studies on two strains of rats, Long-Evans and Han/Wistar (the Han/Wistar being >300 times more resistant to TCDD toxicity) could not demonstrate significant differences in the TCDD-induced changes in vitamin A in the liver, kidney, testicles, or serum after a sublethal dose (4 μg/kg) (Pohjanvirta et al., 1990). These findings show that the correlations between TCDD-induced lethality and changes in vitamin A status found among other species also apply to these strains of rats.

The interaction of 3,4,3′,4′-TCB with vitamin A has been studied by Brouwer and Van den Berg (1983, 1984, 1986), Brouwer et al. (1985, 1986a, b), and Brouwer (1987).

The effects of TCB on vitamin A differ in many respects from those of TCDD. TCB is rapidly converted in vivo into a polar 5-OH-TCB metabolite, which binds with a relatively high affinity to TTR. As a consequence of this interaction, the physiological functions of TTR in retinoid and thyroid hormone transport are severely affected in TCB-exposed animals. The model proposed by Brouwer (1987) may explain some of the characteristic toxicological lesions related to PCB exposure. This mechanism of action seems to be clearly separated from the Ah receptor-mediated toxicity of CDDs and CDFs. Hydroxylated metabolites of TCDD have also been demonstrated to bind in a similar manner to TTR (Lans et al., 1992). However, due to the very slow metabolism of TCDD (or other 2,3,7,8-substituted CDDs/CDFs), this mechanism of toxicity probably plays a very minor role in the toxicity.

Taken together, these data indicate that TCDD interferes with the storage mechanism for vitamin A. Because supplementation of dietary vitamin A seems to be unable to counteract all of the observed toxic effects, this would imply either that the effect on vitamin A storage is secondary to TCDD toxicity or that the cellular utilization of vitamin A is affected by TCDD.

3.5.8. Lipid Peroxidation

Lipid peroxidation and oxidative stress have been indicated as factors that affect the acute toxicity of TCDD (WHO/IPCS, 1989; Wahba et al., 1989a, b, 1990a, b; Pohjnavirta et al., 1989; Alsharif et al., 1990; Stohs et al., 1990). Among the effects noted have been membrane lipid peroxidation, decreased membrane fluidity, and increased incidence of single-strand breaks in DNA. Studies relating these observations to the Ah receptor have not been performed. However, when considering the available data on TCDD and lipid peroxidation, it is not possible to attempt to define a relationship between lipid peroxidation and TCDD-induced lethality.

3.6. MECHANISMS OF TOXICITY

Despite extensive research to elucidate the ultimate event(s) underlying the toxic action of TCDD, definite information is not yet available. The toxicity of TCDD apparently depends on the fact that the four lateral positions of the molecule are occupied by chlorine. Toxicity decreases with decreasing lateral substitution and increasing total chlorine substitution. TCDD toxicity involves many different types of symptoms; these symptoms vary from species to species and from tissue to tissue, both quantitatively and qualitatively. Furthermore, age- and sex-related differences in sensitivity have been reported. Another characteristic of TCDD toxicity is the delay before all the endpoints of toxicity are manifested (from 2 weeks to 2 months), which is seen in all species.

Polymorphism in the Ah locus, which has been suggested to be the structural gene for the cytosolic receptor, seems to determine the sensitivity of genetically different strains of mice to TCDD and congeners. Ah-responsive strains of mice (e.g., C57Bl/6) are characterized by high hepatic levels of the TCDD-receptor protein, highly elevated levels of hepatic cytochrome P-4501A1 and associated enzyme activities in response to treatment with 3-MC (3-methylcholanthrene), and sensitivity to the ulcerative action of DMBA (7,12-dimethylbenz(a)anthracene) on the skin. Ah-nonresponsive mice (e.g., DBA/2) lack these characteristics.

Based on these findings, several genetic studies have been performed to elucidate the role of the receptor in TCDD toxicity. In contrast to 3-MC, TCDD induces AHH activity and several toxic effects both in Ah-responsive and Ah-nonresponsive strains of mice. However, the dose required to produce the effect in an Ah-nonresponsive strain is approximately 10-fold greater than that needed in an Ah-responsive strain. This indicates that the Ah-nonresponsive strain also contains the TCDD receptor but this receptor is defective (Okey and Vella, 1982). Data from studies of DBA/2 mice given either single or multiple doses of TCDD (Jones and Sweeney, 1980; Smith et al., 1981) suggest that the LD_{50} in this strain of mice is at least fivefold greater than the values recorded for the C57Bl/6 and C57Bl/10 strains (Jones and Greig, 1975; Smith et al., 1981; Vos et al., 1974). TCDD-induced hepatic porphyria has also been shown to segregate with the Ah locus in mice (Jones and Sweeney, 1980). The correlative differences between the C57Bl/6 and DBA/2 strains of

mice, in terms of altered specific binding of TCDD and sensitivity to this compound, may be unique and may not be applicable to other species (Gasiewicz and Rucci, 1984).

In a genetic-crossing experiment between Long-Evans and Han/Wistar rats (Pohjanvirta, 1990), it was demonstrated that the F_1 offspring were as resistant to TCDD toxicity as the Han/Wistar rats (LD_{50}, >3,000 μg/kg). Further studies on the F_2 generation indicated that the distribution of resistant and susceptible phenotypes was consistent with inheritance regulated by two (possibly three) autosomal genes displaying complete dominance, independent segregation, and an additive coeffect. Thus, in contrast to the findings in mice, TCDD resistance seems to be a dominant trait in the rat.

Less convincing evidence for the model of a receptor-mediated toxicity of TCDD arises from studies of the toxicity, receptor levels, and/or enzyme induction of TCDD in various species, tissues, and cell cultures. Despite enormous variability in the recorded LD_{50} values for the guinea pig, rat, mouse, rabbit, and hamster, the amounts and physical properties of the hepatic as well as extrahepatic receptors are comparable in these species (Gasiewicz and Rucci, 1984; Poland and Knutson, 1982). Furthermore, although the recorded LD_{50} values for TCDD vary >100 times between the chick embryo, the C3H/HeN mouse, and the SD rat, the ED_{50} doses for AHH induction in these species are comparable (Poland and Glover, 1974). Even between strains of rats with a difference of >300 times in LD_{50}, no differences in enzyme induction could be demonstrated (Pohjanvirta et al., 1988). In the guinea pig, the most TCDD-susceptible species, AHH induction is not a prominent symptom, even at lethal doses of TCDD. A number of cell types, including primary cultures and established and transformed cell lines from several species and tissues, are inducible for AHH activity, indicating the presence of the receptor, yet toxicity is not expressed in these systems (Knutson and Poland, 1980a). The available data thus suggest that the receptor for TCDD may be a prerequisite but is not sufficient in itself for the mediation of toxicity.

TCDD toxicity mimics in many respects endocrine imbalance, although evidence indicating a direct involvement of hormones in the toxic action of TCDD does not exist. However, the studies by Lucier et al. (1991) clearly indicate the importance of interactions with estrogen regulation.

The most reliable and consistent symptom of TCDD toxicity among all experimental animals is that of weight loss. The cause of the body weight loss seems to be reduced food intake apparently occurring secondarily to a physiological adjustment that reduces the body weight to a maintenance level that is lower than normal. The physiological trigger for this body weight set-point might be a target for TCDD.

The ability of TCDD to impair vitamin A storage may be responsible for some of the toxic effects produced by TCDD.

3.7. CONCLUSIONS

From the complex picture that evolves from the above outlined data, it is amply evident that TCDD elicits a plethora of toxic responses, both after short-term and long-term exposure. The lowest doses (single or repeated) that have been demonstrated to elicit various biological responses in certain animals have been compiled in Table 3-4. The analysis of the various signs and symptoms that occur in various species and strains may lead to the following conclusions:

- When comparing species and strains, it is clearly evident that there are enormous differences in the sensitivity to specific TCDD-induced toxicities. This conclusion is valid for almost all the responses studied. Qualitatively, however, there seems to be fairly good agreement between the types of responses that can be observed (i.e., almost all responses can be produced in every species and strain if the right dose is chosen). In highly sensitive species (e.g., the guinea pig), lethality may prevent a response from occurring.

- Our present knowledge rules out enzyme induction, as such, as being the cause of toxicity and death. Although the toxicokinetics of TCDD vary between species, these differences are not sufficient to explain the variabilities in sensitivity to TCDD toxicity (see Chapter 1). The available data indicate an involvement of TCDD in processes regulating cellular differentiation and/or division as well as those controlling estrogen homeostasis. Alterations in the regulation of such processes, which are not equally active in all cells throughout the organism, would be expected to result in effects that vary among tissues as well as among species.

Table 3-4. Lowest Effect Levels for Biological Responses of 2,3,7,8-TCDD in Experimental Animals

Species	Dose or concentration and duration	Effect	Reference
Guinea pigs	2.0 μg/kg-single oral dose	acute lethality (single dose LD_{50})	McConnell et al., 1978a McNulty, 1977
Rhesus monkey	1.0 μg/kg-single oral dose	acute (systemic) toxicity	McNulty, 1977
Sprague-Dawley rat	2.0 ng/kg-single oral dose[a]	induction of AHH (CYP1A1)	Kitchin and Woods, 1979
Marmoset monkey	3.0 ng/kg-single oral dose	induction of N-demethylation (CYP1A2)	Kruger et al., 1990
Guinea pig	1 ng/kg-day for 8 weeks	immunosuppression (decreased response to tetanus toxin)	Zinkl et al., 1973
C57B/6 mouse	1 ng/kg-week for 4 weeks intraperitoneally	immunosuppression (decreased generation of CTL)	Clark et al., 1983
Rhesus monkey	500 ppt in diet for 9 months (12 ng/kg-day); 2 ppb in diet for 61 days (50 ng/kg-day)	chronic lethality	Allen et al., 1977; McNulty, 1977
Rhesus monkey	50 ppt in diet for 20 months (1.5 ng/kg-day)	chronic toxicity (hair loss)	Schantz et al., 1978
Sprague-Dawley rat	10 ng/kg-day for 2 years in feed	porphyrin metabolism	Kociba et al., 1978

[a]0.6 ng/kg = no effect level

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- The overwhelming number of toxic responses to TCDD (including lethality) typically show a delay in their appearance, which supports the assumption that these responses are not the result of a direct insult from the compound.

- The induction of hepatic cytochrome P-450-dependent monooxygenases (mainly CYP1A1) is one of the hallmarks of TCDD exposure. This effect has been demonstrated to be mediated through the interaction with a specific protein called the Ah receptor. This process covers binding of TCDD to the receptor, followed by binding of the receptor-ligand complex to DNA recognition sites leading to expression of specific genes and translation of their protein products, which then mediate their biological effects.

- Studies in congenic mice that are Ah responsive or Ah nonresponsive have demonstrated that the majority of TCDD-induced toxic responses segregate quantitatively with the Ah locus. However, the amount of Ah receptor expressed in most laboratory species and strains is rather comparable. The Ah receptor is thus unlikely to be the only determinant of TCDD-induced toxicity. Rather, it has to be assumed that the species and strain differences are confined to the latter parts of the receptor-mediated chain of events (i.e., binding of the receptor-ligand complex to DNA and the subsequent expression of specific genes). Another explanation may be that the binding affinity of the Ah receptor is different or defective. In addition, some of the responses may be secondary in the sense that they are caused by the altered homeostasis of endogenous compounds caused by the TCDD-induced increased activities of various enzymes.

- It has repeatedly been reported as the current opinion that all known effects of TCDD are probably Ah receptor mediated (e.g., Roberts, 1991). However, except for the chain of events leading to the induction of certain enzymes, clear evidence for such a conclusion is still lacking. Nevertheless, the studies in congenic mice in combination with the usually rather strong correlation between enzyme induction and various other TCDD-induced toxic responses make the assumption rather likely. Further support for the probability of a receptor-mediated process is provided by the very strong structure-activity relationships that have been demonstrated between various CDDs/CDFs and a variety of toxic responses.

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4. IMMUNOTOXICITY

4.1. INTRODUCTION

Concern over the potential toxic effects of chemicals on the immune system arises from the critical role that the immune system plays in maintaining health. It is well recognized that suppressed immunological function can result in increased incidence and severity of infectious diseases as well as some types of cancer. Conversely, the inappropriate enhancement of immune function or the generation of misdirected immune responses can precipitate or exacerbate the development of allergic and autoimmune diseases. Thus, suppression as well as enhancement of immune function are considered to represent potential immunotoxic effects of chemicals.

The immune system consists of a complex network of cells and soluble mediators that interact in a highly regulated manner to generate immune responses of appropriate magnitude and duration. Consequently, comprehensive evaluation of immunotoxicity must include specific assessments of multiple functional parameters on a kinetic basis. In addition, because an immune response develops in a time-dependent manner relative to antigen exposure, the immunotoxicity of a chemical can be profoundly influenced by the timing of chemical exposure relative to antigen challenge. Consideration of these levels of complexity involved in immunotoxicology assessment is critical for interpretation of the effects of chemical exposure on immune function.

Extensive evidence has accumulated over the past 20 years to demonstrate that the immune system is a target for toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related halogenated aromatic hydrocarbons (HAHs), including the polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs). This evidence was derived from numerous studies in various animal species, primarily rodents, but also guinea pigs, rabbits, monkeys, marmosets, and cattle. Epidemiological studies also provide evidence for the immunotoxicity of HAHs in humans. In animals, relatively high doses of HAHs produce lymphoid tissue depletion, except in the thymus where cellular depletion occurs at lower doses. Alterations in specific immune

effector functions and increased susceptibility to infectious disease have been identified at doses of TCDD well below those that cause lymphoid tissue depletion. Both cell-mediated and humoral immune responses are suppressed following TCDD exposure, suggesting that there are multiple cellular targets within the immune system that are altered by TCDD. Evidence also suggests that the immune system is indirectly targeted by TCDD-induced changes in nonlymphoid tissues. In addition, in parallel with increased understanding of the cellular and molecular mechanisms involved in immunity, studies on TCDD are beginning to establish biochemical and molecular mechanisms of TCDD immunotoxicity. These advances will be highlighted in this document.

There is an enormous literature based on descriptive studies on the immunotoxic effects of TCDD and related HAHs in laboratory animals. Unfortunately, due to widely differing experimental designs, exposure protocols, and immunologic assays used, it has been very difficult to define a "TCDD-induced immunotoxic syndrome" in a single species, let alone across species. Until recently (Smialowicz et al., 1994), there was only one report that directly compared the effects of TCDD on the immune system of rats, mice, and guinea pigs, and even then, different immunologic parameters were assessed, and different antigens were used in the different species (Vos et al., 1973). In that study, the delayed-type hypersensitivity (DTH) response to tuberculin was evaluated in guinea pigs and rats for assessment of cell-mediated immunity, while the graft versus host (GVH) response was measured in mice. A decreased DTH response to tuberculin was observed in guinea pigs following 8 weekly doses of 40 ng/kg TCDD (total dose, 320 ng/kg), while the DTH response of rats to tuberculin was unaffected by 6 weekly doses of 5 μg/kg TCDD (total dose, 30,000 ng/kg). The GVH response in mice was suppressed by 4 weekly doses of 5 μg/kg TCDD (total dose, 20,000 ng/kg). The greater sensitivity of guinea pigs compared with rats and mice to the immunosuppressive effects of TCDD is consistent with the greater sensitivity of guinea pigs to other toxic effects of TCDD (McConnell et al., 1978; Poland and Knutson, 1982). Although these results appear to suggest that cell-mediated immunity in mice is more sensitive to TCDD than in rats, no studies have directly compared cell-mediated immunity in rats and mice using the same antigens and end points. There is, however, a recent study in which a direct comparison was made of the effects that TCDD

has on humoral immunity in rats and mice (Smialowicz et al., 1994). In this study the primary plaque-forming cell response to sheep red blood cells (SRBCs) was suppressed in B6C3F$_1$ mice (ED_{50} of 0.68 μg/kg TCDD), while the anti-SRBC response was enhanced in Fischer 344 rats at a dose as high as 30 μg/kg TCDD. In another study in mice, the DTH response to oxazolone was suppressed by 4 weekly doses of 4 μg/kg TCDD (total dose, 16,000 ng/kg), while the DTH response to SRBC was unaffected by a tenfold higher dose of TCDD (Clark et al., 1981), illustrating that DTH responses to different antigens are not equally sensitive to TCDD-induced suppression, even in the same species. When PCB and PBB studies are considered, variable effects on DTH and other immune reactions are also apparent (Fraker, 1980; Vos and van Driel-Grootenhuis, 1972; Luster et al., 1980b; Thomas and Hinsdill, 1978). Because the exact basis for the interstudy variability is not known, it would serve no useful purpose in terms of risk assessment to catalog all of the effects of TCDD and other HAHs on the immune system that have been reported. Several comprehensive reviews have been published on the immunotoxic effects of HAHs in general (Kerkvliet, 1984; Vos and Luster, 1989) and TCDD in particular (Holsapple et al., 1991a, b). The reader is also referred to the previous EPA TCDD risk assessment document, Appendix E (Sonawane et al., 1988) for another perspective on TCDD immunotoxicity. The present document will not reiterate this extensive literature, but rather, will emphasize more recent developments in the field of HAH immunotoxicity that may assist in the risk assessment process. Gaps in our knowledge that require further research will also be identified.

4.2. ROLE OF THE AH LOCUS IN HAH IMMUNOTOXICITY

One of the most important advances in the study of HAH toxicity in recent years has been the elucidation of a genetic basis for sensitivity to the toxicity of these chemicals, which may ultimately provide a logical explanation for much of the controversial data in the literature regarding HAH toxicity in different species and in different tissues of the same species. In this regard, many of the biochemical and toxic effects of HAHs appear to be mediated via binding to an intracellular protein known as the aryl hydrocarbon (Ah) or TCDD receptor in a process similar to steroid hormone receptor-mediated responses (Poland

and Knutson, 1982; Cuthill et al., 1988). Ah receptor activation follows stereospecific ligand binding; interaction of the receptor-ligand complex with dioxin-response elements (DREs) in the genome induces the transcription of the structural genes encoding mRNA for CYP1A1 enzyme activity (i.e., CYP1A1) as well as the expression of additional unidentified genes, the products of which are hypothesized to mediate HAH toxicity (Whitlock, 1990). Differences in toxic potency between various HAH congeners generally correlate with differences in Ah receptor-binding affinities. The most toxic HAH congeners are approximate stereoisomers of 2,3,7,8-TCDD and are halogen substituted in at least three of the four lateral positions in the aromatic ring system.

In mice, allelic variation at the Ah locus has been described (Poland et al., 1987; Poland and Glover, 1990). The different alleles code for Ah receptors that differ in their ability to bind TCDD and thus help to explain the different sensitivities of various inbred mouse strains to TCDD toxicity. Ah^{bb} C57Bl/6 (B6) mice represent the prototype "responsive" strain and are the most sensitive to TCDD toxicity, while Ah^{dd} DBA/2 (D2) mice represent the prototypic "nonresponsive" strain and require higher doses of TCDD to produce the same toxic effect. Recently, congenic Ah^{dd} mice on a B6 background were derived that differ from conventional B6 mice primarily at the Ah locus. The spectrum of biochemical and toxic responses to TCDD exposure was similar in both strains, but the doses needed to bring about the responses were significantly higher in congenic mice homozygous for the Ah^{d} allele as compared with mice carrying two Ah^{b} alleles (Birnbaum et al., 1990; Kerkvliet et al., 1990a).

Two lines of evidence have been used to investigate the Ah receptor dependence of the acute immunotoxicity of TCDD and related HAHs: (1) comparative studies using PCDD, PCDF, and PCB congeners that differ in their binding affinity for the Ah receptor and (2) studies using mice of different genetic background known to differ at the Ah locus. Vecchi et al. (1983) were the first to report that the antibody response to sheep erythrocytes (SRBCs) was differentially suppressed by TCDD in B6 mice as compared with D2 mice, with D2 mice requiring approximately a 10 times higher dose to produce the same degree of suppression. Immunosuppression in F1 and backcross mice supported the role of the Ah locus in the expression of TCDD immunotoxicity. 2,3,7,8-TCDF was significantly less

potent than TCDD and showed a similar differential immunosuppressive effect in B6 and D2 mice. At the same time, Silkworth and Grabstein (1982) reported a B6 versus D2 strain-dependent difference in sensitivity to suppression of the anti-SRBC response by 3,4,3′,4′-tetrachlorobiphenyl, a ligand for the Ah receptor. In comparison, the 2,5,2′,5′-tetrachlorobiphenyl isomer, which lacks affinity for the Ah receptor, was not immunosuppressive in either B6 or D2 mice. Structure-activity relationships were extended by Kerkvliet et al. (1985) in studies that compared the immunosuppressive potency of the chlorinated dioxin and furan isomers that contaminate technical grade pentachlorophenol. The 1,2,3,6,7,8-hexachlorinated dibenzo-*p*-dioxin (HxCDD), 1,2,3,4,6,7,8-heptachlorinated dibenzo-*p*-dioxin (HpCDD), and 1,2,3,4,6,7,8-heptachlorinated dibenzofuran (HpCDF) isomers, which bind the receptor, were all significantly immunosuppressive. The dose of each isomer that produced 50 percent suppression of the anti-SRBC response (ID_{50}) was 7.1, 85, and 208 μg/kg for HxCDD, HpCDD, and HpCDF, respectively (Figure 4-1). The ID_{50} for TCDD was 0.65 μg/kg based on the data of Vecchi et al. (1980). In contrast, octachlorodibenzo-*p*-dioxin (OCDD), which does not bind the receptor, was not immunosuppressive at a dose as high as 500 μg/kg (Kerkvliet et al., 1985). More extensive structure-dependent immunosuppressive activities of technical grade PCB mixtures (Davis and Safe, 1990), PCB congeners (Davis and Safe, 1989), and PCDF congeners (Davis and Safe, 1988) have also been reported. Results of these studies using different HAH congeners are summarized in Table 4-1.

The role of the Ah receptor in suppression of the anti-SRBC response has recently been verified in studies using B6 mice congenic at the Ah locus (Kerkvliet et al., 1990a). As expected, congenic Ah^{dd}-B6 mice were significantly less sensitive to TCDD-induced immune suppression as compared with wild-type Ah^{bb}-B6 mice. Unexpectedly, however, the dose response in congenic B6-Ah^{dd} mice appeared to be bimodal, with a portion of the response sensitive to suppression by low doses of TCDD. Because of the bimodal response, the data did not permit extrapolation of an ID_{50} dose in the congenic mice. The results were interpreted to suggest potential non-Ah receptor-mediated immunosuppressive effects. It should be noted, however, that recent studies by Silkworth et al. (1993) using rederived congenic Ah^{dd}-B6 mice have not corroborated a bimodal dose response. The issue of Ah

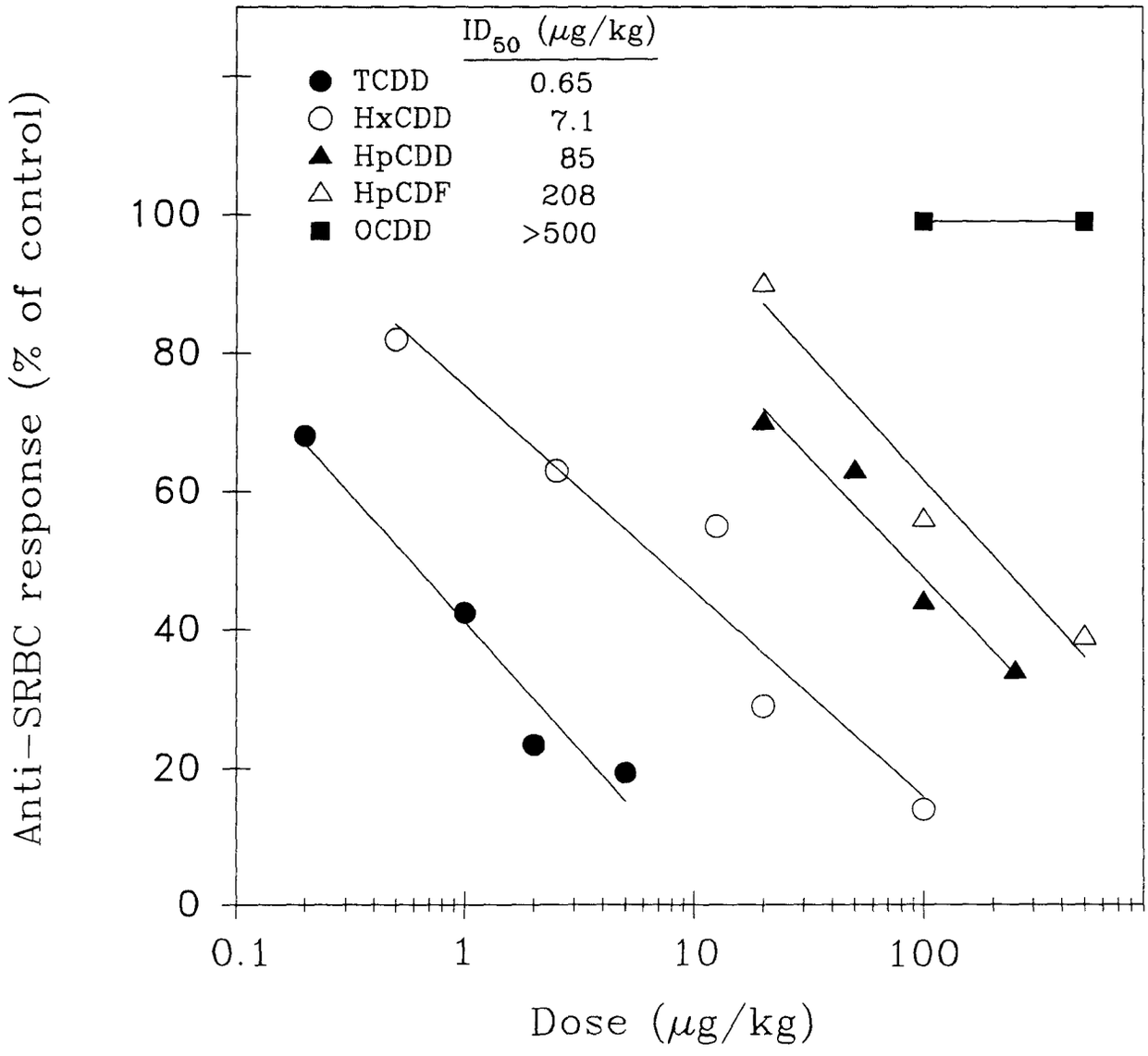

Figure 4-1. Structure-dependent immunotoxicity of some polychlorinated dioxin and furan isomers. Immunotoxicity assessed by suppression of the splenic antibody response to SRBC (modified from Kerkvliet et al., 1985).

Table 4-1. Toxic Equivalency Factors (TEFs) for Polychlorinated Dioxins, Furans, and Biphenyls Based on the Acute Single Dose ID_{50} for Suppression of the PFC Response to SRBCs in Ah-responsive B6 Mice

Congener	ID_{50}	Reference	TEF
2,3,7,8-TCDD	0.74 μg/kg	Kerkvliet and Brauner, 1990a	1.0
"	0.65 μg/kg	Vecchi et al., 1980	(based on mean ID_{50}
"	0.77 μg/kg	Davis and Safe, 1988	of 0.7 ± 0.07 μg/kg)
"	0.60 μg/kg	Kerkvliet et al., 1990a	
1,2,3,6,7,8-HxCDD	7.1 μg/kg	Kerkvliet et al., 1985	0.1
1,2,3,4,6,7,8-HpCDD	85.0 μg/kg	Kerkvliet et al., 1985	8.2×10^{-3}
OCDD	>500 μg/kg	Kerkvliet et al., 1985	$<1.4 \times 1^{-3}$
2,3,4,7,8-PCDF	1.0 μg/kg	Davis and Safe, 1988	0.7
2,3,7,8-TCDF	4.3 μg/kg	Davis and Safe, 1988	1.6×10^{-1}
1,2,3,4,6,7,8-HpCDF	208 μg/kg	Kerkvliet et al., 1985	3.4×10^{-3}
1,2,3,7,9-PenCDF	239 μg/kg	Davis and Safe, 1988	2.9×10^{-3}
1,3,6,8-TCDF	11 mg/kg	Davis and Safe, 1988	6.4×10^{-5}
3,4,3',4'-TCB	28 mg/kg[a]	Silkworth and Grabstein, 1982	2.5×10^{-5}
2,3,4,5,3',4'-HxCB	0.7 mg/kg	Davis and Safe, 1990	1.0×10^{-3}
2,3,4,5,3',4'-HxCB	36 mg/kg[a]	Silkworth et al., 1984	1.9×10^{-5}
2,4,3',4',5',6'-HxCB	43 mg/kg	Davis and Safe, 1990	1.6×10^{-5}
2,3,4,3',5'-PenCB	65 mg/kg	Ibid.	1.1×10^{-5}
2,3,4,5,3',5'-HxCB	72 mg/kg	Ibid.	9.7×10^{-6}
2,4,2',4'-TCB	>100 mg/kg	Silkworth et al., 1984	$<7.0 \times 10^{-6}$
2,4,5,2',4',6'-HxCB	>360 mg/kg	Davis and Safe, 1990	$<1.9 \times 10^{-6}$
2,4,6,2',4',6'-HxCB	>360 mg/kg	Ibid.	$<1.9 \times 10^{-6}$
2,4,5,2',4',5'-HxCB	>360 mg/kg	Biegel et al., 1989	$<1.9 \times 10^{-6}$
Aroclor 1260	104 mg/kg	Davis and Safe, 1989	6.7×10^{-6}

Table 4-1. (continued)

Congener	ID_{50}	Reference	TEF
Aroclor 1254	118 mg/kg	Ibid.	5.9×10^{-6}
Aroclor 1254	207 mg/kg	Lubet et al., 1986	3.4×10^{-6}
Aroclor 1248	190 mg/kg	Davis and Safe, 1989	3.7×10^{-6}
Aroclor 1242	391 mg/kg	Ibid.	1.8×10^{-6}
Aroclor 1016	408 mg/kg	Ibid.	1.7×10^{-6}
Aroclor 1232	464 mg/kg	Ibid.	1.5×10^{-6}

[a] Interpolated from two data points.

receptor-independent immunotoxicity will be discussed in detail in a subsequent section of this document.

Ah receptor dependency of HAH immunotoxicity has also been demonstrated in mice using other immunologic responses. For example, Kerkvliet et al. (1990a) reported that the ID_{50} for suppression of the antibody response to lipopolysaccharide (TNP-LPS) in Ah^{bb}-B6 mice was 7.0 μg/kg compared with a significantly higher ID_{50} of 30 μg/kg in congenic Ah^{dd}-B6 mice. Because the antibody response to TNP-LPS shows little requirement for macrophages or T helper cells (Jelinek and Lipsky, 1987), these results suggest an Ah receptor-dependent B cell response. In terms of cytotoxic T cells, Clark et al. (1983) was first to report data suggesting that TCDD and PCB isomers suppressed in vitro cytotoxic T lymphocyte (CTL) responses of B6 and D2 mice through an Ah receptor-dependent mechanism. Subsequently, Kerkvliet et al. (1990b) reported that B6 mice congenic at the Ah locus showed Ah-dependent sensitivity to suppression of the CTL response following exposure to either TCDD or 3,4,5,3′,4′,5′-hexachlorobiphenyl (HxCB). Furthermore, the potency of TCDD and of three HxCB congeners to suppress the CTL response of Ah^{bb}-B6 mice directly correlated with their relative binding affinities for the Ah receptor (Table 4-2, from Kerkvliet et al., 1990b). The ID_{50} of TCDD for suppression of the CTL response in B6 mice was 7.0 μg/kg.[1]

In summary, the data relating HAH immunotoxicity, at least in part, to Ah receptor-dependent events are convincing. However, it should be emphasized that all of the data have been obtained from studies in inbred mice using an acute or subacute exposure regimen. Except for thymic atrophy, structure-immunotoxicity relationships in other species, including rats, have not been established, and the availability of inbred strains of other species with defined Ah genotype are not currently available. The importance of Ah

[1]The dose of TCDD required to suppress the CTL response reported by Kerkvliet et al. (1990b) is significantly greater than that reported by Clark et al. (1981), who reported CTL suppression following 4 weekly doses of 0.1 μg/kg TCDD. Clark et al. (1983) also reported that doses of TCDD as low as 4 ng/kg to B6 mice suppressed the in vitro generation of CTL and that the suppression was Ah dependent. The potency of TCDD described in Clark's studies has not been corroborated by other laboratories. For example, the in vivo and in vitro CTL response in B6 mice was not affected at doses ranging from 0.01 to 3.0 μg/kg at weekly intervals for 4 weeks (Hanson and Smialowicz, 1994).

Table 4-2. TEFs Based on the ID_{50} for Suppression of Alloantigen (P815)-Specific CTL Response in C57Bl/6 Mice

Congener	ID_{50}	Reference	TEF
TCDD	7 μg/kg	Kerkvliet et al., 1990b	1.0
3,4,5,3′,4′,5′-HxCB	7 mg/kg	Ibid.	1×10^{-3}
2,3,4,5,3′,4′-HxCB	70 mg/kg[a]	Ibid.	1×10^{-4}
2,4,5,2′,4′,5′-HxCB	>300 mg/kg	Ibid.	$<2.3 \times 10^{-5}$

[a] Interpolated from two data points.

receptor-mediated events in chronic, low-level HAH immunotoxicity also remains to be established. Morris et al. (1992) have recently reported that the sensitivity of D2 mice to TCDD-induced suppression of the anti-SRBC response increased significantly when TCDD was administered daily over 2 weeks rather than as an acute single dose. Unfortunately, in these studies, the lowest dose of TCDD produced near-maximum suppression of the anti-SRBC response of $B6C3F_1$ mice in the acute exposure model, precluding the detection of any similar increase in sensitivity of the $B6C3F_1$ mice to chronic dosing. In contrast to these findings, Vecchi et al. (1983) reported that multiple exposures to TCDD (2 μg/kg for 5 weeks or 0.5 μg/kg for 8 weeks) did not increase the sensitivity of D2 mice to suppression of the anti-SRBC response. Thus, the basis for any change in potency resulting from multiple treatment or chronic exposure to TCDD and the role of Ah receptor-mediated events in the phenomenon remain to be elucidated.

4.3. TOXIC EQUIVALENCY FACTORS FOR IMMUNOTOXICITY

Based on the available data from mice, the majority of the immunotoxic effects of HAHs appear to be mediated via the Ah receptor. Thus, the toxicity of different HAH congeners can be compared by calculating toxic equivalence factors (TEFs). TEFs based on acute, single-dose exposure (oral or i.p.) of B6 mice to various HAHs for suppression of the anti-SRBC response and the CTL response are presented in Tables 4-1 and 4-2, respectively. As shown in Table 4-1, the potency of TCDD to suppress the antibody response to SRBCs has been reported by several laboratories, with remarkable agreement[2] in the ID_{50} value of 0.7 μg/kg in B6 mice. The ID_{50} of $B6C3F_1$ mice is similar (<1 μg/kg;

[2]Several laboratories have reported that the antibody response to SRBC is sensitive to suppression following acute exposure to TCDD, either i.p. or orally, at doses <1 μg/kg. In contrast, Clark et al. (1981) reported that 4 weekly i.p. doses of 10 but not 1 or 0.1 μg/kg TCDD significantly suppressed the anti-SRBC response in B6 mice. The chronic-dosing protocol used by Clark does not readily explain his decreased potency because Vecchi et al. (1983) reported that 5 weekly doses of 2 μg/kg or 8 weekly doses of 0.5 μg/kg TCDD significantly suppressed the anti-SRBC response. Likewise, when total doses of 0.2 or 1 μg/kg TCDD were given as a single dose or divided into 5 daily doses, the divided dose produced slightly more suppression than the single dose (see Table 4-3). In addition, the route of antigen challenge (intravenous [used by Clark] vs. intraperitoneal [used by Vecchi]) does not appear to greatly influence the degree of suppression of the anti-SRBC response produced by TCDD (see Table 4-4). Thus, the basis for the discrepancies between the data of Clark et al. (1981, 1983) and other laboratories regarding the potency of TCDD to suppress the anti-SRBC response is unknown.

Table 4-3. Effect of Single vs. Multiple Dosing With TCDD on Suppression of the Antibody Response to SRBCs in C57Bl/6 Mice

Total Dose (μg/kg)	Plaque-Forming Cells/10^6 Spleen Cells (Mean ± SD)	
	Single[a]	Multiple[b]
0	2,460 ± 657	3,846 ± 1618
0.2	1,879 ± 445 (76)	2,356 ± 592 (61)
1.0	1,293 ± 285 (52)[c]	1,143 ± 208 (30)[c]

[a] Total dose of TCDD given once 2 days prior to SRBC injection.
[b] Total dose of TCDD divided into five equal doses administered on days -7 to -2 prior to SRBC injection.
[c] $p < 0.01$.

Table 4-4. Influence of Route of Antigen Challenge on Suppression of the Antibody Response to SRBCs in C57Bl/6 Mice[a]

Dose of TCDD (μg/kg)	Plaque-Forming Cells/10^6 Spleen Cells (Mean ± SD)	
	IV	IP
0	1,151 ± 367	1,812 ± 872
0.2	623 ± 324 (54)	1,197 ± 519 (66)
1.0	466 ± 212 (40)[b]	697 ± 163 (38)[b]

[a] 2.5×10^8 SRBCs were injected intravenously (IV) or intraperitoneally (IP) 2 days after oral dosing with TCDD. Plaque-forming cells were measured 5 days later.
[b] $p < 0.01$.

House et al., 1990; Smialowicz et al., 1994) or slightly higher (1.2 μg/kg; Holsapple et al., 1986a) in comparison with B6 mice. These data thus provide a well-defined base value to use in calculating TEFs for other HAH congeners in the context of suppression of the anti-SRBC response.

However, in contrast to the reproducible data on TCDD, the accuracy of the derived TEFs for other HAH congeners shown in Table 4-1 is difficult to evaluate because few congeners have been examined in more than one study. In the few cases where the same congener has been evaluated independently, discrepancies in the data exist. For example, both Davis and Safe (1990) and Silkworth et al. (1984) evaluated the potency of the 2,3,4,5,3′,4′-HxCB congener in the anti-SRBC response. Based on the ID_{50}s from the two data sets, the respective TEFs differ by almost two orders of magnitude (1×10^{-3} vs. 2×10^{-5}). When the same congener was compared with TCDD for suppression of the CTL response, the TEF was 1×10^{-4} (Table 4-2). The basis for these discrepancies is unknown. Thus, the database for TEF comparisons using immunotoxicity data must be expanded considerably before TEFs can be used with confidence in risk assessment.

4.4. INTERACTIONS BETWEEN HAHs

If the immunotoxicity of TCDD and structurally related HAHs is dependent on Ah receptor-mediated mechanisms, then coexposure to subsaturating levels of more than one Ah receptor ligand should produce additive effects. An additive interaction has been demonstrated in mice coexposed to 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD, two relatively strong Ah receptor ligands (Kerkvliet et al., 1985). On the other hand, Davis and Safe (1988, 1989) have reported that coexposure of mice to an immunotoxic dose of TCDD and a subimmunotoxic dose of different commercial Aroclors or different PCB congeners resulted in partial antagonism of TCDD suppression of the anti-SRBC response. In limited studies, an apparently similar antagonism was observed following coexposure to 2,3,7,8-TCDF (10 μg/kg) and TCDD (1.2 μg/kg) (Rizzardini et al., 1983). The mechanism for the antagonism has not been fully elucidated, but the effects are consistent with competition for binding at the Ah receptor because the weaker agonist was administered in excess compared with TCDD. Furthermore, Silkworth et al. (1988) and Silkworth and

O'Keefe (1992) have shown that the immunotoxicity of TCDD can be modified by coexposure to other HAHs present as cocontaminants of actual environmental samples from Love Canal, New York. Such interactions complicate hazard assessment of mixtures based on TEFs and may preclude dependence on TEFs without biological response evaluation for risk assessment.

4.5. SENSITIVE TARGETS FOR HAH IMMUNOTOXICITY

Despite considerable investigation, the cells that are altered by HAH exposure leading to suppressed immune function have not been unequivocally identified. The main reason for the lack of definitive progress in this area is the conflicting data reported from different laboratories regarding the ability of TCDD to suppress lymphocyte functions when examined "ex vivo" or in vitro. As discussed in a subsequent section of this document, the in vitro effects of TCDD are greatly influenced by the in vitro culture conditions, which may explain the discrepancies in effects observed in different laboratories.

In contrast to in vitro studies, the in vivo immunotoxicity of TCDD, expressed in terms of suppression of the anti-SRBC response of B6 or B6C3F_1 mice, is highly reproducible between laboratories. Because the magnitude of the anti-SRBC response depends on the concerted interactions of antigen-presenting cells (APC), regulatory T cells (helper and suppressor), and B cells, this response has been used most widely to evaluate target cell sensitivity to HAHs. In addition, the antibody response to SRBCs can be modulated by many nonimmunological factors, including hormonal and nutritional variables, and HAHs are known to affect numerous endocrine and metabolic functions. These latter effects will be apparent only in in vivo studies, while only direct effects of HAHs on APC and lymphocyte functions would be evident following in vitro exposure to HAHs. To date, direct in vitro effects of TCDD on purified B cell activity have been reported (Holsapple et al., 1986a; Morris et al., 1991; Luster et al., 1988), while direct effects on macrophages and T cells in vitro have not been described. (The in vitro effects of TCDD will be discussed in more detail in a subsequent section of this document.)

Kerkvliet and Brauner (1987) compared the sensitivity of antibody responses to antigens that differ in their requirements for APC and T cells as an in vivo approach to

evaluate the cellular targets of 1,2,3,4,6,7,8-HpCDD humoral immunotoxicity. The T helper cell independent (TI) antigens, DNP-Ficoll and TNP-LPS, were used in these studies. These TI antigens differ from each other in their requirement for APC (higher for DNP-Ficoll) and their sensitivity to regulatory (amplifier and suppressor) T cell influence (DNP-Ficoll is sensitive, TNP-LPS is not) (Braley-Mullen, 1982). Obviously, all antibody responses require B cell differentiation into antibody-secreting plasma cells. Although HpCDD produced dose-dependent suppression of the antibody response to all three antigens, sensitivity to suppression directly correlated with the sensitivity of the response to T cell regulation. The ID_{50}s were 53, 127, and 516 μg/kg for SRBC, DNP-Ficoll, and TNP-LPS, respectively. These results were interpreted as follows: If one assumes that B cell function is targeted in the TNP-LPS response, then regulatory T cells and/or APC may represent the more sensitive target in the SRBC and DNP-Ficoll responses. The difference in sensitivity between the SRBC and DNP-Ficoll responses suggests that the T helper cell may be a particularly sensitive target. The differential sensitivity of the antibody responses to TNP-LPS versus SRBC has been corroborated in TCDD-treated mice (House et al., 1990; Kerkvliet et al., 1990a). Thus, the exquisite in vivo sensitivity of the antibody response to SRBC would appear to depend on the T cell and/or APC components of the response rather than the B cell, unless the B cells that respond to SRBC are different from the B cells that respond to TNP-LPS. Currently, evidence for such a difference is lacking. However, this interpretation conflicts with the ex vivo data of Dooley and Holsapple (1988). Using separated spleen T cells, B cells, and adherent cells from vehicle- and TCDD-treated mice, they reported that B cells from TCDD-treated mice were functionally compromised in in vitro antibody responses but T cells and macrophages were not. The basis for these discrepant findings has not been established. However, it is possible that the effects of TCDD on T cells are indirectly induced following antigen exposure such that removal of the cells from the TCDD environment of the host prior to antigen challenge would preclude detection of T cell dysfunction. This interpretation is supported by the findings of Tomar and Kerkvliet (1991) that spleen cells taken from TCDD-treated mice were not compromised in their ability to reconstitute the antibody response of lethally irradiated mice. This

interpretation is also consistent with the reported lack of direct effects of TCDD and other HAHs on T cells in vitro (Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988a).

Although the direct effects of TCDD on T cells in vitro have not been demonstrated, it is clear that functional T cell responses generated in vivo are compromised following in vivo exposure. Nude mice that are congenitally T cell deficient are significantly less sensitive to HpCDD-induced immunotoxicity when compared with their T cell-competent littermates (Kerkvliet and Brauner, 1987). Likewise, exposure to TCDD or HxCB suppresses the development of CTL activity following alloantigen challenge (Kerkvliet et al., 1990b). The influence of TCDD exposure on regulatory T cell functions has been addressed in a limited number of studies. Clark et al. (1981) first proposed that T suppressor cells were induced by TCDD in the thymus that were responsible for the suppressed CTL response. However, increased suppressor cell activity in peripheral lymphoid tissue was not observed in mice exposed to TCDD (Dooley et al., 1990) or 3,4,5,3′,4′,5′-HxCB (Kerkvliet and Baecher-Steppan, 1988b). In terms of T helper cell activity, Tomar and Kerkvliet (1991) reported that a dose of 5 μg/kg TCDD suppressed the in vivo generation of carrier-specific T helper cells. Lundberg et al. (1990) reported that thymocytes from B6 mice treated with TCDD (50 μg/kg) were less capable of providing help for an in vitro anti-SRBC response. However, Clark et al. (1983) reported in ex vivo studies that T cells from TCDD-treated mice produced normal levels of IL-2. The in vivo effect of TCDD on the production of IL-2 as well as other lymphokines important in the development of an antibody response (e.g., IL-4, IL-5) have not been reported.

The influence of TCDD exposure on B cell function has been addressed primarily in in vitro studies. The issue is difficult to address in vivo given that most B cell responses (except perhaps anti-LPS responses) are dependent on interactions with T cells and macrophages. In vitro studies have described the direct effects of TCDD on the activation and differentiation of purified B cells (Luster et al., 1988; Morris et al., 1991). These studies suggest that TCDD inhibits the terminal differentiation of B cells via alteration of an early activation event (Luster et al., 1988). Increased phosphorylation and tyrosine kinase activity in TCDD-treated B cells may underlie this B cell dysfunction (Kramer et al., 1987; Clark et al., 1991a).

Macrophage functions have also been examined following TCDD exposure and generally found to be resistant to suppression by TCDD when assessed ex vivo. Macrophage-mediated phagocytosis, macrophage-mediated tumor cell cytolysis or cytostasis, oxidative reactions of neutrophils and macrophages, and natural killer (NK) cell activity were not suppressed following TCDD exposure, with doses as high as 30 μg/kg failing to suppress NK and macrophage functions (Vos et al., 1978; Mantovani et al., 1980). A potentially important exception is the reported selective inhibition of phorbol ester-activated antitumor cytolytic and cytostatic activity of neutrophils by TCDD (Ackermann et al., 1989).

On the other hand, it is interesting to note that the pathology associated with TCDD toxicity often includes neutrophilia and an inflammatory response in liver and skin characterized by activated macrophage and neutrophil accumulation (Weissberg and Zinkl, 1973; Vos et al., 1973; Vos et al., 1974; Puhvel and Sakamoto, 1988). Although these observations may simply reflect a normal inflammatory response to tissue injury, there is some preliminary experimental evidence that suggests inflammatory cells may be activated by TCDD exposure. For example, Alsharif et al. (1990) recently reported that TCDD increased superoxide anion production in rat peritoneal macrophages. In addition, it has been shown that TCDD exposure results in an enhanced inflammatory response following SRBC challenge (Kerkvliet and Brauner, 1990b; Kerkvliet and Oughton, 1993). This effect of TCDD was characterized by a twofold to fourfold increase in the number of neutrophils and macrophages locally infiltrating the intraperitoneal site of SRBC injection. However, the kinetics of the cellular influx was not altered by TCDD. Likewise, the expression of macrophage activation markers (I-A and F4/80) and the antigen-presenting function of the peritoneal exudate cells were unaltered by TCDD. Thus, the effect of TCDD appeared to reflect a quantitative rather than a qualitative change in the inflammatory response. Importantly, TCDD-induced suppression of the anti-SRBC response could not be overcome by increasing the amount of antigen used for sensitization, suggesting that enhanced antigen clearance/degradation by the increased numbers of phagocytic cells (e.g., decreased antigen load) was not responsible for the decreased antibody response in TCDD-treated mice. Thus, the relationship, if any, between the inflammatory and immune effects of TCDD remains to be elucidated.

One mechanism by which TCDD and related HAHs may augment inflammatory responses is via enhanced production of inflammatory mediators such as interleukin 1 (IL-1) and tumor necrosis factor (TNF). Recent evidence suggests that the long-recognized hypersusceptibility of TCDD- and PCB-treated animals to endotoxin (lipopolysaccharide [LPS]) (Thomas and Hinsdill, 1978, 1979; Vos et al., 1978; Loose et al., 1979) may be related to an increased production of TNF and/or IL-6 in the chemically treated animals (Clark et al., 1991b; Taylor et al., 1990; Hoglen et al., 1992). The ability of methylprednisolone to reverse the mortality associated with TCDD/endotoxin treatment is also consistent with an inflammatory response (Rosenthal et al., 1989). Similarly, increased inflammatory mediator production may underlie the enhanced rat paw edema response to carrageenan and dextran in TCDD-treated rats (Theobald et al., 1983; Katz et al., 1984). Limited preliminary data are available to indicate that the production of inflammatory mediators such as TNF (Taylor et al., 1990; Clark et al., 1990b) and IL-6 (Hoglen et al., 1992) may be increased in HAH-treated animals. Serum complement activity, on the other hand, has been reported to be suppressed in dioxin-treated mice (White et al., 1986), although enhanced activity was reported at the lowest exposure level when 1,2,3,6,7,8-HxCDD was tested. A primary effect of TCDD on IL-1 is supported by the recent findings of Sutter et al. (1991) that the IL-1β gene contains a DRE. Likewise, Steppan and Kerkvliet (1991) have reported that under some exposure conditions TCDD increased the level of mRNA for IL-1 in TCDD-treated IC21 cells, a macrophage cell line derived from B6 mice. On the other hand, House et al. (1990) reported that inflammatory macrophages obtained from TCDD-treated mice produced control levels of IL-1 when examined ex vivo. Thus, the effect of TCDD on inflammatory mediator production may be a "priming effect" and require coexposure to antigen or LPS. The influence of TCDD on inflammatory mediator production and action is an important area for further study.

Because the rapid influx of phagocytic cells to the site of pathogen invasion is an important factor in host resistance to infection, the ability of TCDD to augment the production of inflammatory chemoattractive mediators would imply that TCDD exposure could result in enhanced host resistance. However, since TCDD exposure is, at the same time, immunosuppressive, resulting in decreased specific immune responses generated by T

and B lymphocytes, the overall impact of TCDD exposure on disease susceptibility will likely vary depending on the nature of the pathogen and the major mode of host response to the specific infectious agent. Such effects may in fact help to explain the disparate effects of TCDD in different host resistance models that previously have been reported.

4.6. INFLUENCE OF TCDD ON HOST RESISTANCE TO DISEASE

The ability of an animal to resist and/or control viral, bacterial, parasitic, and neoplastic diseases is determined by both nonspecific and specific immunological functions. Decreased functional activity in any immunological compartment may result in increased susceptibility to infectious and neoplastic diseases. In terms of risk assessment, host resistance is often accorded the "bottom line" in terms of relevant immunotoxic end points. Animal host resistance models that mimic human disease are available and have been used to assess the effect of TCDD on altered host resistance.

TCDD exposure increases susceptibility to challenge with the gram-negative bacterium *Salmonella*. TCDD was given per os at 0.5 to 20 μg/kg once a week for 4 weeks to male 4-week-old C57Bl/6Jfh (J67) mice and challenged 2 days after the fourth dose (when mice were 8 weeks old) with either *Salmonella bern* or *Herpesvirus suis* (also known as pseudorabies virus). Results with *S. bern* indicated that there was an increased mortality at 1 μg TCDD/kg (total dose of 4 μg/kg) and a reduced time to death after bacterial challenge with 5 μg TCDD/kg (total dose of 20 μg/kg). In contrast, the same doses of TCDD did not alter the time to death or the incidence of mortality following *Herpesvirus suis* infection (Thigpen et al., 1975). A TCDD feeding study by Hinsdill et al. (1980) also demonstrated increased susceptibility of 7-week-old Swiss Webster outbred female mice to *S. typhimurium* var. *copenhagen*. Mice were fed control feed or feed containing 10, 50, or 100 ppb TCDD for 8 weeks, after which they were injected intravenously with $10^{3.5}$ *S. typhimurium* var. *copenhagen*. Results indicated that 50 and 100 ppb TCDD increased mortality from *Salmonella* and shortened the time to death, while 10 ppb caused an increased bacteremia. Vos et al. (1978) reported that TCDD resulted in a reduced resistance to endotoxin (*E. coli* O 127:B 8 lipopolysaccharide) and suggested that the increased susceptibility to *Salmonella*

caused by TCDD may be due to the lipopolysaccharide or endotoxin of this gram-negative bacterium. Vos et al. (1978) demonstrated reduced resistance to endotoxin with a single oral dose of 100 μg TCDD/kg using 3- to 4-week-old outbred female mice and challenged with endotoxin 5 days later. Vos et al. (1978) also reported enhanced mortality from the intravenous injection of endotoxin 2 days after the final oral dose of TCDD (1.5, 5, 15, or 50 μg/kg; once a week for 4 weeks) in 3- to 4-week-old male outbred Swiss mice. These studies indicate a reduced resistance to endotoxin after single or multiple doses of TCDD. Thomas and Hinsdill (1979), using *S. typhimurium* lipopolysaccharide, demonstrated a reduced resistance to endotoxin in the offspring of female Swiss Webster mice fed TCDD prior to mating, during gestation, and between parturition and weaning. Rosenthal et al. (1989) used female $B6C3F_1$, DBA/2, as well as congenic mice to demonstrate that acute doses of 50, 100, or 200 μg TCDD per os increased endotoxin-induced mortality in $B6C3F_1$ mice, which was associated with hepatotoxicity and decreased clearance of the endotoxin. D2 and Ah^{dd} congenic mice were relatively resistant to this effect, implicating Ah receptor-dependent mechanisms in endotoxin hypersensitivity.

White et al. (1986) reported that *Streptococcus pneumoniae*, a gram-positive bacterium that does not contain endotoxin, caused increased mortality in 5- to 6-week-old female $B6C3F_1$ mice after subchronic oral administration of TCDD (1 μg/kg for 14 days) and challenged with *S. pneumoniae* intraperitoneally 1 day after the last treatment. The 1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin (HCDD) isomer also resulted in a dose-dependent increase in susceptibility to *S. pneumoniae*.

Enhanced susceptibility to viral disease has also been reported after TCDD administration. Clark et al. (1983) injected TCDD intraperitoneally once a week for 4 weeks and challenged mice 7 to 22 days later with *Herpes simplex* type II strain 33 virus. Mice receiving TCDD at 0.04, 0.4, or 4.0 μg/kg weekly (total dose of 160, 1,600, and 16,000 ng/kg) all had significantly enhanced mortality to Herpesvirus type II infection. House et al. (1990) also reported an enhanced susceptibility to viral infection following low-level single-dose TCDD administration intraperitoneally. $B6C3F_1$ female mice, 6 to 8 weeks of age, were challenged with influenza A/Taiwan/1/64 (H2N2) virus 7 to 10 days following TCDD.

TCDD administration at 10, 1.0, and 0.1 μg/kg decreased resistance to virus. The lowest observable effect level was 0.1 μg/kg, making this one of the most sensitive end points for TCDD immunotoxicity. These results have recently been verified for 0.1 μg/kg and extended to show enhanced mortality to influenza virus at 0.01 μg/kg by Burleson et al. (submitted). TCDD treatment of rats significantly augmented influenza virus replication in the lungs. This effect was correlated with a significant suppression of virus-augmented but not spontaneous NK cell activity (Yang et al., submitted).

TCDD exposure also results in more severe parasitic diseases. Tucker et al. (1986) studied the effects of TCDD administration on *Plasmodium yoelii* 17 XNL, a nonlethal strain of malaria, in 6- to 8-week-old $B6C3F_1$ female mice. A single dose of TCDD at 5 μg/kg or 10 μg/kg per os resulted in increased susceptibility to *P. yoelii*. The peak parasitemia was greater and of longer duration in TCDD-treated animals than in controls, the difference being significant at 5 μg/kg on day 10 and at 10 μg/kg on days 12 and 14. A single dose of TCDD at 10 or 30 μg/kg intraperitoneally 7 days prior to infection of $B6C3F_1$ mice with *Trichinella spiralis* resulted in delayed onset of adult parasite elimination and at 1.0 μg/kg TCDD suppressed the proliferative response of splenocyte and mesenteric lymph node cells stimulated with *T. spiralis* antigen (Luebke et al., 1994). Tissue levels of TCDD were also higher in infected versus noninfected mice in this study. In a separate study, TCDD at 1, 10, or 30 μg/kg administered intraperitoneally 7 days prior to infection of Fischer 344 rats with *T. spiralis* did not affect adult parasite elimination. Furthermore, proliferative responses of lymphocytes from rats dosed at 30 μg/kg and stimulated with parasite antigen were enhanced in contrast to the suppression observed in $B6C3F_1$ mice (Luebke et al., submitted).

Luster et al. (1980a) demonstrated enhanced growth of transplanted tumors in mice treated with TCDD at doses of 1.0 or 5.0 μg/kg in $B6C3F_1$ mice. Mothers were given TCDD by gavage at day 14 of gestation and again on days 1, 7, and 14 following birth; host resistance studies were performed 6 to 8 weeks after weaning. This exposure protocol resulted in an increased incidence of PYB6 tumors in pups from dams receiving repeated doses of 1.0, but not 5.0, μg TCDD/kg.

Although it is clear that TCDD adversely affects numerous host resistance models detailed above, the effects of TCDD on susceptibility to *Listeria monocytogenes* infections are ambiguous. The disparate results may reflect different study designs, including dose, route, single versus multiple administrations, mouse strain, age, or sex. However, it is clear that TCDD, under certain conditions, results in increased susceptibility to *Listeria*. Hinsdill et al. (1980) reported the increased susceptibility of 7-week-old Swiss Webster outbred female mice to *Listeria*. Mice were fed control feed or feed containing 10 or 50 ppb TCDD for 8 weeks, after which they were injected intravenously with 10^5 *Listeria*. Results indicated that the 50-ppb diet increased bacteremia and mortality. Luster et al. (1980a) used doses of 1.0 or 5.0 μg TCDD/kg in B6C3F$_1$ mice. Mothers were given TCDD by gavage at day 14 of gestation and again on days 1, 7, and 14 following birth, and host resistance studies were performed 6 to 8 weeks after weaning. This exposure protocol resulted in an increased susceptibility to *Listeria* in pups from dams receiving repeated doses of 5.0 μg TCDD/kg. However, Vos et al. (1978) reported that oral administration of 50 μg TCDD/kg once a week for 4 weeks to 3- to 4-week-old male Swiss mice followed by intravenous challenge 4 days after the last dose with *Listeria* had no effect on nonspecific phagocytosis and killing of *Listeria*. House et al. (1990) used B6C3F$_1$ female mice, 6 to 8 weeks of age and challenged intravenously with *Listeria* 7 to 10 days following a single dose of TCDD at 10, 1.0, and 0.1 μg/kg. TCDD did not enhance mortality to *Listeria*.

In summary, results from host resistance studies provide evidence that exposure to TCDD results in increased susceptibility to bacterial, viral, parasitic, and neoplastic disease. These effects are observed at low doses and likely result from TCDD-induced suppression of immunological function. However, it is interesting that the role of the Ah receptor has not been addressed in terms of host resistance models except in studies on endotoxin hypersensitivity by Rosenthal et al. (1989). Furthermore, the specific immunological functions targeted by TCDD in each of the host resistance models remain to be fully defined.

4.7. IN VITRO IMMUNOTOXIC EFFECTS OF HAHs

Investigators in the field of TCDD immunotoxicity have long acknowledged the difficulties in consistently demonstrating the immunotoxicity of TCDD when cells from treated animals are tested ex vivo or when TCDD is added to culture in vitro. Although effects following in vitro and ex vivo exposure to TCDD or related HAHs on lymphocyte functions have been reported (Tucker et al., 1986; Luster et al., 1988; Dooley and Holsapple, 1988), other laboratories have failed to observe suppression with in vitro or ex vivo exposure to HAHs (Lundberg et al., 1990; Clark et al., 1981; Kerkvliet and Baecher-Steppan, 1988b). In addition, the effects of TCDD seen in vitro are sometimes inconsistent with those observed after in vivo assessment of immunotoxicity. For example, the rank order of sensitivity to suppression of T helper cell-dependent and T helper cell-independent antibody responses seen in vivo (Kerkvliet and Brauner, 1987; Kerkvliet et al., 1990a; House et al., 1990) is not seen in vitro (Holsapple et al., 1986a; Tucker et al., 1986), suggesting different cellular targets may be affected following in vitro exposure to TCDD. More importantly, some data suggest that suppression of the in vitro antibody response may occur independent of the Ah receptor. Tucker et al. (1986) and Holsapple et al. (1986a) reported that direct addition of TCDD in vitro suppressed the antibody response to SRBCs. However, based on the response of cells from congenic mice as well as a limited structure-activity study, the data of Tucker et al. (1986) supported an Ah receptor-dependent suppression, while the data of Holsapple et al. (1986a) did not. In the latter study, the magnitude of suppression was comparable using cells from responsive $B6C3F_1$ or congenic heterozygous (B6-Ah^{bd}) mice compared with nonresponsive D2 or homozygous B6-Ah^{dd} mice. In addition, they reported that the 2,7-dichlorodibenzo-*p*-dioxin congener, which lacks affinity for the Ah receptor, was equipotent with TCDD in suppressing the in vitro response.

In other studies, Davis and Safe (1991) directly compared the in vitro structure-immunotoxicity relationships for a series of HAH congeners that show $>14{,}900$-fold difference in in vivo immunotoxic potency. Results of these studies indicated that all of the congeners were equipotent in vitro and produced a similar concentration-dependent suppression of the in vitro anti-SRBC response using cells from either B6 or D2 mice. Coexposure to the Ah receptor antagonist α-napthoflavone

antagonized the immunosuppression induced by either TCDD or 1,3,6,8-TCDF (a weak Ah receptor agonist). Collectively, the results supported a mechanism of suppression in vitro that was independent of the Ah receptor.

The basis for these variable effects of TCDD in vitro currently are not known. However, recent studies by Morris et al. (1991) demonstrated that the in vitro effects of TCDD on the anti-SRBC response were critically dependent on the type and concentration of the serum used in the in vitro culture. Only 3 of 23 lots of serum were able to support a full dose-responsive suppression, and in serum-free cultures, TCDD caused a fifteenfold enhancement of the anti-SRBC response. Thus, differences in media components used in in vitro cultures may account for the different effects seen in vitro in different laboratories. Other factors such as the TCDD carrier/solvent used, the calcium content of the media, or procedures used for preparation of spleen cell suspensions may all contribute to variable effects of TCDD in vitro.

The obvious question relates to the relevance of the in vitro findings to the in vivo immunotoxicity. In this respect, it is important to note that the concentrations of TCDD required for in vitro suppression of immune function (1-30 $\times$ 10^{-9} M) of murine lymphocytes is several orders of magnitude higher than the concentration found in lymphoid tissues following exposure in vivo to an immunotoxic dose of TCDD (Neumann et al., 1992). The amount of TCDD associated with isolated spleen cells obtained from mice 2 days following treatment with 5 μg/kg ^{3}H-TCDD was 2 $\times$ 10^{-15} M per 10^{7} spleen cells. Importantly, as much as 50 percent of the radioactivity associated with whole spleen tissue was recovered in the stromal and/or capsular material (i.e., splenic tissue that resisted passage through the mesh screens used for preparation of spleen cell suspensions). These findings suggest that (1) the most potent effects of TCDD on immune function in vivo may be induced indirectly by effects on nonlymphoid cells, or (2) based on the delivered dose of TCDD, this molecule is more toxic than previously thought. Alternatively, TCDD effects in vivo on nonlymphoid cells may amplify the direct effects of TCDD on lymphoid tissue. Certainly, additional studies are needed to elucidate the serum components that are permissive for suppression or enhancement of immune responses in vitro and to determine their relevance to in vivo

conditions. Such studies are also likely to provide insight into the mechanisms of TCDD interaction with lymphoid cells.

4.8. INDIRECT MECHANISMS OF HAH IMMUNOTOXICITY

The difficulty in demonstrating consistent, direct effects of TCDD in vitro on lymphocytes, the dependence of those effects on serum components, and the requirement for high concentrations of TCDD are all consistent with an indirect mechanism of TCDD on the immune system. One potentially important indirect mechanism is via effects on the endocrine system. Several endocrine hormones have been shown to regulate immune responses, including glucocorticoids, sex steroids, thyroxine, growth hormone, and prolactin. Importantly, TCDD and other HAHs have been shown to alter the activity of all of these hormones (see Chapter 5, Developmental and Reproductive Toxicity).

Kerkvliet et al. (1990b) reported that exposure of mice to 3,4,5,3′,4′,5′-HxCB followed by injection of P815 allogeneic tumor cells induced a dose-dependent elevation of serum corticosterone concentrations that correlated with the dose-dependent suppression of the anti-P815 CTL response. However, because adrenalectomy or treatment with the glucocorticoid receptor antagonist RU38486 failed to protect mice from the immunosuppressive effect of HxCB (DeKrey et al., 1990; DeKrey et al., in press), a role for the elevated CS in the suppression of the CTL response seems unlikely. Adrenalectomy and hypophysectomy also failed to prevent TCDD-induced thymic atrophy in rats (van Logten et al., 1980).

Using the P815 allogeneic tumor model, Kerkvliet and Baecher-Steppan (1988a) reported that male mice were more sensitive than female mice to suppression of the CTL response by HxCB (Kerkvliet and Baecher-Steppan, 1988a). Castration of male mice partially ameliorated the immunosuppressive effects of HxCB (DeKrey et al., 1992; DeKrey et al., in press), suggesting a role for testosterone in suppression of the CTL response.

Pazdernik and Rozman (1985) suggest that thyroid hormones may play a role in TCDD immunotoxicity based on the finding that radiothyroidectomy prevented the suppression of the anti-SRBC response in rats treated with TCDD. However, because

thyroidectomy alone suppressed immune function, the significance of the findings requires further study.

4.9. ROLE OF THE THYMUS IN HAH IMMUNOTOXICITY

Thymic involution is one of the hallmarks of exposure to TCDD and related HAHs in all species examined. In mice, thymic involution occurs by an Ah receptor-dependent mechanism (Poland and Knutson, 1982). Because the thymus plays a critical role in the ontogeny of T lymphocytes, thymic involution is often referred to as an immunotoxic effect. However, although an intact thymus is crucial to the developing immune system during the prenatal and early postnatal period of rodents as well as during the prenatal period of humans, the physiological role played by the thymus in adult life has not been established. In animal models, adult thymectomy has little effect on the quantity or quality of T lymphocytes, which have already matured and populated the secondary lymphoid organs (Benjamini and Leskowitz, 1991). Likewise, in humans, childhood and adult thymectomy produces no clearly identifiable adverse consequences in terms of altered immune function, although some might argue that such studies have not been done. Based on this knowledge, it is not surprising that a direct relationship between the effects of TCDD on the thymus and immune suppression has not been established in studies using adult animals. In fact, adult thymectomy prior to HAH exposure did not modify TCDD- or HpCDD-induced suppression of the anti-SRBC response (Tucker et al., 1986; Kerkvliet and Brauner, 1987). Furthermore, suppression of immune responses occurs at dose levels of HAH significantly lower than those required to induce thymic atrophy (Vos et al., 1978; Silkworth and Antrim, 1985; Holsapple et al., 1986b; Tucker et al., 1986; Kerkvliet and Brauner, 1990a). Thus, it is clear that thymic involution does not represent a surrogate marker for TCDD immunotoxicity in adult animals. On the other hand, it is possible that chronic exposure to TCDD resulting in a chronic thymic atrophy may produce more delayed, subtle effects on immune function not yet identified (Clarke and MacLennan, 1986).

In contrast to adult animals, congenital thymic aplasia or neonatal thymectomy results in severe reduction in the number and function of T lymphocytes and produces a potentially lethal wasting disease (Benjamini and Leskowitz, 1991). Similarly, there is evidence from

studies carried out in the 1970s that rodents exposed to TCDD or PCBs during the prenatal or neonatal period are more sensitive to immune suppression as compared with rodents exposed as adults and that the prenatal effects are more selective for cell-mediated immunity (Vos and Moore, 1974; Faith and Moore, 1977; Luster et al., 1980a). TCDD has also been shown to alter thymocyte differentiation in vitro in cell cultures (Greenlee et al., 1985; Cook et al., 1987) and organ cultures (Dencker et al., 1985; d'Argy et al., 1989) as well as in vivo following prenatal exposure to TCDD (Blaylock et al., 1992). These observations suggest that altered thymic T cell maturation induced by TCDD in the thymus may play an important role in the suppressed immune function of prenatally exposed animals. However, because TCDD also influences B cell development in the bursa of chick embryos (Nikolaidis et al., 1990) as well as lymphocyte stem cells in the fetal liver and bone marrow of mice (Fine et al., 1989, 1990), other mechanisms of immunotoxicity are also likely to be important.

4.10. IMMUNOTOXICITY FOLLOWING PRENATAL/NEONATAL EXPOSURE TO HAHs

The reported increase in susceptibility of very young animals to HAH immunotoxicity necessitates a close examination of the available literature on prenatal or neonatal immunotoxic effects. Several studies have examined immune function in mice, rats, and guinea pigs following exposure to TCDD or PCB during fetal development (Vos et al., 1973; Vos and Moore, 1974; Thomas and Hinsdill, 1979; Luster et al., 1980a).

The results of three major studies in which exposure of the progeny occurred via placental transfer and lactation are summarized in Table 4-5. The most sensitive indicator of TCDD immunotoxicity in these studies was an increase in the growth of transplanted tumor cells in the offspring of $B6C3F_1$ mice (Ah responsive strain) treated with 1 μg/kg TCDD at 4 weekly intervals. (Total TCDD dose to dam was 4 μg/kg; dose to offspring was not determined.) The offspring of Swiss mice fed a diet containing 1 ppb TCDD for 7 weeks showed enhanced mortality following endotoxin challenge, while the plaque-forming cell response to SRBCs and delayed hypersensitivity response were suppressed in offspring of mice fed 5.0-ppb TCDD diets. (Estimated daily dose to 20 g dam consuming 5 g of 5-ppb

Table 4-5. Immunotoxic Effects of TCDD in the Offspring Following Prenatal/Neonatal Exposure to TCDD

Protocol[a]	End points	Effect	LOAEL*
Study #1:			
Pregnant B6 or	PYB6 tumor incidence	Increased	1 μg/kg × 4
$B6C3F_1$ mice given 1, 2, 5,	Allograft rejection time	Increased	2 μg/kg × 4
or 15 μg/kg TCDD orally	Body, thymus, spleen wt	Decreased	5 μg/kg × 4
on day -7, 0, +7, +14	Bone marrow cellularity	Decreased	5 μg/kg × 4
relative to parturition	T cell blastogenesis	Decreased	5 μg/kg × 4
on day 0	*Listeria monocytogenes*-induced mortality	Decreased	5 μg/kg × 4
	Bone marrow colony formation (CFU-S)	Decreased	5 μg/kg × 4
	LPS blastogenesis	--	>15 μg/kg × 4
	Anti-SRBC serum titers	--	>15 μg/kg × 4
Study #2:			
Pregnant Swiss mice	Endotoxin mortality	Increased	1.0 ppb diet
fed diets containing	Thymus weight	Decreased	2.5 ppb diet
1.0, 2.5, or 5.0 ppb	PFC response to SRBC	Decreased	5.0 ppb diet
TCDD for 7 weeks prenatally	DTH response	Decreased	5.0 ppb diet
and postnatally	Anti-SRBC serum titers	--	>5.0 ppb diet
	T and B cell blastogenesis	--	>5.0 ppb diet
	Listeria-induced mortality	--	>5.0 ppb diet
Study #3:			
Pregnant Fischer 344	Allograft rejection time	Increased	5 μg/kg × 4
rats given 1 or 5 μg/kg	T cell blastogenesis	Decreased	5 μg/kg × 4
TCDD orally on day -3, 0,	DTH response	Decreased	5 μg/kg × 4
+7, and +14 relative to	*Listeria*-induced mortality	Decreased	5 μg/kg × 4
parturition on day 0	Body and thymus weight	Decreased	5 μg/kg × 4
	Anti-BGG serum titers	--	>5 μg/kg × 4

[a]Study #1 (Vos and Moore, 1974; Luster et al., 1980a); Study #2 (Thomas and Hinsdill, 1979); Study #3 (Vos and Moore, 1974; Faith and Moore, 1977).

*Abbreviations used: LOAEL - lowest observable adverse effect level; BGG - bovine gamma globulin; LPS - lipopolysaccharide; PHA - phytohemagglutinin; Con A - Concanavalin A; SRBC - sheep red blood cell; DTH - delayed-type hypersensitivity; PFC - plaque-forming cell; CFU-S - colony-forming units-spleen.

TCDD diet is equivalent to 1.25 μg/kg TCDD/day.) Rats appeared to be more resistant to the immunotoxic effects of prenatal or neonatal exposure to TCDD based on the finding that 5, but not 1, μg/kg TCDD given four times at weekly intervals produced immunotoxicity in the offspring. Immunotoxic end points that were unaffected by the highest exposure levels in these studies included blastogenesis induced by LPS and serum antibody titers to SRBCs and bovine gamma globulin (BGG).

Two recent studies have examined immune function in offspring of female mice exposed to TCDD (Holladay et al., 1991) or PCB (Kanechlor 500) (Takagi et al., 1987) but that were cross-fostered to unexposed lactating mice at birth. Thus, exposure was limited to in utero exposure. (It is important to recognize that rodents are born with an immature immune system that matures in the first few weeks following birth. In contrast, the human immune system is considered to be more mature at birth.) B6 mice exposed to 3.0 μg/kg TCDD on gestational days 6 to 14 gave birth to offspring that had significant thymic atrophy and hypoplasia measured on gestational day 18 or on day 6 postnatally. The thymic effects were no longer apparent by day 14. At 7 to 8 weeks postnatally, mitogen responses and antibody plaque-forming cell response to SRBCs were unaltered, while the CTL response was significantly suppressed compared with controls (Holladay et al., 1991). These results suggest a selectivity of prenatal TCDD on the CTL and not the T helper cells involved in the antibody response to SRBCs. In contrast to these results, Takagi et al. (1987) exposed female C3H mice per os to 50 mg/kg Kanechlor 500 twice per week for 4 weeks, at which time steady-state tissue levels were noted. The offspring derived from mating to unexposed males had an unaltered antibody response to the T-independent antigen DNP-dextran. On the other hand, carrier-primed T helper cell activity assessed by adoptive transfer was significantly suppressed by PCB exposure when assessed 4 and 7 weeks after birth but fully recovered by 11 weeks. Together, these studies confirm prior studies to indicate that T cell function is selectively altered by HAH when exposure is prenatal. Although both T helper cells and CTL show altered function, T helper cell activity may recover faster than CTL function.

Fine et al. (1990) reported on TCDD levels in offspring following maternal treatment with TCDD (10 μg/kg) on gestational day 14. The fetal liver had the highest concentration

on gestational day 18 (235 fg/mg), which declined slightly by postnatal day 6 to around 100 fg/mg. The concentration of TCDD in the thymus on gestational day 18 was 140 fg/mg, which declined to 20 fg/mg on day 6 after birth. (These thymic TCDD concentrations are equivalent to 60 to 425 pM, assuming 1 kg of tissue is equivalent to 1 liter of water.) TCDD concentrations in the spleen remained constant at about 40 fg/mg during the same time frame, while bone marrow concentrations were very low (about 3 fg/mg). These concentrations of TCDD were associated with thymic atrophy (Fine et al., 1989) and significant reduction in the ability of prothymocytes in liver and bone marrow to repopulate an irradiated thymus (Fine et al., 1990).

4.11. IMMUNOTOXICITY OF HAHs IN NONHUMAN PRIMATES

A limited number of studies using nonhuman primates as surrogate models for humans have been conducted to assess HAH immunotoxicity. Immunological effects were described in rhesus monkeys and their offspring chronically exposed to TCDD at levels of 5 or 25 ppt for 4 years (Hong et al., 1989). In the mothers, the total number of T cells increased in monkeys fed 25 ppt TCDD, with a selective increase in $CD8^+$ cells and a decrease in $CD4^+$ cells. However, no significant effect on T cell function was established when assessed as proliferation response to mitogens, alloantigens, or xenoantigens. NK cell activity and production of antibodies to tetanus immunization were normal. In the offspring of TCDD-exposed dams examined 4 years after exposure, a significantly increased antibody response to tetanus toxoid immunization was observed that correlated with TCDD tissue levels. The body burden of TCDD in the offspring ranged from a low of 290 ppt to a high of 1,400 ppt. Interestingly, there was no strict correlation between exposure levels and resulting body burden.

In other TCDD studies, a single injection of TCDD in marmosets (*Callithrix jacchus*) resulted in a delayed decrease in the percentage of $CD4^+$ T cells and $CD20^+$ B cells in the blood and an increase in the percentage of $CD8^+$ cells (Neubert et al., 1990). The total number of T cells was not significantly altered by TCDD exposure. The $CD4^+$ subset most affected was the $CDw29^+$ "helper-inducer" or "memory" subset, with significant effects observed after a TCDD dose of 10 ng/kg. The no-observed-effect level (NOEL) for this

effect was 3 ng/kg TCDD. Concomitant with suppression of the CDw29 subset in TCDD-treated animals, the percentage of $CD4^+CD45RA^+$ cells increased. This subset has been classified as "suppressor-inducer" or "naive" cells. The changes in the T cell subsets were intensified following in vitro culture of the cells with mitogen (Neubert et al., 1991). Interestingly, however, a recent study from the same laboratory reported that chronic exposure of young marmosets to very low levels of TCDD (0.3 ng/kg/week for 24 weeks) produced the opposite effect on the $CD4^+CDw29^+$ subset, resulting in a significant increase in this population (Neubert et al., 1992). Concomitantly, the $CD4^+CD45RA^+$ subset decreased. Upon transfer of the animals to a higher dose of TCDD (1.5 ng/kg/week) for 3 weeks, the enhancement effect was reversed, and suppression of the $CD4^+CDw29^+$ subset was observed, with maximum suppression after 6 weeks of exposure to the higher dose. In addition, the $CD8^+CD56^+$ T cytotoxic T cell subset was transiently increased but normalized even though TCDD dosing continued. After discontinuation of dosing, the reduction in the percentage and absolute number of $CD4^+CDw29^+$ cells persisted for 5 weeks, reaching normal range 7 weeks later. These results led the authors to conclude that "extrapolations of the results obtained at higher doses to very low exposures is not justified with respect to the effects induced by TCDD on the immune system of marmosets."

The immunomodulatory effects of chronic low-level PCB exposure in monkeys has also been investigated. In early studies, Thomas and Hinsdill (1978) reported that rhesus monkeys fed diets containing 2.5 or 5 mg/kg of Aroclor 1248 had significantly suppressed antibody response to SRBCs but not to tetanus toxoid (TT). These monkeys also had chloracne, alopecia, and facial edema. Similarly, exposure of cynomolgus monkeys to Aroclor 1254 (100 or 400 μg/kg/day) for 3 months suppressed antibody responses to SRBCs but not TT (Truelove et al., 1982). Suppressive effects on anti-SRBC responses were more severe in cynomolgus monkeys when the PCB mixture contained PCDFs (Hori et al., 1982). Tryphonas et al. (1989; 1991a, b) have recently reported results of studies in rhesus monkeys exposed chronically to Aroclor 1254 (5-80 μg/kg/day) for 23 or 55 months. These exposures resulted in steady-state blood PCB levels that ranged from a mean low of 0.01 ± 0.001 ppm in the 5 μg/kg group to a mean high of 0.11 ± 0.01 ppm in the 80 μg/kg group. The only consistently altered immune parameter was the primary and anamnestic antibody responses to

SRBCs, which were suppressed in a dose-dependent manner. In contrast, the antibody response to pneumococcus vaccine antigen measured at 55 months of exposure was not significantly altered. At 23 months, the percentage of T helper cells in the blood was significantly decreased in the 80 μg/kg group, and the percentage and absolute number of T suppressor cells were increased; however, these effects were not apparent at 55 months of exposure (Tryphonas et al., 1991b). Lymphoproliferative responses to PHA and Con A were not significantly altered at 23 months but were dose dependently suppressed at 55 months. Proliferation to alloantigens was not significantly altered. Likewise, serum immunoglobulin and hydrocortisone levels did not differ between treatment groups. After 55 months, the chemiluminescent response (time to peak) of monocytes was slower in PCB-exposed cells. Also noted at 55 months was a significant elevation in serum hemolytic complement levels, a dose-related increase in NK cell activity, and a dose-related increase in thymosin alpha-1 levels but not thymosin beta-4 levels (Tryphonas et al., 1991a). Effects on interferon levels were inconsistent, and TNF production was not altered.

The studies in nonhuman primates are important from the standpoint that the antibody response to SRBCs emerges as the only immunological parameter consistently suppressed by HAH in several different animal species. A notable exception is the recent report that TCDD does not suppress the antibody response to SRBCs in rats (Smialowicz et al., 1994). Other immunological end points such as total T cell numbers, percentages of T cell subsets, lymphoproliferative responses, and DTH responses are inconsistently increased or decreased in various studies. At the present time, it is not clear why the antibody response to SRBCs is most consistently altered by HAH exposure in many different species. The sensitivity of the anti-SRBC response does not appear to be due solely to the T cell dependency of the response because antibody responses to other T-dependent antigens (e.g., TT, BGG) are not suppressed and may be enhanced following HAH exposure. It is possible that the particulate nature of the SRBC antigens is an important factor even though a mechanistic basis for this is not readily apparent. The sensitivity of the technique used to quantify the antibody response may also contribute to apparent increased sensitivity of the SRBC model, which is most often measured as the PFC response rather than serum antibody titers that are usually more variable. Nonetheless, the finding that the SRBC response is also suppressed in nonhuman

primates exposed to PCB lends support to the use of the anti-SRBC data generated in mice to calculate TEFs for immunotoxicity.

4.12. IMMUNOTOXICITY OF HAHs IN HUMANS

The immunotoxicity of TCDD and related HAHs in humans has been the subject of several studies derived from accidental and occupational exposures to PCBs, PBBs, and TCDD. Immunological assessment was carried out on patients who consumed acnegenic and hepatotoxic doses of PCDF- and PCB-contaminated rice oil in Taiwan in 1979. Clinical symptoms were primarily related to increased frequency of various kinds of infection, especially of the respiratory tract and skin (Lu and Wu, 1985). Immunologic effects included decreased serum IgA and IgM, but not IgG, decreased percentage of T cells in blood related to decreased $CD4^+$ T helper cells and increased $CD8^+$ T suppressor cells, and suppressed dermal delayed-type hypersensitivity responses to streptokinase-streptodornase and tuberculin antigens (reviewed by Lu and Wu, 1985). The percentage of anergic patients increased, and the degree of induration decreased with increased PCB concentration in the blood. In contrast, lymphoproliferative responses of peripheral blood lymphocyte (PBL) to PHA, pokeweed mitogen (PWM), and tuberculin, but not Con A, were significantly augmented in PCB-exposed patients. PCB concentrations in the blood ranged from 3 to 1,156 ppb, with a mean of 89 $\pm$ 6.9 ppb. The oil was contaminated at PCB concentrations of 4.8 to 204.9 ppm, with a mean of 52 $\pm$ 39 ppm.

Immunotoxic effects were also described in Michigan dairy farmers exposed to PBBs via contaminated dairy products and meat in 1973 (Bekesi et al., 1979). Like PCB-exposed patients, the percentage and absolute numbers of T cells in peripheral blood of PBB-exposed farmers were significantly reduced compared with a control group. However, in contrast to PCB, lymphoproliferation responses to PHA, PWM, and allogeneic leukocytes were significantly decreased in PBB-exposed persons. Also in contrast to PCB, skin testing using standard recall antigens indicated that PBB-exposed Michigan dairy farmers had significantly increased responses, particularly to candida and Varidase. Tissue levels of PBB in the subjects were not determined in these studies.

Webb et al. (1989) reported the findings from immunologic assessment of 41 persons from Missouri with documented adipose tissue levels of TCDD resulting from occupational, recreational, or residential exposure. Of the participants, 16 had tissue TCDD levels less than 20 ppt; 13 had levels between 20 and 60 ppt; and 12 had levels greater than 60 ppt. The highest level was 750 ppt. Data were analyzed by multiple regression based on adipose tissue level and the clinical dependent variable. Increased TCDD levels were correlated with an increased percentage and total number of T lymphocytes. $CD8^+$ and $T11^+$ T cells accounted for the increase, while $CD4^+$ T cells were not altered in percent or number. Lymphoproliferative responses to Con A, PHA, PWM, or TT were unaltered, as was the cytotoxic T cell response. Serum IgA, but not IgG, was increased. No adverse clinical disease was associated with TCDD levels in these subjects. Only 2 of the 41 subjects reported a history of chloracne. These findings differ from those reported for the Quail Run Mobile Home Park residents (tissue levels unknown) in which decreased T cell numbers (T3, CD4, and T11) and suppressed cell-mediated immunity were reported (Hoffman et al., 1986). However, subsequent retesting of these anergic subjects failed to confirm the anergy (Evans et al., 1988). On the other hand, when serum from some of these individuals was tested for levels of the thymic peptide, thymosin alpha-1, the entire frequency distribution for the TCDD-exposed group was shifted toward lower thymosin alpha-1 levels (Stehr-Green et al., 1989). The statistically significant difference between the TCDD-exposed persons and controls remained after controlling for age, sex, and socioeconomic status, with a trend of decreasing thymosin alpha-1 levels with increasing number of years of residence in the TCDD-contaminated residential area. The thymosin alpha-1 levels were not correlated with changes in other immune system parameters nor with any increased incidence of clinically diagnosed immune suppression. The decrease in thymosin alpha-1 levels in humans contrasts with the increase in thymosin alpha-1 seen in PCB-treated monkeys (Tryphonas et al., 1991b). Finally, Mocarelli et al. (1986) reported studies on the immune status of 44 children, 20 of whom had chloracne, who were exposed to TCDD following an explosion at a herbicide factory in Seveso, Italy. No abnormalities were found in the following parameters: serum immunoglobulin concentrations, levels of circulating complement, or lymphoproliferative responses to T and B cell mitogens. Interestingly, in a study conducted

6 years after the explosion, a different cohort of TCDD-exposed children exhibited a significant increase in complement protein levels, which correlated with the incidence of chloracne as well as increased numbers of peripheral blood lymphocytes and increased lymphoproliferative responses (Tognoni and Bonaccorsi, 1982). However, no specific health problems were correlated with dioxin exposure in these children.

It is readily apparent that no clear pattern of immunotoxicity to HAH emerges from these studies in humans. In some cases, T cell numbers increase; in others, they decrease. The findings are not unlike the varied and often conflicting reports found in the literature regarding animal studies of HAH immunotoxicity. The basis for the lack of consistent, significant exposure-related effects is unknown and may be dependent on several factors. Most notable in this regard are the generic difficulties in assessing subclinical immunomodulation, particularly in outbred human populations. Most immunological assays have a very broad range of normal responses reducing the sensitivity to detect small changes. Similarly, the assays used to examine immune function in humans exposed to TCDD and related HAH have unfortunately been based to a greater extent on what was clinically "doable" (e.g., mitogen responsiveness) rather than on assays that have been shown to be sensitive to TCDD in animal studies. Thus, the lack of consistent or significant immunotoxic effects in humans resulting from TCDD exposure may be as much a function of the assays used as the immune status of the cohort. In addition, few studies have examined the immune status of individuals with known, documented exposure to HAH. Rather, cohorts based on presumption of exposure have been studied. There is some evidence to suggest that the lack of significant effects may sometimes be due to the inclusion of subjects that had little or no actual exposure to TCDD (Webb et al., 1989). Likewise, the important role that Ah phenotype plays in TCDD immunotoxicity has not been considered when addressing human sensitivity. Whether there are human equivalents of murine Ah^{bb} and Ah^{dd} types is not known. Finally, in most studies, the assessment of immune function in exposed populations was carried out long after exposure to TCDD ceased. Thus, recovery from the immunotoxic effects of TCDD may have occurred.

Recently, several laboratories have examined the direct effects of TCDD on human lymphocytes. Neubert et al. (1991) reported decreased PBL subpopulations from humans

and nonhuman primates cultured in the presence of TCDD. $CD4^+CD29^+$ helper-inducer/memory T cells and $CD20^+$ B cells were dose dependently decreased in PWM-stimulated cultures of human PBLs at concentrations as low as 10^{-12}-10^{-14} M TCDD. However, an attempt to corroborate these findings failed to detect any suppression in human PBL subpopulations, including $CD4^+CD29^+$ helper-inducer/memory T cells and $CD19^+$ B cells, at TCDD concentrations ranging from 10^{-7} to 10^{-14} M (Lang et al., 1994). Furthermore, PWM- or anti-CD3-stimulated lymphocyte proliferation was not altered by TCDD at concentrations of 10^{-7} to 10^{-11} M (Lang et al., 1994). Similar results were obtained by Wood et al. (1992) who reported that exposure of human tonsillar lymphocytes (HTLs) to concentrations of 3×10^{-8} to 3×10^{-10} M TCDD did not affect either PWM-induced proliferation or IgM antibody production. In contrast, 3×10^{-8} to 3×10^{-10} M TCDD suppressed toxic shock syndrome toxin (TSST-1)-induced IgM secretion of HTL B cells (Wood and Holsapple, 1993). The sensitivity of the HTL B cells to TCDD suppression of TSST-1-induced IgM secretion, however, was found to be highly variable among the different donors. In a separate study, concentrations of 3×10^{-8} to 3×10^{-10} M TCDD suppressed the background proliferation and IgM secretion of low-density, but not high-density, HTL B cells (Wood et al., 1993). TCDD also suppressed LPS plus T cell replacement factor-stimulated proliferation and IgG secretion of low-density, but not high-density, HTL B cells. These results suggest that TCDD may have a direct effect on HTL low-density B cells and that this lymphocyte subpopulation may be a sensitive target for TCDD. Further work is necessary to corroborate these findings.

In summary, based on animal data, one might speculate that any future study to determine HAH immunotoxicity in humans should evaluate their antibody response to SRBC. However, it should be emphasized that even the relatively low exposure levels that have been shown to suppress the anti-SRBC response in nonhuman primates resulted in blood and tissue PCB or TCDD levels that far exceed the levels measured in humans in most studies published to date involving environmental exposure. Thus, even the anti-SRBC response may not have been sensitive enough to demonstrate immune suppression in these cohorts. Given the current lack of data correlating clinical immunological end points with immune status in humans (except in cases of overt immune deficiencies), massive retrospective studies

of poorly defined exposure groups cannot be justified to try to "prove" that immune modulation has occurred in these people. Rather, such efforts would be better directed toward the establishment of a broad database of normal values for the clinical immunology end points that may be of use in immunotoxicity assessments. In conjunction with this effort, research must focus on the definition of sensitive end points (i.e., biomarkers) of immune dysfunction in humans so that, in the future, emergency response teams could respond rapidly to accidental exposures to assess the immunological status of the exposed persons. To validate these biomarkers, there is a parallel need for animal research to identify TCDD-sensitive immune end points in animals that can also be measured in humans to establish correlative changes in the biomarker and immune function. In particular, it will be important to determine in animal models how well changes in immune function in the lymphoid organs (e.g., spleen, lymph nodes) correlate with changes in the expression of lymphocyte subset/activation markers in peripheral blood. Until such correlations are established, the interpretation of changes observed in subsets or activation markers in human peripheral blood lymphocytes in terms of health risk will be limited to speculation. Research must also continue to develop well-defined animal models using multiple animal species that will lead to an understanding of the underlying mechanisms of HAH immunotoxicity. For example, there is a clear need to document Ah receptor involvement in the immunotoxicity of TCDD and related HAHs in species other than mice. These studies need to go beyond descriptive immunotoxicity assessment to determine the mechanistic basis for differences in species sensitivity to TCDD immunotoxicity following both acute and chronic exposure. In the interim, the available database derived from well-controlled animal studies on HAH immunotoxicity can be used for establishment of no-effect levels and acceptable exposure levels for human risk assessment of TCDD using the same procedures that are used for other noncarcinogenic toxic end points. Because the antibody response to SRBC has been shown to be dose dependently suppressed by TCDD and related HAHs in several animal species, this database is best suited for current application to risk assessment.

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5. DEVELOPMENTAL AND REPRODUCTIVE TOXICITY*

5.1. INTRODUCTION

The potential for dioxins and related compounds to cause reproductive and developmental toxicity has been recognized for many years. Recent laboratory studies have broadened our knowledge in this area and suggest that altered development may be among the most sensitive TCDD endpoints. This chapter reviews much of the literature on dioxin's developmental and reproductive toxicity but is not intended to be exhaustive. Special emphasis is placed on that part of the database that has accumulated since the last major EPA review of this topic (Kimmel, 1988). In addition, the database is viewed in light of the Ah receptor model of TCDD action that is being examined for its applicability in the current EPA risk assessment.

To focus the analysis of the database, the chapter is divided into developmental toxicity and male and female reproductive toxicity. The authors recognize the interrelatedness of developmental and reproductive events at all levels of biological complexity. Therefore, the reader should not view the chapter subheadings within each of these divisions as defining discrete endpoints that are exclusive of other endpoints. For example, the effects of TCDD on circulating levels of sex hormones or on responsiveness to sex hormones may be translated into reproductive dysfunction if exposure occurs in adulthood or abnormal development of sexual behavior if exposure occurs perinatally. Likewise, even though organ structure and growth are considered separate manifestations in developmental toxicity that are associated with perinatal exposure to TCDD, the normal development of an organ is dependent on normal growth processes, and inhibiting perinatal growth can significantly disrupt the structural integrity of an organ system.

2,3,7,8-TCDD is one of 75 possible CDD congeners. It is one of the most potent of the CDDs, BDDs, CDFs, BDFs, PCBs, and PBBs and as such serves as the prototype

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congener for investigating the toxicity elicited by these classes of chemicals. Developmental and reproductive toxicity is generally believed to be caused by the parent compound; there is no evidence that TCDD metabolites are involved. The toxic potency of TCDD is due to the number and position of chlorine substitutions on the dibenzo-*p*-dioxin molecule. CDD congeners with decreased lateral (2, 3, 7, and 8) or increased nonlateral chlorine and bromine substituents are less potent than TCDD (Safe, 1990); however, most of these congeners will produce toxicity, and the pattern of responses within animals of the same species, strain, sex, and age will generally be similar to that of TCDD (McConnell and Moore, 1979; Poland and Knutson, 1982). PCB congeners with zero or one *ortho* chlorines, two *para* chlorines, and at least two *meta* chlorines can assume a coplanar conformation sterically similar to TCDD and also produce a pattern of toxic responses similar to that of TCDD. In contrast, PCB congeners with two or more *ortho* chlorines cannot assume a coplanar conformation and do not resemble TCDD in toxicity (Poland and Knutson, 1982; Safe, 1990).

CDD and CDF congeners chlorinated in the lateral positions, as compared with those lacking chlorines in the 2, 3, 7, and 8 positions, are preferentially bioaccumulated by fish, reptiles, birds, and mammals (Stalling et al., 1983; Cook et al., 1991; U.S. EPA, 1991). Furthermore, coplanar PCBs and/or monoortho-chlorine-substituted analogs of the coplanar PCBs bioaccumulate in fish, wildlife, and humans (Tanabe, 1988; Kannan et al., 1988; Mac et al., 1988; Kubiak et al., 1989; Smith et al., 1990). This is of concern because combined effects of the lateral-substituted CDD, BDD, CDF, BDF, PCB, and PBB congeners acting through an Ah receptor mechanism have the potential of decreasing feral fish and wildlife populations secondary to developmental and reproductive toxicity (Gilbertson, 1989; Walker and Peterson, 1991; Walker et al., 1991; Cook et al., 1991). Humans are not exempt from the developmental and reproductive effects of complex halogenated aromatic hydrocarbon mixtures. Such mixtures that contain both TCDD-like congeners and non-TCDD-like congeners have been implicated in causing developmental and reproductive toxicity in the Yusho and Yu-Cheng poisoning incidents in Japan and Taiwan (Kuratsune, 1989; Hsu et al., 1985; Rogan, 1989). Thus, exposure to TCDD-like congeners is a health concern for

humans as well as for domestic animals, fish, and wildlife, although the relative contributions of TCDD-like and non-TCDD-like congeners are not known in some exposure situations.

A mechanism of action that CDD, BDD, CDF, BDF, PCB, and PBB congeners substituted in the lateral positions have in common is that they bind to the Ah receptor, which then binds to a translocating protein that carries the activated TCDD receptor complex into the nucleus. These activated TCDD receptor complexes bind to specific sequences of DNA referred to as dioxin-responsive enhancers (DREs), resulting in alterations in gene transcription. There is evidence that this Ah receptor mechanism, explained in detail in an earlier chapter, may be involved in the antiestrogenic action of TCDD and in its ability to produce the structural malformations of cleft palate and hydronephrosis in mice. However, its role in producing other signs of developmental and reproductive toxicity is not firmly established, leaving open the possibility that some TCDD effects are not Ah receptor mediated.

5.2. DEVELOPMENTAL TOXICITY

The manifestations of developmental toxicity from exposure to TCDD have been divided into three categories for convenience in assessing the database with respect to an Ah receptor-mediated response. These categories include death/growth/clinical signs, structural malformations, and functional alterations. Exposure-related effects on death/growth/clinical signs are described for fish, birds, laboratory mammals, and humans along with structure-activity results that are consistent with, but do not prove, an Ah receptor-mediated mechanism. Structural malformations, particularly cleft palate formation and hydronephrosis, occur in mice. In other mammalian species, however, postnatal functional alterations, some of which may be irreversible, are the most sensitive adverse developmental effects of TCDD-like congeners. These include effects on the male and female reproductive systems in rats and object learning behavior in monkeys.

5.2.1. Death/Growth/Clinical Signs

5.2.1.1. *Fish*

Early life stages of fish appear to be more sensitive to TCDD-induced mortality than adults. This is suggested by the LD_{50} of TCDD in rainbow trout sac fry (0.4 μg/kg egg weight) being 25 times less than that in juvenile rainbow trout (10 μg/kg body weight) (Walker and Peterson, 1991; Kleeman et al., 1988). The significance of this finding is that early life stage mortality caused by high concentrations of TCDD-like congeners in fish eggs may pose the greatest risk to feral fish populations (Walker and Peterson, 1991; Cook et al., 1991). Cooper (1989) reviewed the developmental toxicity of CDDs and CDFs in fish, and Cook et al. (1991) discussed components of an aquatic ecological risk assessment for TCDD in fish. The reader is referred to this literature for more indepth coverage than is presented here.

TCDD is directly toxic to early life stages of fish. This has been demonstrated for Japanese medaka, pike, rainbow trout, and lake trout exposed as fertilized eggs to graded concentrations of waterborne TCDD. In these species, TCDD causes an overt toxicity syndrome characterized by edema, hemorrhages, and arrested growth and development culminating in death (Helder, 1980, 1981; Wisk and Cooper, 1990a; Spitsbergen et al., 1991; Walker et al., 1991; Walker and Peterson, 1991). Histopathologic evaluation of lake trout embryos and sac fry has shown this syndrome to be essentially identical to that of blue sac disease (Helder, 1981; Spitsbergen et al., 1991). Following egg exposure to TCDD, signs of toxicity are not detected in medaka until after the liver rudiment forms (Wisk and Cooper, 1990a), and in lake trout toxicity is first detected ~ 1 week prior to hatching but becomes fully manifest during the sac fry stage (Spitsbergen et al., 1991; Walker et al., 1991). Among all fish species investigated thus far, lake trout are the most sensitive to TCDD developmental toxicity. Following exposure of fertilized lake trout eggs to graded waterborne concentrations of TCDD, the NOAEL for sac fry mortality is 34 pg TCDD/g egg, the LOAEL is 55 pg TCDD/g egg, and the egg TCDD concentration that causes 50 percent mortality above control at swim up (LD_{50}) is 65 pg TCDD/g egg (Walker et al., 1991). Thus, TCDD is a potent developmental toxicant in fish and the effect is not secondary to maternal toxicity.

The Ah receptor has not been identified in early life stages of fish; however, it is assumed to be present because PCBs induce hepatic cytochrome P-4501A1 in lake trout and brook trout embryos and fry (Binder and Stegeman, 1983; Binder and Lech, 1984). The Ah receptor has been identified in adult rainbow trout liver (Heilmann et al., 1988) and in a rainbow trout hepatoma cell line (Lorenzen and Okey, 1990). CDD and CDF congeners that are approximate isostereomers of TCDD produce essentially the same pattern of toxic responses as TCDD in early life stages of medaka and rainbow trout, suggesting that they may act through a common mechanism (Wisk and Cooper, 1990b; Walker and Peterson, 1991). Also, in rainbow trout their potencies relative to TCDD (i.e., toxic equivalency factors, TEFs) for causing early life stage mortality (TCDD LD_{50}/congener LD_{50}) are in the same range as those proposed for human health risk assessment based on a diverse spectrum of acute and subchronic toxicity tests in mammalian species (Safe, 1990; Walker and Peterson, 1991). However, for the coplanar PCBs and monoortho-chlorinated analogs of the coplanar PCBs, TEFs based on early life stage mortality in rainbow trout are 1/14 to 1/80 less (Walker and Peterson, 1991) than the TEFs proposed for risk assessment (Safe, 1990).

5.2.1.2. *Birds*

Bird embryos are also more sensitive to TCDD toxicity than adults. The LD_{50} of TCDD in the chicken embryo (0.25 μg/kg egg weight) is 100 to 200 times less than the TCDD dose that causes mortality in adult chickens (25-50 μg/kg body weight) (Greig et al., 1973; Allred and Strange, 1977). The LD_{50} of TCDD injected into fertilized ring-necked pheasant eggs (1.1-1.8 μg/kg egg weight) is 14 to 23 times less than the TCDD dose that causes 75 percent mortality in ring-necked hen pheasants (25 μg/kg body weight) (Nosek et al., 1993).

Among bird species, most developmental toxicity research has been done on chickens. Injection of TCDD or its approximate isostereomers into fertilized chicken eggs causes a toxicity syndrome in the embryo characterized by pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in the thymus and bursa of Fabricius, microphthalmia, beak deformities, cardiovascular malformations, and mortality (Cheung et al., 1981a,b; Brunstrom and Darnerud, 1983; Rifkind et al., 1985; Brunstrom and Lund,

1988; Brunstrom and Andersson, 1988; Nikolaidis et al., 1988a,b). On the other hand, injection of a coplanar PCB into fertilized turkey eggs at a dose high enough to cause microphthalmia, beak deformities, and embryo mortality did not produce liver lesions, edema, or thymic hypoplasia, all hallmark signs of TCDD toxicity in the chicken embryo (Brunstrom and Lund, 1988). This disparity in signs of TCDD embryotoxicity among bird species is not unique to the turkey and chicken. In fertilized eggs of ring-necked pheasants and eastern bluebirds, injection of TCDD produces embryo mortality, but all of the other signs of toxicity seen in the chicken embryo are absent, including cardiovascular malformations (Thiel et al., 1988; Martin et al., 1989; Nosek et al., 1993). Thus, in bird embryos the signs of toxicity elicited by TCDD and its approximate isostereomers are highly species dependent; the only toxic effect common to all bird species is embryo mortality.

There is evidence in chicken embryos that the Ah receptor may be involved in producing developmental toxicity. The Ah receptor has been detected in chicken embryos (Denison et al., 1986; Brunstrom and Lund, 1988) and the rank order potency of PCB congeners for producing chicken embryo mortality (3,3′,4,4′,5-PCB > 3,3′,4,4′-TCB > 3,3′,4,4′,5,5′-HCB > 2,3,3′,4,4′-PCB > 2,3,4,4′,5-PCB, with 2,2′,4,5′-TCB, 2,2′,4,4′,5,5′-HCB, and 2,2′,3,3′,6,6′-HCB being inactive) is similar to that for a classic Ah receptor-mediated response in the chicken embryo cytochrome P-4501A1 induction (Rifkind et al., 1985; Brunstrom and Andersson, 1988; Brunstrom, 1989). However, although induction of cytochrome P-4501A1 and toxicity may both be part of a pleiotropic response linked to the Ah receptor, they are not otherwise causally related. This is demonstrated by the nonsteroidal anti-inflammatory drug benoxoprofen that suppresses 3,3′,4,4′-TCB-induced toxicity in the chicken embryo without altering its ability to induce microsomal enzyme activity (Rifkind and Muschick, 1983). Also, for 3,3′,4,4′-TCB, 3,3′,4,4′,5,5′-HCB, and TCDD there is a marked dissociation of the dose-response relationship for lethality and enzyme induction in the chicken embryo (Rifkind et al., 1985).

A decreased activity of uroporphyrinogen decarboxylase (URO-D) and an increased accumulation of uroporphyrins are effects that are readily produced by exposure of cultured chicken embryo liver cells to TCDD, 3,3′,4,4′-TCB, and other PCBs (Sinclair et al., 1984; Marks, 1985; Lambrecht et al., 1988). Coplanar PCB congeners are more potent inhibitors

of URO-D activity in cultured chicken embryo liver cells than are noncoplanar PCB congeners (Sassa et al., 1986), suggesting an Ah receptor-mediated mechanism. Unlike the results in cultured cells, however, a lethal dose of TCDD (6 nmol/egg) does not affect URO-D activity or cause an increased accumulation of uroporphyrins in chicken embryos (Rifkind et al., 1985). Thus, TCDD-induced lethality in chicken embryos is not associated with the effects of TCDD on URO-D activity, even though a decrease in URO-D activity might be expected to occur if a sufficient dose of TCDD could be reached without being lethal.

The chicken embryo heart is a target organ for TCDD and other halogenated aromatic hydrocarbons that act by an Ah receptor mechanism. The classic sign of chick embryo toxicity involving the heart is pericardial edema. However, TCDD has other effects on the chick embryo heart that are less well known. These include its ability to produce cardiovascular malformations and to increase cardiac release of arachidonic acid metabolites. When fertilized chicken eggs are injected with graded doses of TCDD, cardiovascular malformations are produced including ventricular septal defects, aortic arch anomalies, and conotruncal malformations. Approximately 1.6 pmol TCDD/egg (9 ng/kg egg, assuming a 55 g egg weight) causes cardiovascular malformations in 46 percent of treated embryos versus 29 percent of control embryos (Cheung et al., 1981a,b). The cardiovascular malformation response may be unique to the chicken embryo because in fertilized ring-necked pheasant and eastern bluebird eggs injected with TCDD the incidence of such malformations is not increased (Thiel et al., 1988; Martin et al., 1989; Nosek et al., 1993).

In the chicken embryo heart, arachidonic acid metabolism is stimulated by TCDD, resulting in increased formation of prostaglandins (Quilley and Rifkind, 1986). Dose-response relationships for the release of immunoreactive PGE_2, $PGF_{2\alpha}$, and TxB_2 from chick embryonic heart are biphasic with an apparent maximally effective dose of 100 pmol TCDD/egg. When the egg TCDD dose is further increased, release of these prostaglandins tends to decline towards levels in control hearts. Biphasic dose response curves for cardiac PGE_2 release also were obtained with 3,3′,4,4′-TCB and 3,3′,4,4′,5,5′-HCB (Quilley and Rifkind, 1986). The thymus and bursa of Fabricius are other TCDD target organs in the chicken embryo. TCDD, 3,3′,4,4′-TCB, and 3,3′,4,4′-TCAOB cause dose-related decreases in the lymphoid development of both of these immune system organs (Nikolaidis et al.,

1988a,b, 1990). Cultured thymus anlage from chick embryos are 100 times more sensitive to TCDD's inhibitory effect on lymphoid development than cultured thymus anlage from turkey and duck embryos (Nikolaidis et al., 1988a). This suggests that the reason thymic atrophy was not seen in turkey embryos at egg doses of 3,3′,4,4′-TCB that were overtly toxic (Brunstrom and Lund, 1988) was not because the turkey embryo thymus was incapable of responding to 3,3′,4,4′-TCB. Rather, turkey embryos appear to be more sensitive to the lethal than to the immunotoxic effect of this coplanar PCB.

Within the same bird species, the signs of developmental toxicity elicited by TCDD and its approximate isostereomers are similar. In the chicken embryo, TCDD, 3,3′,4,4′,5-PCB, 3,3′,4,4′-TCB, and 3,3′,4,4′,5,5′-HCB all cause pericardial and subcutaneous edema, liver lesions, micropthalmia, beak deformities, and mortality, and TCDD, 3,3′,4,4′-TCB, and 3,3′,4,4′-TCAOB inhibit lymphoid development (Cheung et al., 1981a; Brunstrom and Andersson, 1988; Nikolaidis et al., 1988a,b). In pheasant embryos, an altogether different pattern of responses is seen. Nevertheless, the TCDD-like congeners injected into fertilized pheasant eggs, TCDD and 3,3′,4,4′-TCB, produce the same pheasant embryo-specific pattern. This pattern consists of embryo mortality in the absence of edema, liver lesions, thymic hypoplasia, and structural malformations (Brunstrom and Reutergardh, 1986; Nosek et al., 1993).

The lethal potency of TCDD and its approximate isostereomers in embryos of different bird species varies widely. The chicken embryo is an outlier in that it is by far the most sensitive of all bird species to TCDD. Turkey, ring-necked pheasant, mallard duck, domestic duck, domestic goose, golden-eye, herring gull, black-headed gull, and eastern bluebird embryos are considerably less sensitive to the embryo lethal effect of TCDD and TCDD-like congeners (Brunstrom and Reutergardh, 1986; Brunstrom and Lund, 1988; Thiel et al., 1988; Martin et al., 1989; Elliott et al., 1989; Nosek et al., 1993). TCDD is 4 to 7 times more potent in causing embryo mortality in chicken than pheasant embryos, and 3,3′,4,4′-TCB is 20 to 100 times more potent in chicken than turkey embryos (Allred and Strange, 1977; Brunstrom and Lund, 1988; Nosek et al., 1989). In chicken embryos, an egg dose of 3,3′,4,4′-TCB of 4 μg/kg increased embryo mortality, whereas an egg dose of 100 μg/kg of the same coplanar PCB had no embryotoxic effect in pheasants and mallard ducks

and a dose of 1,000 μg/kg egg had no effect on embryo mortality in domestic ducks, domestic geese, golden eyes, herring gulls, and black-headed gulls (Brunstrom, 1988; Brunstrom and Reutergardh, 1986). In contrast to the above species differences, the potency of 3,3′,4,4′-TCB in causing embryo mortality among different strains of chickens is quite similar, with the LD_{50} in six different strains varying less than fourfold (Brunstrom, 1988).

Graded doses of TCDD have been administered to fertilized eastern bluebird and ring-necked pheasant eggs for the purpose of determining an LOAEL and NOAEL for embryotoxicity. Mortality was the most sensitive embryotoxic effect in both species. For eastern bluebirds, the LOAEL was 10,000 pg TCDD/g egg and the NOAEL was 1,000 pg TCDD/g egg (Martin et al., 1989). For ring-necked pheasants, the LOAEL was 1,000 pg TCDD/g egg and the NOAEL was 100 pg TCDD/g egg. The LD_{50} for embryo mortality in the ring-necked pheasant is 1,354 pg TCDD/g egg when the dose is injected into the egg albumin and 2,182 pg TCDD/g egg when the dose is injected into the egg yolk (Nosek et al., 1993). In contrast, for chickens the LD_{50} for embryo mortality is 240 pg TCDD/g egg (Allred and Strange, 1977).

5.2.1.3. *Laboratory Mammals*

When exposed to TCDD during adulthood, laboratory mammals display wide differences in the LD_{50} of TCDD. It is interesting to note, however, that when exposure occurs during prenatal development, the potency of TCDD tends to be more similar across species. The LD_{50} of TCDD in adult hamsters, 1,157 to 5,051 μg/kg, makes adult hamsters three orders of magnitude more resistant to TCDD-induced lethality than are adult guinea pigs (Olson et al., 1980; Henck et al., 1981). Yet, a maternal dose of 18 μg TCDD/kg can increase the incidence of prenatal mortality in the hamster embryo/fetus. Since this dose is only twelvefold larger than the dose (1.5 μg TCDD/kg) that increases the incidence of prenatal mortality in the guinea pig, the hamster embryo/fetus approaches other rodent species in its sensitivity to TCDD-induced lethality (Olson and McGarrigle, 1990, 1991). Thus, the magnitude of the species differences in lethal potency of TCDD is affected by the timing of TCDD exposure during the life history of the animal.

Exposure to TCDD during pregnancy causes prenatal mortality in the monkey, guinea pig, rabbit, rat, hamster, and mouse (Table 5-1). Given a particular dosage regimen, the response is dose related and there appear to be species and/or strain differences in susceptibility to TCDD-induced prenatal mortality. The rank order of susceptibility from the most sensitive to least sensitive species would appear to be monkey = guinea pig > rabbit = rat = hamster > mouse. However, an important caveat must be applied to the information presented in Table 5-1; i.e., that the time period during which exposure of the embryo/fetus to TCDD occurs is just as important a determinant of prenatal mortality as is the dose of TCDD administered. This point will be illustrated in the text that follows when prenatal mortality is described for different strains of mice.

It is important to note that the concept of a critical time period for exposure makes the analysis of lethality data in the embryo/fetus qualitatively different from that which might be applied to similar data in adult animals. For example, a common dosing regimen used in mice, rats, and rabbits (Table 5-1) is to administer 10 daily doses of TCDD to the pregnant dam on days ~6 to 15 of gestation. This dosing regimen is expected to cover the critical period of early development that results in the greatest incidence of prenatal toxicity. In nearly all species of adult laboratory mammals, however, a single lethal dose of TCDD would be expected to produce a similar delayed-onset death regardless of the age of the adult animal. Susceptibility to TCDD-induced prenatal mortality, in contrast, may be greatly dependent on the age of the embryo/fetus. In this case, multiple doses of TCDD that cover this critical period might result in prenatal mortality, whereas a single dose might miss the critical time and not result in prenatal mortality.

The following paragraphs illustrate a type of analysis using an index of cumulative maternal dose similar to the type of analysis that might be applied to lethality data resulting from multiple dosing of adult animals. After presenting the results of applying this type of analysis to prenatal mortality data from different species, the caveat of critical time dependence will be applied to the data obtained by using different strains of mice. This will illustrate the importance of considering dosage regimen when evaluating prenatal mortality data that are available in the literature. In this case, a difference of 1 gestational day might be critically important. It turns out that the form of analysis using cumulative maternal dose

Table 5-1. Relationship Between Maternal Toxicity and Prenatal Mortality in Laboratory Mammals Exposed to TCDD During Gestation

Species/Strain	Daily TCDD Dose (μg/kg/day)	Cumulative TCDD Dose (μg/kg)	Overt Maternal Toxicity[a]	Percent Prenatal Mortality[b]	Reference
Monkey/rhesus		0[c]	-	25	McNulty, 1984
		0.2	-	25	
		1	+[d]	81	
		5	+[d]	100	
Guinea pig/Hartley		0[e]	-	-	Olson and McGarrigle, 1991
		0.15	-	-	
		1.5	+	+	
Rabbit/New Zealand	0[f]	0	-	7	Giavini et al., 1982b
	0.1	1	-	12	
	0.25	2.5	+	42	
	0.5	5	+	22	
	1	10	+	100	
Rat/Wistar	0[f]	0	-	3	Khera and Ruddick, 1973
	0.125	1.25	-	1	
	0.25	2.5	-	2	
	0.5	5	-	9	
	1	10	±	8	
	1	10	+	36[g]	
	2	20	+	53[g]	
	4	40	+	100[g]	
Rat/Sprague-Dawley	0[f]	0	-	25	Sparschu et al., 1971
	0.03	0.3	-	21	
	0.125	1.25	-	15	
	0.5	5	+	41[g]	
	2	20	+	95[g]	
	8	80	+	100[g]	
Hamster/Golden Syrian		0[h]	-	-	Olson and McGarrigle, 1991
		1.5	-	-	
		3	-	-	
		6	-	-	
		18	-	58	
Mouse/CD-1	0[i]	0	-	7	Courtney, 1976
	25	250	-	6	
	50	500	-	13	
	100	1,000	-	14	
	200	2,000	+	87	
	400	4,000	+	97	

[a]Decreased body weight gain or marked edema compared with vehicle dosed controls. A (+) or (-) indicates the presence or absence of an effect.
[b]Percentage of absorptions plus late gestational deaths relative to all implantations. A (+) or (-) indicates the presence or absence of an effect.
[c]TCDD administered in a single or divided doses between gestational days 20 and 40.
[d]Effects include thickening and reddening of the eyelids, weight loss, dryness and granularity of the skin, loss of hair, and in some cases anemia, purpura, and bleeding from the nose and mouth.
[e]Single dose of TCDD administered on gestational day 14.
[f]TCDD administered daily on days 6 to 15 of gestation.
[g]Significant at $p < 0.05$.
[h]Single dose of TCDD administered on gestational days 7 or 9.
[i]TCDD administered daily on days 7 to 16 of gestation.

Source: Couture et al., 1990a.

may give the greatest possible degree of species variation. As such, different species may actually be more similar with respect to susceptibility to prenatal mortality than would be apparent from the results of this type of an analysis.

Using the cumulative dose data that are given in Table 5-1, there appears to be a tenfold to twentyfold difference in the fetolethal potency of TCDD when the monkey/guinea pig is compared with the rabbit/rat/hamster. In the CD-1 mouse treated with TCDD on gestational days 7 to 16, it appears that a daily dose of 200 μg TCDD/kg is required to significantly increase prenatal mortality. Given a ~5.5-day half-life of TCDD in the pregnant dam (Weber and Birnbaum, 1985), the pregnant CD-1 mouse would be exposed to a maximal accumulated dose, of ~1,200 μg TCDD/kg by the lowest dosage regimen that significantly increased prenatal mortality. Therefore, by using the index of cumulative dose, the CD-1 mouse would appear to be ~1,200-fold less sensitive than the monkey/guinea pig for TCDD-induced prenatal mortality. However, in NMRI mice administered TCDD only on day 6 of gestation, prenatal mortality begins to increase after a single dose of 45 μg TCDD/kg (Neubert and Dillman, 1972). The NMRI embryo/fetus is less susceptible to TCDD-induced prenatal mortality when the TCDD is administered on later gestational days up to day 15. Thus, there appears to be only an approximate 45-fold difference between the monkey/guinea pig and the NMRI mouse when the NMRI embryo/fetus is exposed specifically on day 6. In C57BL/6 mice, prenatal mortality is significantly increased after a single maternal dose of 24 μg TCDD/kg given on gestational day 6 (Couture et al., 1990b). This mouse strain, therefore, is about twentyfold to thirtyfold less sensitive to TCDD-induced prenatal mortality than is the monkey/guinea pig when exposed specifically on day 6. As with the NMRI mouse, there was little or no increase in prenatal mortality for the C57BL/6 strain when TCDD was administered to the pregnant dam on gestational days 8, 10, 12, or 14.

Mammalian pregnancies (including the human) are characterized by critical periods or "windows" during which the embryo/fetus exhibits different susceptibilities and responses to chemical exposures. As the embryo matures into a fetus and a neonate capable of extrauterine life, the responses to chemical insult shift from a predominance of death and structural alterations to changes in growth and functional maturation. The susceptibility of

any particular endpoint depends on the developmental state of that endpoint at the time of exposure. The embryo/fetus is constantly changing at all biological levels (e.g., cellular, tissue, organism), and the mechanisms of action, response, and repair of a particular endpoint at the time of exposure are the determinants of whether a response to a given exposure will result in a developmental alteration or not.

The concept of a critical window for TCDD-induced lethality in the embryo/fetus suggests an explanation for the apparent insensitivity of the CD-1 embryo/fetus exposed to cumulative doses of TCDD. It could very well be that the critical window for prenatal mortality in the mouse occurs on or before gestational day 6. If the embryo/fetus is not exposed to TCDD by gestational day 6, much larger doses of TCDD are required to produce prenatal mortality. Given that exposure of the pregnant CD-1 dams did not begin until gestational day 7, this interpretation is consistent with the ability of a single 24 μg TCDD/kg dose to increase the incidence of prenatal mortality when administered to pregnant C57BL/6 mice on gestational day 6, but not when administered on gestational days 8, 10, 12, or 14 (Couture et al., 1990b). Similarly, Neubert and Dillman (1972) found that the largest increase in prenatal mortality occurred when a single dose of TCDD was given on gestational day 6 compared with prenatal mortality when the TCDD dose was administered on one of gestational days 7 to 15. In addition, this would suggest that the CD-1 embryo/fetus does not have quite the relative insensitivity to the lethal effects of TCDD compared with the embryo/fetus of other species that would be indicated by using cumulative maternal dose as the index of exposure.

It should be noted that the concept of a critical window for prenatal mortality could potentially alter all of the species comparisons made previously that were based on the cumulative maternal doses shown in Table 5-1. If this turned out to be the case, then the true differences between species with respect to their susceptibility to TCDD-induced prenatal mortality could be substantially less than those indicated by using the cumulative maternal dose. This, of course, would involve a comparison between species using only single doses of TCDD given during the critical time period for each species. At the present time, it is not possible to make such a comparison from the information available in the literature.

Similar to fish and birds, the mammalian embryo/fetus is more sensitive to the lethal action of TCDD than the adult. The maternal dose of TCDD that causes 58 percent fetal mortality in hamsters is 64 to 280 times less than the LD_{50} of TCDD in adult hamsters (Olson et al., 1980; Henck et al., 1981; Olson et al., 1990). In Sprague-Dawley rats, the cumulative maternal dose of TCDD that causes 41 percent prenatal mortality is 5 to 10 times less than the approximate LD_{50} of TCDD in adult rats of the same strain (Sparschu et al., 1971; Seefeld et al., 1984). In rhesus monkeys, the cumulative maternal TCDD dose that causes 81 percent prenatal mortality is 6 and 25 times less, respectively, than the lowest TCDD dose reported to cause mortality in 1-year-old and adult rhesus monkeys (McNulty, 1977, 1985; Seefeld et al., 1979).

A general finding in all nonprimate laboratory mammals, with the possible exception of the hamster, is that TCDD-induced prenatal mortality is most commonly associated with maternal toxicity that is not severe enough to result in maternal lethality. This is seen in Table 5-1 for the guinea pig, rabbit, rat, and mouse. In each species, the dose-response relationship for maternal toxicity, indicated by decreased maternal weight gain and/or marked subcutaneous edema of the dam, is essentially the same as that for increased prenatal mortality. What this means is that there may be an association between the fetolethal effect of TCDD and maternal toxicity in all of these species. Even in the hamster, where maternal toxicity is far less severe, fetuses exhibit increases in neutrophilic metameylocytes and bands, and increases in leukocyte number and bands are also found in maternal blood (Olson and McGarrigle, 1991). More recently in mice, it has been shown that TCDD exposure causes rupture of the embryo-maternal vascular barrier, which results in hemorrhage of fetal blood into the maternal circulation (Khera, 1992). It is not known whether these extraembryonic hematologic changes are contributory to or coincidental with developmental toxicity in these species. However, their occurrence reinforces the concept that prenatal mortality can be associated with maternal toxicity.

In rhesus monkeys, on the other hand, fewer data are available to make the association between prenatal mortality and maternal toxicity. Although only small numbers of monkeys have been studied to date, the results following dietary exposure to 25 ppt TCDD (Bowman et al., 1989b; Schantz and Bowman, 1989) and 50 ppt TCDD (Allen et al.,

1977, 1979; Barsotti et al., 1979; Schantz et al., 1979) before and during pregnancy suggest that TCDD-induced prenatal mortality can occur in monkeys in the absence of overt toxic effects on the mother (see Section 5.3.1). In other studies, developmental toxicity in monkeys exposed to a total cumulative maternal dose of 1 μg TCDD/kg administered during the first trimester indicated a high incidence of prenatal mortality (McNulty, 1984, 1985). However, maternal toxicity occurred in some but not all of the mothers exposed. In these monkeys, 13 of 16 pregnancies resulted in prenatal mortality. Within 20 to 147 days after aborting, 8 of the 13 females that had aborted showed signs of maternal toxicity and three of these monkeys died. Thus, the remaining 5 of 13 instances of prenatal mortality apparently occurred in the absence of overt maternal toxicity. The results of these studies indicate that some levels of TCDD exposure can result in prenatal mortality in monkeys even though overt toxicity seems absent in the mother. As will be described (Section 5.3.1.1), however, only limited attention has been given to female reproductive toxicity in general and to the effects of maternal toxicity during pregnancy on fetal development in particular. Therefore, the relationship between maternal toxicity and prenatal mortality in the monkey is not well established. The integrity of the embryo-vascular barrier, for example, has not been evaluated after TCDD exposure.

Gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses in most laboratory mammals consisting of thymic hypoplasia, subcutaneous edema, decreased fetal growth, and prenatal mortality. Added to these common fetotoxic effects are other effects of TCDD that are highly species-specific. Examples of the latter are cleft palate formation in the mouse and intestinal hemorrhage in the rat. Table 5-2 shows those maternal and fetal toxic responses that are produced by gestational exposure to TCDD in various species of laboratory mammals. In the mouse, hydronephrosis is the most sensitive effect of prenatal toxicity, followed by cleft palate formation and atrophy of the thymus at higher doses, and by subcutaneous edema and mortality at maternally toxic doses (Couture et al., 1990a; Courtney, 1976; Courtney and Moore, 1971; Neubert and Dillman, 1972). In the rat, TCDD prenatal toxicity is manifested by intestinal hemorrhage, subcutaneous edema, decreased fetal growth, and mortality (Sparschu et al., 1971; Khera and Ruddick, 1973). Structural abnormalities do occur in the rat but only at relatively large doses (Couture et al.,

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Table 5-2. Developmental Toxicity Following Gestational Exposure to 2,3,7,8-TCDD

Species/Strain	Daily Dose[a]	Treatment Days[b]	Sacrifice Day[b]	Maternal Effects[c]	Embryo/Fetal Effects[c]	Reference
Mice/C57BL/6N	0, 1, or 3 μg/kg	10, 10-13	18	NR	Increase in cleft palate and hydronephrosis	Moore et al., 1973
Mice/C57BL/6N	0, 12, 17, or 22 μg/kg	10	18	Increase in liver-to-body weight ratio	Increase in cleft palate and hydronephrosis	Weber et al., 1985
Mice/C57BL/6N	0, 3, or 12 μg/kg	11, 10-13	18	Increase in liver-to-body weight ratio	Increase in cleft palate and hydronephrosis	Birnbaum et al., 1985
Mice/C57BL/6N	0 or 3 μg/kg	10-13	18	Increase in liver-to-body weight ratio	Increase in hydronephrosis	Birnbaum et al., 1986
Mice/C57BL/6N	0, 6, 9, 12, 15, or 18 μg/kg	10, 12	18	Increase in liver-to-body weight ratio and weight gain	Increase in cleft palate and hydronephrosis	Birnbaum et al., 1989
Mice/C57BL/6J	0 or 3 μg/kg (subcutaneous)	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971
Mice/C57BL/6J	20 μg/kg	10	17	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal body weight	Haake et al., 1987
Mice/C57BL/6J	0, 0.5, 1, 2, or 4 μg/kg	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal body weight	Silkworth et al., 1989
Mice/NMR	0.3, 3, 4, 5, or 9 μg/kg	6-15	18	NR	Increase in cleft palate and fetal mortality; decrease in fetal body weight	Neubert and Dillman, 1972
Mice/CF-1	0, 0.001, 0.01, 0.1, or 1.3 μg/kg	6-15	NR	None	Increase in cleft palate and hydronephrosis	Smith et al.. 1976
Mice/DBA	0 or 3 μg/kg (subcutaneous)	6-15	18	Increase in liver-to-body weight ratio	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971
Mice/DBA	0, 0.5, 2, 4, or 8 μg/kg	6-15	18	Increase in liver-to-body weight ratio; decrease in thymus-to-body weight ratio	Increase in cleft palate and hydronephrosis	Silkworth et al., 1989
Mice/CD-1	0, 1, or 3 μg/kg (subcutaneous)	6-15	17	None	Increase in cleft palate and kidney anomaly	Courtney and Moore, 1971

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Table 5-2 (continued)

Species/Strain	Daily Dose[a]	Treatment Days[b]	Sacrifice Day[b]	Maternal Effects[c]	Embryo/Fetal Effects[c]	Reference
Mice/CD-1	0, 25, 50, 100, 200, or 400 μg/kg	6-15	17	Increase in liver-to-body weight ratio	Increase in cleft palate, hydronephrosis, and fetal mortality	Courtney, 1976
Rats/CD	0 or 0.5 μg/kg 2 μg/kg (subcutaneous)	6-15 9-10 or 13-14	20	None at 0.5 μg/kg NR at 2 μg/kg	Increase in kidney anomaly	Courtney and Moore, 1971
Rats/Sprague-Dawley	0, 0.125, 0.5, or 2 μg/kg	0-2	20	Decrease in weight gain	Decrease in fetal body weight	Giavini et al., 1982a
Rats/Sprague-Dawley	0.03, 0.125, 0.5, 2, or 8 μg/kg	6-15	21	Decrease in weight gain; toxicity	Increase in fetal mortality, resorptions, edema, and gastrointestinal hemorrhage	Sparschu et al., 1971
Rats/Wistar	0, 0.125, 0.25, 0.5, 1, 2, 4, 8, or 16 μg/kg	5-14	21	Toxicity	Increase in fetal mortality, edema and gastrointestinal hemorrhage; decrease in fetal weight	Khera and Ruddick, 1973
Guinea pigs/Hartley	0, 0.15, or 1.5 μg/kg	14	58	Increase in mortality; toxicity	Increase in fetal mortality	Olson and McGarrigle, 1990
Hamsters/Golden Syrian	0, 1.5, 3, 6, or 18 μg/kg	7, 9	15	Increase in liver-to-body weight ratio	Increase in fetal mortality, hydronephrosis, and renal congestion; decrease in thymus size	Olson and McGarrigle, 1990
Rabbits/New Zealand	0, 0.1, 0.25, 0.5, or 1 μg/kg	6-15	28	Decrease in weight gain; toxicity	Increase in fetal mortality and resorptions; extra ribs	Giavini et al. 1982b
Monkeys/rhesus	0, 5, 25, 50, or 500 ppt 7 months before and during pregnancy	Chronic	--	Increase in mortality; toxicity	Increase in fetal mortality	Allen et al., 1979; Bowman et al., 1989
Monkeys/rhesus	0, 0.2[d], 1[d], 1[e], or 5[d] μg/kg	20-40	--	Increase in mortality; toxicity	Increase in fetal mortality	McNulty, 1985

[a]Oral exposure unless otherwise noted.
[b]All days adjusted to reflect plug day—gestation day 0.
[c]Effects reported are only those that were statistically significant.
[d]Cumulative dose divided into nine oral doses administered between days 20 and 40 of gestation; two to four monkeys/dose.
[e]Three animals given single oral dose, on either gestation days 25, 30, 35, or 40; 12 monkeys total.
NR = Not reported.

Source: Couture et al., 1990a.

1990a). In the hamster fetus, hydronephrosis and renal congestion are the most sensitive effects, followed by subcutaneous edema and mortality at fetolethal doses (Olson and McGarrigle, 1991). In the rabbit, an increased incidence of extra ribs and prenatal mortality is found (Giavini et al., 1982b), and in the guinea pig and rhesus monkey, prenatal mortality is seen (Olson and McGarrigle, 1991; McNulty, 1984).

5.2.1.4. *Structure-Activity Relationships in Laboratory Mammals*

The structure-activity relationship for developmental toxicity in laboratory mammals is generally similar to that for Ah receptor binding. Gestational treatment of rats with CDD congeners that do not bind the Ah receptor, 2-MCDD, 2,7-DCDD, 2,3-DCDD, or 1,2,3,4-TCDD, do not cause TCDD-like fetotoxic effects (Khera and Ruddick, 1973). On the other hand, hexachlorodibenzo-*p*-dioxin, which has intrinsic Ah receptor activity, produces fetotoxic responses in rats that are essentially identical to those of TCDD (Schwetz et al., 1973). Similarly, when administered to pregnant rhesus monkeys or CD-1 mice, PCB congeners that act by an Ah receptor-mediated mechanism, 3,3′,4,4′-TCB and 3,3′,4,4′,5,5′-HCB, cause the same type of fetotoxic effects as TCDD. In contrast, 4,4′-DCB, 3,3′,5,5′-TCB, 2,2′,4,4′,5,5′-HCB, 2,2′,4,4′,6,6′-HCB, and 2,2′,3,3′,5,5′-HCB, which have essentially no or a very weak affinity for the Ah receptor, do not produce a TCDD-like pattern of prenatal toxicity in mice (Marks and Staples, 1980; Marks et al., 1981, 1989; McNulty, 1985). Thus, most structure activity results for overt fetotoxic effects of the halogenated aromatic hydrocarbons are consistent with an Ah receptor-mediated mechanism. Nevertheless, one finding that stands out as being inconsistent is that 2,2′,3,3′,4,4′-HCB, which has a very weak if any affinity for binding to the Ah receptor, causes the same pattern of fetotoxic effects in mice as TCDD (Marks and Staples, 1980).

5.2.1.5. *Humans*

In the Yusho and Yu-Cheng poisoning episodes, developmental toxicity was reported in babies born to affected mothers who consumed rice oil contaminated with PCBs, CDFs, and PCQs (Hsu et al., 1985; Yamashita and Hayashi, 1985; Kuratsune, 1989; Rogan, 1989). In these incidents, it is essentially impossible to determine the contribution of TCDD-like

versus non-TCDD-like congeners to the fetal/neonatal toxicity. Nevertheless, high perinatal mortality was observed among hyperpigmented infants born to affected Yu-Cheng women who themselves did not experience increased mortality (Hsu et al., 1985). Thus, in humans the developing embryo/fetus may be more sensitive than the intoxicated mother to mortality caused by halogenated aromatic hydrocarbons.

In most cases, women who had affected children in the Yusho and Yu-Cheng episodes had chloracne themselves (Rogan, 1982). Based on this evidence, Rogan (1982) suggested that "exposure to amounts insufficient to produce some effect on the mother probably lessens the chance of fetopathy considerably." In support of this interpretation, overt signs of halogenated aromatic hydrocarbon toxicity were not observed in infants born to apparently unaffected mothers in the Seveso, Italy, and Times Beach, Missouri, TCDD incidents (Reggiani, 1989; Hoffman and Stehr-Green, 1989).

In laboratory mammals, the studies summarized previously in Table 5-1 have indicated an apparent association between prenatal mortality and maternal toxicity in nonprimate species. However, some TCDD-exposed rhesus monkeys were not able to carry their pregnancies to term even in the absence of any overt signs of maternal toxicity. This result in monkeys indicates that the relationship between maternal toxicity and any prenatal toxic effects on the human embryo/fetus must be cautiously defined. More data may be required to determine whether there is any association between overt maternal toxicity and embryo/fetal toxicity in both monkeys and humans.

Effects of chemical exposure on normal development of the human fetus can have four outcomes depending on the dose and time during gestation when exposure occurs: fetal death, growth retardation, structural malformations, and organ system dysfunction. In the Yusho and/or Yu-Cheng incidents, all of these outcomes were found (Yamashita and Hayashi, 1985; Kuratsune, 1989; Rogan, 1989). Increased prenatal mortality and low birthweight suggesting fetal growth retardation were observed in affected Yusho and Yu-Cheng women (Wong and Hwang, 1981; Law et al., 1981; Yamashita and Hayashi, 1985; Hsu et al., 1985; Miller, 1985; Lan et al., 1989; Rogan et al., 1988). A structural malformation, rocker bottom heel, was observed in Yusho infants (Yamashita and Hayashi, 1985). Organ dysfunction involving the CNS that was characterized by delays in attaining

developmental milestones and by neurobehavioral abnormalities was reported in Yu-Cheng children exposed transplacentally (Rogan et al., 1988).

Organs and tissues that originate from embryonic ectoderm are well-known targets for toxicity following exposure to TCDD-like halogenated aromatic hydrocarbons. For example, treatment of adult monkeys with TCDD results in effects involving the skin, meibomian glands, and nails (Allen et al., 1977). Similarly, a hallmark sign of fetal/neonatal toxicity in the Yusho and Yu-Cheng episodes is an ectodermal dysplasia syndrome. It is characterized by hyperpigmentation of the skin and mucous membranes, hyperpigmentation and deformation of fingernails and toenails, hypersecretion of the meibomian glands, conjunctivitis, gingival hyperplasia, presence of erupted teeth in newborn infants, altered eruption of permanent teeth, missing permanent teeth, and abnormally shaped tooth roots (Taki et al., 1969; Yamaguchi et al., 1971; Funatsu et al., 1971; Wong and Hwang, 1981; Hsu et al., 1985; Yamashita and Hayashi, 1985; Rogan et al., 1988; Kuratsune, 1989; Rogan, 1989; Lan et al., 1989). Accelerated tooth eruption has been observed in newborn mice exposed to TCDD by lactation (Madhukar et al., 1984), as well as in the human infants mentioned above. In addition, other effects have been reported in Yusho and Yu-Cheng exposed infants that resemble those observed following TCDD exposure in adult monkeys. These include subcutaneous edema of the face and eyelids (Allen et al., 1977; Moore et al., 1979; Law et al., 1981; Yamashita and Hayashi, 1985; Rogan et al., 1988). Also, larger and wider fontanels and abnormal lung auscultation were found in the human infants (Law et al., 1981; Yamashita and Hayashi, 1985; Rogan et al., 1988). The similarities between certain effects reported in human infants exposed during the Yusho and Yu-Cheng incidents, as well as in adult monkeys and neonatal mice exposed to TCDD, enhance the probability that certain effects reported in human infants were caused by the TCDD-like PCB and CDF congeners in the contaminated rice oil ingested by the mothers of these infants.

Although chloracne is the most often cited effect of TCDD exposure involving the skin in adult humans, has an animal correlate in the hairless mouse, and can be studied by using a mouse teratoma cell line in tissue culture (Poland and Knutson, 1982), it has rarely been recognized in the TCDD literature that the nervous system, like the skin, is derived from embryonic ectoderm (Balinsky, 1970). As will be described in Section 5.2.3.2,

neurobehavioral effects occur following transplacental and neonatal exposure to TCDD-like congeners in mice, as well as transplacental exposure to TCDD itself in monkeys. In addition, in some of the Yu-Cheng children that were exposed transplacentally to PCBs, PCDFs, and PCQs there was a clinical impression of developmental delay or psychomotor delay including impairment of intellectual development (Rogan et al., 1988). As there is a clustering of effects due to TCDD-induced toxicity in organs derived from ectoderm, it is possible to speculate that direct effects of TCDD-like congeners on the central nervous system are responsible for some of the neurobehavioral effects observed in these children. Effects of TCDD on EGF receptors are associated with certain aspects of the ectodermal dysplasia syndrome such as hyperkeratinization of the skin (Osborne and Greenlee, 1985) and accelerated tooth eruption (Madhukar et al., 1984). Decreased autophosphorylation of the EGF receptor in human placentas is associated with decreased birthweight in infants born to exposed mothers 4 years after the initial Yu-Cheng exposure incident (Sunahara et al., 1987). This last result supports the earlier conclusion that careful study is needed to define the relationship between maternal toxicity, placental toxicity, and developmental toxicity in humans. In addition, further research is needed to characterize and elucidate the mechanisms by which TCDD affects the nervous system.

5.2.2. Structural Malformations

Developmental effects consisting of cleft palate, hydronephrosis, and thymic hypoplasia are produced in mice following in utero exposure to halogenated dibenzo-*p*-dioxin, dibenzofuran, biphenyl, and naphthalene congeners, which bind stereospecifically to the Ah receptor (Weber et al., 1985; Birnbaum et al., 1987a,b, 1991). Of these effects in the mouse, cleft palate is less responsive than hydronephrosis, as the latter is induced in the absence of cleft palate (Couture et al., 1990b). Both responses can be induced at TCDD doses that are not otherwise overtly toxic (Couture et al., 1990a). The potency of TCDD for producing teratogenesis in the mouse is clearly evident when one considers that only 0.0003 percent of a maternally administered dose can be isolated from the fetal palatal shelves or kidneys. More specifically, a maternal TCDD dose of 30 μg/kg administered on gestational day 11 results in a tissue concentration of 0.65 pg TCDD/mg in the palatal shelves 3 days

after dosing, and the same tissue concentration of TCDD is present in the kidneys at that time (Abbott et al., 1989).

Susceptibility to the developmental actions of TCDD in mice depends on two factors: genotype of the fetus and stage of development at the time of exposure. The Ah receptor is thought to mediate the developmental effects of TCDD (Poland and Knutson, 1982). Mouse strains that produce Ah receptors with relatively high affinity for TCDD respond to lower doses of TCDD than mouse strains that produce relatively low-affinity Ah receptors (Poland and Glover, 1980; Hassoun et al., 1984a). Thus, one genetically encoded parameter that determines the responsiveness of different mouse strains is the Ah receptor protein itself.

The differences that exist between mouse strains with respect to developmental responsiveness to these chemicals are not absolute, as all strains, including those with Ah receptors of relatively low affinity, respond when exposed to sufficiently large doses during the critical period of organogenesis (Birnbaum, 1991). In the mouse, the peak times of fetal sensitivity vary slightly depending on which developmental effect is used as the endpoint. However, exposure between days 6 and 15 of gestation will produce teratogenesis (Couture et al., 1990a,b).

In inbred strains of mice, the developmental response, characterized by altered cellular proliferation, metaplasia, and modified terminal differentiation of epithelial tissues (Poland and Knutson, 1982), is extremely organ specific, occurring only in the palate, kidney, and thymus (Birnbaum, 1991). Pharmacokinetic differences are not responsible for this high degree of tissue specificity, and Ah receptors are not found exclusively in the affected organs (Carlstedt-Duke, 1979; Gasiewicz et al., 1983). Therefore, other factors intrinsic to the palate, kidney, and thymus appear to play a role along with the Ah receptors in these tissues in producing the structural malformations. For certain developmental effects, the time at which exposure occurs is important, as there may be a critical period during which the toxicant must be present in order to produce the effect. This critical period can be different for different organs and tissues.

Differences exist between mammalian species with respect to susceptibility to the developmental effects of TCDD. Although genetic differences between species or strains might affect absorption, biotransformation, and/or elimination of TCDD by the maternal

system and its absorption across the placenta, such species differences do not account for the lack of cleft palate formation in species other than mice (Birnbaum, 1991). Rather, the species differences in susceptibility to cleft palate formation appear due to differences in the interaction between TCDD and the developing palatal shelves themselves. This is demonstrated by the occurrence of similar responses when palatal shelves from different species are exposed to TCDD in organ culture (Abbott et al., 1989; Abbott and Birnbaum, 1990a, 1991). The key difference is that much higher concentrations of TCDD are required to elicit essentially the same palatal response that is seen in the mouse in other species (Table 5-3).

With respect to the occurrence of similar developmental effects in mammalian species other than the mouse, no other species develop cleft palate except at maternal doses that are fetotoxic and maternally toxic (Couture et al., 1990a; Birnbaum, 1991). In mice and hamsters, hydronephrosis can be elicited at TCDD doses that are neither fetotoxic nor maternally toxic (Olson and McGarrigle, 1991), whereas thymic hypoplasia is a fetal response to TCDD observed in virtually all laboratory mammalian species that have been tested (Vos and Moore, 1974). Studies in humans have not clearly identified an association between TCDD exposure and structural malformations (Fara and Del Corno, 1985; Mastroiacovo et al., 1988; Stockbauer et al., 1988; Reggiani, 1989).

5.2.2.1. *Cleft Palate*

5.2.2.1.1. ***Characterization of TCDD effect.*** Palatal shelves in the mouse originate as outgrowths of the maxillary process. Eventually, they come to lie vertically within the oral cavity on both sides of the tongue. In order to form the barrier between the oral and nasal cavities, the shelves in the mouse must reorient themselves from a vertical direction to a horizontal direction. Once they come together horizontally, their medial aspects bring apposing epithelia into close contact (Coleman, 1965; Greene and Pratt, 1976). At this stage, the apposing medial edge epithelia of the separate palatal shelves each consist of an outer layer of periderm that overlays a strata of cuboidal-shaped basal cells. These basal cells, in turn, rest on top of a continuous basal lamina. There is a sloughing of the outer periderm cells followed by the formation of junctions between the newly apposing basal

Table 5-3. TCDD Responsiveness of Palatal Shelves From the Mouse, Rat, and Human in Organ Culture

Species	Molar Concentration of TCDD		
	Induction of Epithelial Proliferation and Prevention of Epithelial-To-Mesenchyme Transformation		
	LOEL	EC_{100}	Cytotoxicity
Mouse	$1x10^{-13}$	$5x10^{-11}$	$1x10^{-10}$
Rat[a]	$1x10^{-10}$	$1x10^{-8}$	$1x10^{-7}$
Human[b]	$5x10^{-11}$	$1x10^{-8}$	$1x10^{-7}$

[a]At the highest concentration tested, 60 percent of the palatal shelves failed to undergo programmed cell death.
[b]One of four shelves responded by failing to undergo programmed cell death at $5x10^{-11}$ M.

Source: Birnbaum, 1991.

epithelial cells. The midline seam so formed consists of the two layers of basal cells, all of which appear healthy, even though the outer periderm cells are shed before adhesion occurs. As fusion proceeds, the bilayer seam breaks up into small islands of cells. Eventually, the basal lamina disappears and the elongating former basal cells within the small islands extend filopodia into the adjacent connective tissue. During this process, the former basal cells lose epithelial characteristics and gain fibroblast-like features. Essentially, the medial edge epithelium is an ectoderm that retains the ability to transform into mesenchymal cells. Upon completion of this epithelial to mesenchyme transformation, the once separate and apposing palatal shelves are fused so that a single continuous tissue is formed (Fitchett and Hay, 1989; Shuler et al., 1991).

Cleft palate can result from a failure of the shelves to grow and come together or a failure of the shelves to fuse once they are in close apposition (Pratt et al., 1985). TCDD and other Ah receptor agonists are unusual inducers of cleft palate because the shelves grow and make contact, but the subsequent processes involving loss of periderm, shelf adhesion, and the epithelial to mesenchyme transformation does not occur. Therefore, a cleft is formed as the palatal shelves continue to grow without fusing. When TCDD is administered to pregnant mice on gestational days 6 to 12, the incidence of cleft palate formation increases with time. However, day 12 is a critical window, after which the incidence of cleft palate formation decreases. No cleft palates are formed when TCDD is administered on day 14 (Couture et al., 1990b).

Palatal shelves of the mouse, rat, and human can be removed from the fetus and placed into organ culture. Under these conditions, when the separate shelves are placed in an apposing condition in vitro, sloughing periderm cells are trapped within the seam (Fitchett and Hay, 1989). Thus, due to the presence of these trapped dead cells, the fusion process was previously believed to require programmed cell death to remove epithelial cells at the fusion seam (Coleman, 1965; Greene and Pratt, 1976; Pratt et al., 1984). However, the newer model, which involves transformation of the basal epithelial cells into mesenchyme rather than their death, is believed to be valid under explant conditions in vitro, as well as in vivo (Fitchett and Hay, 1989). When exposed to TCDD as explants in vitro, the palatal shelves of the mouse, rat, and human all respond to TCDD in a similar way by retaining

medial epithelial cells that proliferate and differentiate into a stratified epithelium (Abbott et al., 1989; Abbott and Birnbaum, 1989, 1990a, 1991). The epithelial to mesenchyme transformation of the basal epithelial cells does not occur, and instead there is a differentiation into a stratified squamous epithelium such that these cells resemble the squamous keratinizing oral cells within the tissue (Birnbaum and Abbott, personal communication).

Table 5-3 shows the lowest TCDD concentration that prevents the epithelial to mesenchyme transformation process in isolated palatal shelves (LOEL), TCDD concentration that produces a 100 percent maximal response (EC_{100}), and lowest concentration of TCDD that produces cytotoxicity. Palatal shelves of rats and humans respond to TCDD in a manner identical to the mouse; however, higher concentrations of TCDD are required to induce the epithelial responses. The relative insensitivity of rat palatal shelves may explain the lack of cleft palate when fetal rats are exposed to nonmaternally toxic doses of TCDD. Sensitivity of human palatal shelves to TCDD in vitro is similar to the rat. This suggests that exposure to maternally toxic and fetotoxic doses of TCDD would be required to cause cleft palate formation in humans.

A disruption in the normal spatial and temporal expression of EGF, TGF-α, TGF-β1, and TGF-β2 correlates with altered proliferation and differentiation in the medial region of the developing palate, resulting in a palatal cleft. Thus, the abnormal proliferation and differentiation of TCDD-exposed medial cells may be related to reduced expression of EGF and TGF-α. Also, decreased levels of immunohistochemically detectable TGF-β1 could contribute to the continued proliferation and altered differentiation of medial cells (Abbott and Birnbaum, 1990b). It is important to note that EGF and TGF-α both exert their actions by binding to EGF receptors. The differentiation of basal cells to a stratified squamous epithelium, which resembles the keratinizing oral epithelium within the developing palate that is mentioned above, is similar to certain effects of TCDD that can be studied in cultured human keratinocytes. These effects in cultured human keratinocytes involve altered EGF binding to those cells. In addition, the Ah receptor is implicated in producing this response in cultured cells (Osborne and Greenlee, 1985). Thus, the mechanisms by which TCDD produces a palatal cleft in the mouse may have similarities to the mechanisms by which

TCDD produces other effects that are part of the ectodermal dysplasia syndrome. This is consistent with the description given by Fitchett and Hay (1989) that the medial edge epithelium within the developing palate is essentially an ectoderm that retains the ability to transform into mesenchymal cells.

5.2.2.1.2. ***Evidence for an Ah receptor mechanism.***

5.2.2.1.2.1. ***Genetic.*** When wild-type C57BL/6 (Ah^bAh^b) mice are crossed with DBA/2 (Ah^dAh^d) mice that contain a mutation at the Ah locus, all of the heterozygous B6D2F1 progeny (Ah^bAh^d) resemble the wild-type parent in that AHH activity is inducible by TCDD and other halogenated aromatic hydrocarbons (Nebert and Gielen, 1972). Test crosses between the B6D2F1 progeny and each original parent strain, and other B6D2F1 progeny mice, demonstrate that in the C57BL/6 and DBA/2 strains, susceptibility to AHH induction segregates as a simple dominant trait in the backcross and F_2 progeny. Thus, the trait of AHH induction is expressed in progeny that contain the Ah^bAh^b and Ah^bAh^d genotypes, but is not expressed in the Ah^dAh^d progeny from these crosses. Certain other effects of TCDD, such as its binding affinity for the hepatic Ah receptor (Okey et al., 1979), thymic atrophy (Poland and Glover, 1980), hepatic porphyria (Jones and Sweeney, 1980), and immunosuppressive effects (Vecchi et al., 1983; Nagarkatti et al., 1984) have been shown in similar genetic crosses and test crosses to segregate with the Ah locus that permits AHH induction. Thus, for these effects of TCDD, genetic evidence demonstrates an involvement of the Ah locus (Poland and Knutson, 1982).

Nebert's group was the first to relate developmental toxicity to the Ah locus in mice (Lambert and Nebert, 1977; Shum et al., 1979). Subsequently, Poland and Glover (1980) administered a single 30 μg TCDD/kg dose to pregnant mice on gestational day 10. It was found that there was a 54 percent incidence of cleft palate in homozygous C57BL/6 (Ah^bAh^b) fetuses, a 13 percent incidence in heterozygous B6D2F1 (C57BL/6 and DBA/2 hybrid, Ah^bAh^d) fetuses, and only a 2 percent incidence in homozygous DBA/2 (Ah^dAh^d) fetuses. This pattern of inheritance, in which the incidence of developmental toxicity in the heterozygous F1 generation is intermediate between that of the homozygous parental strains, is consistent with the autosomal dominant pattern of inheritance described for AHH

inducibility and the Ah locus (Nebert and Gielen, 1972), even if dominance is incomplete in the case of developmental toxicity. However, the pattern of inheritance for developmental toxicity described when Poland and Glover (1980) crossed C57BL/6 and DBA/2 mice is not sufficient proof that the Ah locus is the genetic locus that controls susceptibility to TCDD-induced developmental toxicity in these mouse strains.

To provide such proof, it is necessary to show genetic linkage between the susceptibility for developmental toxicity and the Ah locus. The standard of proof would be that developmental toxicity and a particular allele at the Ah locus must always segregate together in genetic crosses because if the loci are the same there can be no recombination between the loci. This is generally accomplished by demonstrating cosegregation between the two loci, not only in crosses between the two homozygous parental strains, which in and of itself is insufficient proof of genetic linkage, but also in test crosses or backcrosses between the heterozygous F1 hybrids with each homozygous parental strain.

It was stated previously that certain effects of TCDD are well known to segregate with the Ah locus due to the results of appropriate crosses and backcrosses between responsive and nonresponsive mouse strains and their hybrid F1 progeny. With this standard of proof in mind, the evidence that specifically links developmental toxicity with the Ah locus can be described. It is intended that this information be provided with a considerable degree of detail, so the reader can independently determine whether the standard of proof has been satisfied by the evidence available.

To strengthen their conclusion based on the results of simple crosses between C57BL/6 and DBA/2 mice, Poland and Glover (1980) planned to perform a backcross between the hybrid B6D2F1 and DBA/2. However, the low incidence of cleft palate in B6D2F1 mice would have required characterizing and phenotyping a prohibitively large number of fetuses. Alternatively, the backcross between B6D2F1 and C57BL/6 was considered, in which Ah^bAh^b and Ah^bAh^d progeny would have been distinguished by the amount of high-affinity specific binding for TCDD in fetal liver. In this case, however, overlap between individual mice would have made the results uncertain in some of the progeny. Therefore, it was not possible to obtain satisfactory results from either backcross.

Instead, Poland and Glover (1980) examined the incidence of cleft palate in 10 inbred strains of mice: 5 strains with high-affinity Ah receptors and 5 strains with low-affinity receptors. In the five latter strains, there was only a 0 to 3 percent incidence of cleft palate formation, whereas four of the five strains with high-affinity Ah receptors developed a ≥ 50 percent incidence. The one strain with high-affinity Ah receptors that did not follow the pattern, CBA strain, is also resistant to cleft palate formation induced by glucocorticoids. Overall, these results indicate that cleft palate formation probably segregates with the Ah locus.

The incidence of cleft palate formation was studied in fetuses from a cross between C57BL/6 and AKR/NBom mice administered 3,3′,4,4′-TCAOB on gestational day 12 (Hassoun et al., 1984b). Although C57BL/6 mice are responsive for AHH induction and cleft palate formation, AKR mice are less responsive, requiring higher doses for both effects. In a manner unlike the result of a cross between C57BL/6 and DBA/2, the incidence of cleft palate formation in the B6AKF1 progeny was <2 percent, showing that nonresponsiveness segregates as the dominant trait when C57BL/6 mice are crossed with AKR mice. Similarly, cleft palate formation was virtually absent in the progeny of a backcross between AKR/NBom and B6AKF1, demonstrating dominance of the noninducible trait. Although Ah phenotyping of the backcross progeny was not performed in this particular study, Robinson et al. (1974) had previously evaluated segregation of the Ah locus in backcrosses between C57BL/6 and AKR/N mice. They found in these two strains that noninducibility for AHH activity segregates as the dominant trait. Thus, inducibility for cleft palate formation and AHH activity both segregate as dominant traits when C57BL/6 mice are crossed with DBA/2, but noninducibility is dominant for both traits when C57BL/6 mice are crossed with AKR/N. These results are consistent with the interpretation that cleft palate induction probably segregates with the Ah locus.

Like Poland and Glover (1980), Hassoun et al. (1984a) were unable to determine whether cleft palate formation segregates with the Ah locus in C57BL/6 and DBA/2 mice by performing simple backcrosses. Instead, they evaluated cosegregation of the Ah locus and 2,3,7,8-TCDF-induced cleft palate formation using a series of recombinant strains called BXD mice. These strains are fixed recombinants produced from an original cross between

the two parental strains C57BL/6J and DBA/2J. Hybrid B6D2F1 mice were crossed to produce F_2 progeny and these were strictly inbred by sister and brother matings into several parallel strains. The mice used in this study were from the F_{42} and F_{58} generations of inbreeding. It was found that the incidence of TCDF-induced cleft palate formation after matings within eight different BXD strains with high-affinity Ah receptors is >85 percent. After similar matings with eight different BXD strains with low-affinity Ah receptors, the incidence of TCDF-induced cleft palate formation is <2 percent. These results of Hassoun et al. (1984a) corroborate those of Poland and Glover (1980) and provide the best evidence currently available that cleft palate formation segregates with the Ah locus. Thus, the Ah locus and the Ah receptor are involved in the formation of palatal clefts that are induced by TCDD-like congeners.

As additional evidence, stereospecific, high-affinity Ah receptors can be isolated from cytosol fractions prepared from embryonic palatal shelves. These receptors are present in palatal shelves of Ah^bAh^b, C57BL/6 fetuses but are not detectable in similar tissue from Ah^dAh^d, AKR/J fetuses (Dencker and Pratt, 1981). However, the significance of this finding may be mitigated to some extent by the following observation. In cytosols prepared from homogenates of whole embryo/fetal tissue (minus head, limbs, tail, and viscera), the concentration of specific binding TCDD receptors is 256 fmol/mg protein in C57BL/6 mice, compared with a concentration of 21 fmol/mg protein in the less responsive DBA/2 strain, 15 fmol/mg protein in the less responsive AKR/J strain, and 19 fmol/mg protein in the less responsive SWR/J strain. However, when embryonic tissue is cultured, the differences between the strains in receptor number are less pronounced, and in the receptors isolated from cultured embryonic cells of different strains, there is only about a twofold difference in the relative binding affinity for ^{3}H-TCDD. The mechanistic reasons for the diminished degree of difference between responsive and less responsive mouse strains during embryonic cell culture are not known (Harper et al., 1991).

The possible influence of maternal toxicity on cleft palate formation was evaluated by performing reciprocal blastocyst transfer experiments using the high-affinity-Ah receptor NMRI and lower affinity-Ah receptor DBA strains of mice (D'Argy et al., 1984). After administration of 30 μg TCDD/kg or 8 mg TCAOB/kg to pregnant dams on gestational day

12, 75 to 100 percent of all NMRI fetuses developed cleft palates. This is true whether the fetuses remained within the uterus of their natural mother or were transferred into the uterus of a DBA mouse. Under the same conditions, none of the 24 DBA fetuses transferred into an NMRI mother developed a cleft palate, even though 89 percent of their NMRI litter mates were affected. Thus, these results, along with the presence of Ah receptors in palatal shelves and responsiveness of palatal shelves in organ culture to TCDD, indicate that cleft palate formation in mice is due to a direct effect of TCDD on the palatal shelf itself and is not secondary to maternal toxicity.

5.2.2.1.2.2. ***Structure activity.*** As genetic evidence in mice indicates that the Ah receptor mediates TCDD-induced cleft palate formation and hydronephrosis (see Section 5.3.2.2.2.1), structure-activity requirements based on Ah receptor-binding characteristics should predict the relative potencies of different agonists for producing cleft palate and hydronephrosis. Of the halogenated aromatic hydrocarbons, TCDD has the greatest affinity for binding to the Ah receptor and it is the most potent teratogen in inbred mouse strains. Table 5-4 shows the relative potencies for cleft palate induction and hydronephrosis in C57BL/6 mice for a number of TCDD-like congeners. As TCDD is the most potent, it is assigned a value of 1.000. When examined by probit analysis, the dose response curve of each congener, compared with all of the others, did not deviate from parallelism. Therefore, the relative potencies of the congeners are valid for any given incidence of cleft palate formation or hydronephrosis. The main finding, however, is that the rank order potency of the various congeners for producing these two developmental effects is generally similar to that for binding to the Ah receptor (see Table 5-4), with the notable exception that the apparent binding affinities for the brominated dibenzofurans have not yet been reported. There are additional ligands for the Ah receptor that cause cleft palate formation in C57BL/6 mice at nonmaternally toxic doses, but they are not listed in the table. These include 3,3′,4,4′-TCAOB (Hassoun et al., 1984a), 3,3′,4,4′-tetrachlorobiphenyl (Marks et al., 1989), 3,3′,4,4′,5,5′-hexachlorobiphenyl (Marks et al., 1981), and a mixture that contained 1,2,3,4,6,7- and 2,3,4,5,6,7-hexabromonaphthalenes (Miller and Birnbaum, 1986).

Table 5-4. Relative Teratogenic Potency of Halogenated Aromatic Hydrocarbon Congeners in C57BL/6 Mice

Congener	Relative Potency (ED_{50} TCDD/ED_{50} congener)	
	Cleft palate	Hydronephrosis
2,3,7,8-TCDD	1.000	1.000
2,3,7,8-TBDD	0.235	0.444
2,3,7,8-TBDF	0.100	0.333
2,3,4,7,8-PeCDF	0.095	0.057
2,3,7,8-TCDF	0.049	0.021
1,2,3,7,8-PeCDF	0.026	0.074
1,2,3,4,7,8-HxCDF	0.010	0.049
2,3,4,7,8-PeBDF	0.005	0.009
1,2,3,7,8-PeBDF	0.004	0.018
2,3,4,5,3′,4′-HxCB	0.0000287	0.0000894

Source: Weber et al., 1985; Birnbaum et al., 1987a,b, 1991.

Also consistent with the structure-activity relationships for binding to the Ah receptor is the finding that a number of hexachlorobiphenyls do not induce cleft palate formation. These congeners either lack sufficient lateral substitution or are substituted in such a manner that they cannot achieve a planar conformation. Included in this category are the diortho and tetraortho chlorine-substituted 2,2′,3,3′,5,5′-, 2,2′,3,3′,6,6′-, 2,2′,4,4′5,5′-, and 2,2′,4,4′,6,6′-hexachlorobiphenyls (Marks and Staples, 1980). In addition, it is consistent with the structure-activity relationships that the monoortho chlorine-substituted 2,3,4,5,3′,4′-HCB is a weak teratogen. Its potency relative to that of TCDD varies from $3x10^{-5}$ to $9x10^{-5}$ for cleft palate formation, AHH induction, and hydronephrosis (see Table 5-4) (Kannan et al., 1988).

A result that would not be expected according to the structure-activity relationships for binding to the Ah receptor is that the diortho chlorine-substituted 2,2′,3,3′,4,4′-hexachlorobiphenyl causes cleft palate formation and hydronephrosis in mice (Marks and Staples, 1980). However, another diortho chlorine-substituted PCB congener, 2,2′,4,4′,5,5′-hexachlorobiphenyl, also can cause hydronephrosis and is a very weak inducer of EROD activity (Biegel et al., 1989; Morrissey et al., 1992). It is consistent with the interpretation that 2,2′,4,4′,5,5′-hexachlorobiphenyl is a partial Ah receptor agonist that it can competitively displace TCDD from the murine hepatic cytosolic receptor and, at large enough doses, can inhibit TCDD-induced cleft palate formation and immunotoxicity in C57BL/6 mice (Biegel et al., 1989; Morrissey et al., 1992). These results suggest that PCB congeners do not have to be in a strictly planar configuration to cause teratogenesis.

5.2.2.1.3. ***Species differences.*** Cleft palate is induced in rats only at maternally toxic TCDD doses that are associated with a high incidence of fetal lethality. Schwetz et al. (1973) reported an increased incidence of cleft palate after maternal administration of 100 μg hexachlorodibenzo-*p*-dioxin/kg/day to Sprague-Dawley rats on days 6 to 15 of gestation. Couture et al. (1989) also observed an increased incidence of cleft palate formation after a single dose of 300 μg/kg of 2,3,4,7,8-pentachlorodibenzofuran given to Fischer 344 rats. Similarly, cleft palate can be produced in fetal hamsters following maternally toxic and fetotoxic doses of TCDD (Olson et al., 1990).

In monkeys, bifid uvula (Zingeser, 1979) and bony defects in the hard palate (McNulty, 1985) were reported, but there were no corresponding soft tissue defects or clefts of the secondary palate. Cleft palates have not been reported in human fetuses of mothers accidentally exposed to TCDD or mixtures of PCBs and CDFs (Fara and Del Corno, 1985; Mastroiacovo et al., 1988; Stockbauer et al., 1988; Rogan, 1989). Thus, sensitivity of the palate in mice to TCDD is unique. In other species, including humans, other forms of fetal toxicity occur at doses lower than those required for cleft palate formation.

5.2.2.2. *Hydronephrosis*

5.2.2.2.1. ***Characterization of TCDD effect.*** Hydronephrosis is the most sensitive developmental response elicited by TCDD in mice. It is produced by maternal doses of TCDD too low to cause palatal clefting and is characterized as a progressive hydronephrosis preferentially occurring in the right kidney, which can be accompanied by hydroureter and/or abnormal nephron development (Courtney and Moore, 1971; Moore et al., 1973; Birnbaum et al., 1985; Weber et al., 1985; Abbott et al., 1987a; 1987b). Hyperplasia of the ureteric lumenal epithelium results in ureteric obstruction. Therefore, the TCDD-induced kidney malformation in the mouse is a true hydronephrosis in that blockage of urine flow results in back pressure damaging or destroying the renal papilla (Abbott et al., 1987a).

When dissected on gestational day 12 from control embryos, isolated ureters exposed to $1x10^{-10}$ M TCDD in vitro display evidence of epithelial cell hyperplasia (Abbott and Birnbaum, 1990c). This is significant in that it shows that the hydronephrosis response is due to a direct effect of TCDD on the ureteric epithelium. Embryonic cell proliferation within the ureter may be regulated by the actions of growth factors, including EGF (Abbott and Birnbaum, 1990c). In control ureteric epithelia, the expression of EGF receptors decreases with advancing development, whereas after TCDD exposure the rate of ^{3}H-thymidine incorporation and expression of EGF receptor does not decline. Therefore, in TCDD-treated mice there is a correlation between excessive proliferation of ureteric epithelial cells and inappropriate expression of EGF receptors.

Other effects of TCDD on the developing kidney involve changes in the extracellular matrix components and basal lamina (Abbott et al., 1987b). In TCDD-exposed fetal kidneys,

extracellular matrix fibers are of a diameter consistent with Type III collagen similar to such fibers in unexposed fetal kidneys. However, the abundance of these Type III collagen fibers is reduced by TCDD treatment. In the developing kidney, these collagen fibers are associated with undifferentiated mesenchymal cells. Similarly, the expression of fibronectin, which is also associated with undifferentiated mesenchymal cells, is decreased by TCDD exposure. In the glomerular basement membrane, the distribution of laminin and Type IV collagen is altered by TCDD exposure. These changes in the glomerular basement membrane may affect the functional integrity of the filtration barrier and could exacerbate the hydronephrosis and hydroureter. The proteins within the extracellular matrix and basal lamina that are altered by TCDD exposure—laminin, fibronectin, and collagen—are considered markers of a commitment to differentiate into epithelial structures. In the mouse embryo/fetus, TCDD exposure also blocks differentiation within the epithelium of the developing palate. Although there are effects of TCDD exposure on EGF in the developing ureter as well as the developing palate, the urinary system, unlike parts of the soft palate, is derived from mesoderm. Thus, it is important to note that the ectodermal-dysplasia syndrome is intended to denote a clustering of effects that appears to involve ectoderm-derived organs. It is not intended to imply that all TCDD-induced developmental toxicity involves organs derived from ectoderm.

5.2.2.2.2. ***Evidence for an Ah receptor mechanism.***

5.2.2.2.2.1. ***Genetic.*** With respect to involvement of the Ah locus in TCDD-induced hydronephrosis, very few genetic studies have been done. Prior to the discovery of the Ah locus, however, Courtney and Moore (1971) reported a 62 percent incidence of hydronephrosis in C57BL/6 mice exposed to a maternal TCDD dose of 3 μg/kg/day on days 6 to 15 of gestation, whereas the incidence in similarly exposed DBA/2 mice was only 26 percent. More recently, Silkworth et al. (1989) reported that when TCDD is administered on gestational days 6 to 15, the incidence of hydronephrosis is dose related. As the maternal dose of TCDD is increased from 0.5 to 4 μg/kg/day, the incidence of hydronephrosis in C57BL/6 mice increases from 31 to 92 percent, whereas in DBA/2 mice the incidence varies from 5 to 37 percent over the same dose range. In DBA/2 mice the incidence of

hydronephrosis increases to 60 percent when the largest dose of TCDD administered is doubled to 8 μg/kg/day (but does not reach the 92 percent level seen in C57BL/6 mice at 4 μg TCDD/kg). Thus, the incidence of hydronephrosis is higher in the mouse strain that produces high-affinity Ah receptors (C57BL/6) compared with that strain (DBA/2) that produces Ah receptors having lower ligand-binding affinity (Okey et al., 1989). The largest dose of TCDD used in these experiments resulted in hydronephrosis of the fetus without affecting the mean body weight or body weight gain of the dam. In the BXD strains (Hassoun et al., 1984a), the incidence of 2,3,7,8-TCDF-induced hydronephrosis is 34 to 48 percent in eight strains with high-affinity Ah receptors and 3 to 4 percent in eight strains with low-affinity Ah receptors. These results obtained in the BXD strains of mice provide the best evidence currently available of an association between the ability of TCDD-like congeners to induce hydronephrosis and the wild-type Ah^b allele. Thus, the Ah locus and the Ah receptor are involved in the hydronephrosis that is induced by TCDD-like congeners.

5.2.2.2.2.2. ***Structure activity.*** The rank order of potencies for various halogenated aromatic hydrocarbon congeners to cause hydronephrosis in mice is consistent with the structure-activity requirements for binding to the Ah receptor (see Table 5-4). This provides further evidence that the Ah receptor mediates the effects of these TCDD-like congeners on the developing mouse kidney.

5.2.2.2.3. ***Species differences.*** Hydronephrosis has been reported after administration of low maternal doses of TCDD to rats and hamsters. Possibly due to the small numbers of fetuses examined, the observed incidences of hydronephrosis in rats after exposure to cumulative maternal doses <2 μg TCDD/kg have not been statistically significant (Courtney and Moore, 1971; Giavini et al., 1983). On the other hand, following a 1.5 μg TCDD/kg dose administered on gestational days 7 and 9, the incidence of hydronephrosis in hamster fetuses was 11 and 4.2 percent, respectively. This is in contrast to an incidence of <1 percent in control hamster fetuses. Accordingly, hydronephrosis is one of the most sensitive indicators of prenatal toxicity in hamsters (Olson and McGarrigle, 1991).

5.2.3. Postnatal Effects

5.2.3.1. *Male Reproductive System*

TCDD has been shown to decrease plasma androgen concentrations in the adult male rat (see 5.3.2.2). Because TCDD is known to be transferred from mother to young in utero and during lactation (Moore et al., 1976; Van den Berg et al., 1987), it can be expected to have an impact on the male reproductive system during early development (Mably et al., 1991). Testosterone and/or its metabolite DHT are essential prenatally and/or early postnatally for imprinting and development of accessory sex organs (Chung and Raymond, 1976; Rajfer and Coffey, 1979; Coffey, 1988) and for initiation of spermatogenesis (Steinberger and Steinberger, 1989). In addition, aromatization of testosterone to 17ß-estradiol within the CNS is required perinatally for the imprinting of typical adult male patterns of reproductive behavior (Gorski, 1974) and LH secretion (Barraclough, 1980). Thus, normal development of male reproductive organs and imprinting of typical adult sexual behavior patterns require sufficient testosterone to be secreted by the fetal and neonatal testis at critical times in early development before and shortly after birth (MacLusky and Naftolin, 1981; Wilson et al., 1981). If perinatal imprinting has failed to occur in the accessory sex organs of a neonatal male rat, the result could be that these organs will not develop a normal trophic response to androgenic stimulation and will not grow in a normal way as the animal becomes sexually mature.

5.2.3.1.1. ***Overt toxicity assessment.*** To determine how the male reproductive system is affected by in utero and lactational TCDD exposure, Mably et al. (1991, 1992a,b,c) treated pregnant rats with a single oral dose of TCDD (0.064, 0.16, 0.4, or 1.0 μg/kg) or vehicle on day 15 of gestation (day 0 = sperm positive). Day 15 was chosen because most organogenesis in the fetus is complete by this time and the hypothalamic/pituitary/testis axis is just beginning to function (Warren et al., 1975, 1984; Aubert et al., 1985). The pups were weaned 21 days after birth. The consequences of this single, maternal TCDD exposure for the male offspring were characterized at various stages of postnatal sexual development.

Mably et al. (1992a) found that TCDD treatment had no effect on daily feed intake during pregnancy and the first 10 days after delivery, nor did it have an effect on the body

weight of dams on day 20 of gestation or on days 1, 7, 14, or 21 postpartum. Treating dams with graded doses of TCDD on day 15 of gestation had no effect on gestation index, length of gestation, or litter size. Except for an 8 percent decrease at the highest maternal dose, TCDD had no effect on live birth index. Neither the 4-day nor 21-day survival index was significantly affected by TCDD. In all dosage groups, the number of dead offspring was equally distributed between males and females and of the females that failed to deliver litters, none were pregnant. Signs of overt toxicity among the offspring were limited to the above mentioned 8 percent decrease in live birth index (highest dose only), initial 10 to 15 percent decreases in body weight (two highest doses), and initial 10 to 20 percent decreases in feed intake (measured for males only, two highest doses). The latter two effects disappeared by early adulthood, after which the body weights of the maternally exposed and nonexposed rats were similar. Gray et al. (1993) reported similar findings for offspring survival and growth following a 1 ug/kg TCDD exposure on day 15 of gestation in the Long Evans Hooded rat. No male offspring with gross external malformations were reported in either of these studies.

5.2.3.1.2. ***Androgenic status.*** The androgenic status of the male offspring can be determined from the structure and function of androgen-dependent systems and from the levels of circulating androgens. Mably et al. (1992a) examined the dose-related effect of TCDD exposure on several androgen-dependent endpoints. Anogenital distance, which is dependent on both circulating androgen concentrations and androgenic responsiveness (Neumann et al., 1970), was reduced in 1- and 4-day-old male pups by a single maternal TCDD dose as low as 0.16 ug/kg, even when slight decreases in body length were considered. Gray et al. (1993) reported a similar reduction in male anogenital distance in the Long Evans rat, although the reduction was smaller and it appeared to be related to a decrease in body weight.

Endpoints of sexual maturation were also affected by TCDD exposure. Testis descent, an androgen-mediated developmental event that normally occurs in rats between 20 and 25 days of age (Rajfer and Walsh, 1977) was delayed up to 1.7 days by a single 0.16 ug/kg dose or above (Mably et al., 1992). At 1 ug/kg, preputial separation was delayed for 3 days (Gray et al., 1993). This latter endpoint was not evaluated at lower exposure levels.

Effects into adulthood were also observed. A single maternal exposure to TCDD on gestation day 15 led to a decrease in juvenile and adult ventral prostate weight and seminal vesicle weight in the offspring (Mably et al., 1992a; Gray et al., 1993). Mably et al. (1992a) examined a variety of androgen-dependent parameters of male offspring from postnatal day 32 to 120. The lowest single maternal TCDD dose employed (0.064 ug/kg) led to a significantly depressed ventral prostate weight at 32 days of age. There were trends (though not statistically significant) for plasma testosterone and DHT concentrations to be decreased at these times, although plasma LH concentrations were generally unaffected. An exception was a 95-percent decrease in plasma LH concentration on postnatal day 32 caused by a maternal TCDD dose of 1.0 μg/kg. The lowest maternal TCDD dose to affect a parameter of androgenic status was the lowest dose tested—0.064 μg/kg. This dose resulted in a significantly depressed ventral prostate weight at 32 days of age. When ventral prostate weight was indexed to body weight, however, 0.16 μg TCDD/kg was the lowest dose that caused a significant reduction in relative ventral prostate weight. Although there was no effect on the body weight of male pups at this dose, 0.16 μg TCDD/kg caused a consistent pattern of effects indicating a depression of androgenic status. The reductions in seminal vesicle and ventral prostate weights may be due to modest reductions in plasma androgen concentrations and/or androgen responsiveness caused by incomplete perinatal imprinting of the accessory sex organs (Mably et al., 1992a). Table 5-5 summarizes these effects (Mably et al., 1991, 1992a).

To determine if in utero and lactational exposure to TCDD produces a perinatal androgenic deficiency, Mably et al. (1991, 1992a) dosed pregnant rats with 1.0 μg TCDD/kg on day 15 of gestation. Plasma testosterone concentrations were greater in control male than in control female fetuses on days 17 to 21 of gestation, particularly during the prenatal testosterone surge (days 17 to 19). On days 18 to 21 of gestation, TCDD exposure reduced the magnitude of this sex-based difference. Postnatally, plasma testosterone concentrations peaked 2 hours after birth in control males, whereas in TCDD-exposed males, the peak did not occur until 4 hours after birth and was only half as large. Contrasting these findings, recent findings reported in abstract form have not confirmed an effect of TCDD on androgen production. Neither Chen et al. (1993), repeating the exact Mably protocol (Mably et al.,

Table 5-5. Effects of In Utero and Lactational TCDD Exposure on Indices of Androgenic Status

Index	Lowest Effective Maternal Dose (μg TCDD/kg)[a]	Maximum Effect[b]
Anogenital distance	0.16 (days 1, 4)	21% decrease (day 1)
Time to testis descent	0.16	1.7 day delay
Plasma testosterone concentration	NS	69% decrease (day 32)
Plasma 5α-dihydrotestosterone concentration	NS	59% decrease (day 49)
Plasma LH concentration	1.0 (day 32)	95% decrease (day 32)
Absolute seminal vesicle weight	0.16 (days 32, 63)	56% decrease (day 49)
Relative seminal vesicle weight[c]	0.16 (day 63)	50% decrease (day 49)
Absolute ventral prostate weight	0.064 (day 32)	60% decrease (day 32)
Relative ventral prostate weight[c]	0.16 (days 32, 63)	53% decrease (day 32)

[a]The lowest dose of TCDD (given on day 15 of gestation) that caused a significant ($p < 0.05$) effect in the male offspring and the day or days at which this dose caused such an effect are shown.
[b]The magnitude of the greatest change seen in response to maternal dosing with 1.0 μg TCDD/kg and the day at which this effect was seen are shown.
[c]Weight of organ divided by body weight of rat.
NS = not statistically significant.

Source: Mably et al., 1991.

1992a), nor Gray et al. (1993), using an identical TCDD exposure in a different rat strain, have demonstrated a significant decrease in plasma or testicular testosterone following TCDD exposure identical to that of Mably et al. (1992a).

5.2.3.1.3. ***Spermatogenesis.*** Mably et al. (1991, 1992c) found that decreased spermatogenesis was among the most sensitive responses of the male rat reproductive system to perinatal TCDD exposure. Testis and epididymis weights and indices of spermatogenesis were determined on postnatal days 32, 49, 63, and 120. Perinatal TCDD exposure caused dose-related decreases in testis and epididymis weights. Weights of the caudal portion of the epididymis where mature sperm are stored prior to ejaculation were decreased the most, by ~45 percent. The number of sperm per cauda epididymis was decreased by 75 and 65 percent on days 63 and 120, respectively, and appeared to be the most sensitive effect of perinatal TCDD exposure on the male reproductive system. Daily sperm production was decreased by ≤43 percent at puberty, day 49, but the decrease was less at sexual maturity, day 120. Seminiferous tubule diameter was decreased at all four developmental stages. Each effect of TCDD was dose related and in all cases a significant decrease was seen in response to the lowest maternal TCDD dose tested, 0.064 μg/kg, during at least one stage of sexual development. In general, the magnitude of the decreases recovered with time, though not completely, suggesting that perinatal TCDD exposure delays sexual maturation. These results are summarized in Table 5-6 (Mably et al., 1991, 1992c). Gray et al. (1993) reported similar findings with respect to daily sperm production and epididymal sperm count but did not observe a statistically significant reduction in either paired testes weight or caudal epididymal weight.

Severe preweaning and/or postweaning undernutrition can affect the reproductive system of adult male rodents, including decreased spermatogenesis (Ghafoorunissa, 1980; Jean-Faucher et al., 1982a,b; Glass et al., 1986). At the two highest maternal TCDD doses in the Mably et al. (1992a) study, the feed consumption and body weight of male offspring were decreased such that the decreases were not more than 21 percent of control values. However, reduction in sex organ weights, epididymal sperm reserves, and spermatogenesis occurred at the two lowest maternal TCDD doses, neither of which reduced feed intake or

Table 5-6. Effects of In Utero and Lactational TCDD Exposure on Indices of Spermatogenic Function and Reproductive Capability

Index	Lowest Effective Maternal Dose (μg TCDD/kg)[a]	Maximum Effect[b]
Testis weight	0.40 (days 32)	17% decrease (day 32)
Epididymis weight	0.064 (days 49, 120)	35% decrease (day 32)
Cauda epididymis weight	0.064 (days 63, 120)	53% decrease (day 63)
Sperm per cauda epididymis	0.064 (days 63, 120)	75% decrease (day 63)
Daily sperm production rate	0.064 (days 63, 120)	43% decrease (day 49)
Seminiferous tubule diameter	0.064 (days 32, 49, 120)	15% decrease (day 32)
Plasma FSH concentration	0.40 (day 32)	15% decrease (day 32)
Leptotene spermatocyte: Sertoli cell ratio	NS	No dose-related effects
Sperm motility; percentage abnormal sperm	NS	No dose-related effects
Fertility	NS	22% decrease (day 70)
Gestation index; litter size; live birth index; pup survival	NS	No dose-related effects

[a]The lowest dose of TCDD (given on day 15 of gestation) that caused a significant ($p<0.05$) effect in the male offspring and the day or days at which this dose caused such an effect are shown.
[b]The magnitude of the greatest change seen in response to maternal dosing with 1.0 μg TCDD/kg and the day at which this effect was seen are shown.
NS = not statistically significant.

Source: Mably et al., 1991.

body weight of the male offspring. Thus, undernutrition cannot account for these reproductive system effects, including the decreases in spermatogenesis observed at the lower maternal TCDD doses (Mably et al., 1992a,c).

Because FSH and testosterone are essential for quantitatively normal spermatogenesis (Steinberger and Steinberger, 1989), an alternative explanation for the decreases in daily sperm production is a decrease in FSH and/or testosterone levels. In rats, the duration of spermatogenesis is 58 days (Blazak et al., 1985; Amann, 1986; Working and Hurtt, 1987), so the decreases in plasma FSH concentrations in 32-day-old male offspring could contribute to the reductions of spermatogenesis when the rats were 49 and 63 days of age. However, the modest depressant effect of perinatal TCDD exposure on plasma FSH concentrations was transitory; no effect was found on plasma FSH levels when the offspring were 49, 63, and 120 days old. It was concluded that reduced spermatogenesis in 120-day-old male rats, perinatally exposed to TCDD, is not due to decreases in plasma FSH levels when the animals were 49 to 120 days of age (Mably et al., 1992c). Likewise, even if the original findings of Mably et al. (1992) are confirmed, the potential reduction in intratesticular testosterone levels following TCDD exposure would not be sufficient to reduce spermatogenesis (Zirkin et al., 1989; Mably et al., 1992c; Gray et al., 1993).

In normal rats, daily sperm production does not reach a maximum until 100 to 125 days of age (Robb et al., 1978), but in rats perinatally exposed to TCDD it takes longer for sperm production to reach the adult level. Furthermore, the length of the delay is directly related to maternal TCDD dose (Mably et al., 1992c), and if the dose is high enough, the reduction in spermatogenesis may be permanent. This is suggested by a maternal TCDD dose of 1.0 μg/kg decreasing daily sperm production in male rat offspring that are 300 days of age (Moore et al., 1992). Since the mechanism by which perinatal TCDD exposure decreases spermatogenesis in adulthood is unknown, it is unclear whether the irreversible effect at the largest maternal dose of 1 μg/kg, which results in depressed feed consumption and decreased body weight, is caused by the same mechanism as that at smaller maternal doses, which do not result in undernutrition and from which the male offspring may eventually recover.

A key observation for postulating mechanisms by which perinatal TCDD exposure reduces spermatogenesis in adulthood is the finding that the ratio of leptotene spermatocytes per Sertoli cell in the testes of 49-, 63-, and 120-day-old rats is not affected by in utero and lactational TCDD exposure even though daily sperm production is reduced (Mably et al., 1992c). Since Sertoli cells provide spermatogenic cells with functional and structural support (Bardin et al., 1988) and the upper limit of daily sperm production in adult rats is directly dependent on the number of Sertoli cells per testis (Russell and Peterson, 1984), three possible mechanisms for the decrease in daily sperm production may be involved. TCDD could increase the degeneration of cells intermediate in development between leptotene spermatocytes and terminal stage spermatids (the cell type used to calculate daily sperm production); decrease postleptotene spermatocyte cell division (meiosis); and/or decrease the number of Sertoli cells per testis (Orth et al., 1988). Elucidating the mechanism by which perinatal TCDD exposure decreases spermatogenesis is important because it is one of the most sensitive responses of the male reproductive system to TCDD.

5.2.3.1.4. ***Epididymis.*** The epididymis has two functions: In proximal regions, spermatozoa mature gaining the capacity for motility and fertility, whereas in distal regions mature sperm are stored before ejaculation (Robaire and Hermo, 1989). Mably et al. (1991, 1992c) found that motility and morphology of sperm taken from the cauda epididymis on postnatal days 63 and 120 were unaffected by perinatal TCDD exposure. Thus, no effect of TCDD on epididymal function was detected. The dose-dependent reduction in epididymis and cauda epididymis weights in postpubertal rats, 63 and 120 days old, can be accounted for, in part, by decreased sperm production. However, in immature males, 32 and 49 days of age, where sperm are not present in the epididymis, the decrease in weights of epididymal tissue, as observed by Mably et al. (1992c), cannot be explained by effects on sperm production. Since epididymal growth is androgen dependent, a TCDD-induced androgenic deficiency and/or decrease in androgen responsiveness of the epididymis could account for decreased size of the organ (Setty and Jehan, 1977; Dhar and Setty, 1990).

5.2.3.1.5. ***Reproductive capability.*** To assess reproductive capability, male rats born to dams given TCDD (0.064, 0.16, 0.40, or 1.0 μg/kg) or vehicle on day 15 of gestation were mated with control virgin females when the males were ~70 and 120 days of age (Mably et al., 1991, 1992c). The fertility index of the males is defined as number of males impregnating females divided by number of males mated. The two highest maternal TCDD doses decreased the fertility index of the male offspring by 11 and 22 percent, respectively. However, these decreases were not statistically significant, and at lower doses, the fertility index was not reduced. Gestation index, defined as the percentage of control dams mated with TCDD-exposed males that delivered at least one live offspring, was also not affected by perinatal TCDD exposure. With respect to progeny of these matings, there was no effect on litter size, live birth index, or 21-day survival index. When perinatal TCDD-exposed males were mated again at 120 days of age, there was no effect on any of these same parameters. Thus, despite pronounced reductions in cauda epididymal sperm reserves, when the TCDD-treated males were mated, perinatal TCDD exposure had little or no effect on fertility of male rats or on survival and growth of their offspring. These results are summarized in Table 5-6 (Mably et al., 1991, 1992c).

Since rats produce and ejaculate 10 times more sperm than is necessary for normal fertility and litter size (Aafjes et al., 1980; Amann, 1982), the absence of a reduction in fertility of male rats exposed perinatally to TCDD is not inconsistent with the substantial reductions in testicular spermatogenesis and epididymal sperm reserves. In contrast, reproductive efficiency in human males is very low, the number of sperm per ejaculate being close to that required for fertility (Working, 1988). Thus, measures of fertility using rats are not appropriate for low-dose extrapolation in humans (Meistrich, 1992). A percentage reduction in daily sperm production in humans, similar in magnitude to that observed in rats (Mably et al., 1991, 1992c) may reduce fertility in men.

5.2.3.1.6. ***Sexual differentiation of the CNS.*** Sexual differentiation of the CNS is dependent on the presence of androgens during early development. In rats, the critical period of sexual differentiation extends from late fetal life through the first week of postnatal life (MacLusky and Naftolin, 1981). In the absence of adequate circulating levels of

testicular androgen during this time, adult rats display high levels of feminine sexual behavior (e.g., lordosis), low levels of masculine sexual behavior, and a cyclic (i.e., feminine) pattern of LH secretion (Gorski, 1974; Barraclough, 1980). In contrast, perinatal androgen exposure of rats will result in the masculinization of sexually dimorphic neural parameters, including reproductive behaviors, regulation of LH secretion, and several morphological indices (Raisman and Field, 1973; Gorski et al., 1978). The mechanism by which androgens cause sexual differentiation of the CNS is not completely understood. In the rat, it appears that 17ß-estradiol, formed by the aromatization of testosterone within the CNS, is one of the principal active steroids responsible for mediating sexual differentiation (McEwen, 1978); however, androgens also are involved.

5.2.3.1.6.1. ***Demasculinization of sexual behavior.*** Mably et al. (1991, 1992b) assessed sexually dimorphic functions in male rats born to dams given graded doses of TCDD or vehicle on day 15 of gestation. Masculine sexual behavior was assessed in male offspring at 60, 75, and 115 days of age by placing a male rat in a cage with a receptive control female and observing the first ejaculatory series and subsequent postejaculatory interval (see Table 5-7). The number of mounts and intromissions (mounts with vaginal penetration) before ejaculation was increased by a maternal TCDD dose of 1.0 μg/kg. The same males exhibited twelvefold and elevenfold increases in mount and intromission latencies, respectively, and a twofold increase in ejaculation latency. All latency effects were dose related and significant at a maternal TCDD dose as low as 0.064 μg/kg (intromission latency) and 0.16 μg/kg (mount and ejaculation latencies). Copulatory rates (number of mounts + intromissions/time from first mount to ejaculation) were decreased to less than 43 percent of the control rate. This effect on copulatory rates was dose related, and a statistically significant effect was observed at maternal TCDD doses as low as 0.16 μg/kg. Postejaculatory intervals were increased 35 percent above the control interval, and a statistically significant effect was observed at maternal doses of TCDD as low as 0.40 μg/kg. Collectively, these results demonstrate that perinatal TCDD exposure demasculinizes sexual behavior.

Table 5-7. Effects of In Utero and Lactational TCDD Exposure on Indices of Sexual Behavior and Regulation of LH Secretion in Adulthood

Index	Lowest Effective Maternal Dose (μg TCDD/kg)[a]	Maximum Effect[b]
Masculine Sexual Behavior[c]		
Mount latency	0.16	1,200% increase
Intromission latency	0.064	1,100% increase
Ejaculatory latency	0.16	97% increase
Number of mounts	0.064	130% increase
Number of intromissions	1.0	38% increase
Copulatory rate (mounts plus intromissions/minute)	0.16	43% decrease
Postejaculatory interval	0.40	35% increase
Feminine Sexual Behavior[d]		
Lordosis quotient[e]	0.16	300% increase
Lordosis intensity score	0.40	50% increase
Regulation of LH Secretion		
LH surge	0.40	460% increase[f]

[a]The lowest dose of TCDD (given on day 15 of gestation) that caused a significant ($p < 0.05$) effect in the male offspring is shown.
[b]The magnitude of the greatest change seen in response to maternal dosing with 1.0 μg TCDD/kg is shown (average of three trials for masculine behavior and two for feminine).
[c]Measured when the rats were ~60, 75, and 115 days of age.
[d]Feminine sexual behavior was measured following castration, estrogen priming, and progesterone administration. The rats were 170 to 184 days old.
[e]Number of times lordosis was displayed in reponse to a mount, divided by the number of times each rat was mounted, times 100.
[f]Since control males do not secrete LH in response to progesterone, this percentage was calculated by comparing peak plasma LH concentrations in TCDD-exposed rats with plasma LH concentrations in control males at the same time after progesterone was administered.

Source: Mably et al., 1991.

Because perinatal exposure to a maternal TCDD dose of 1.0 μg/kg has no effect on the open field locomotor activity of adult male rats (Schantz et al., 1991), the increased mount, intromission, and ejaculation latencies appear to be specific for these masculine sexual behaviors, not secondary to a depressant effect of TCDD on motor activity. The reported postpubertal plasma testosterone and DHT concentrations in litter mates of the rats evaluated for masculine sexual behavior were as low as 56 and 62 percent, respectively, of controls (Mably et al., 1991, 1992a). However, plasma testosterone concentrations that were only 33 percent of controls are still sufficient to masculinize sexual behavior of adult male rats (Demassa et al., 1977). Therefore, the modest reductions in adult plasma androgen concentrations following perinatal TCDD exposure were not of sufficient magnitude to demasculinize sexual behavior.

Reductions in perinatal androgenic stimulation can inhibit penile development and subsequent sensitivity to sexual stimulation in adulthood (Nadler, 1969; Södersten and Hansen, 1978). Therefore, the demasculinization of sexual behavior could, to some extent, be secondary to decreased androgen-dependent penile development. However, perinatal TCDD exposure had no effect on gross appearance of the rat penis. In addition, TCDD-exposed males exhibited deficits in such masculine sexual behaviors as mount latency and postejaculatory interval, which do not depend on stimulation of the penis for expression (Sachs and Barfeld, 1976). Thus, although some effects of TCDD, such as decreased copulatory rate and prolonged latency until ejaculation, could be due to reduced sensitivity of the penis to sexual stimulation, the twelvefold increase in mount latency and increase in postejaculatory interval cannot be explained by this mechanism.

5.2.3.1.6.2. ***Feminization of sexual behavior.*** Mably et al. (1991, 1992b) determined if the potential of adult male rats to display feminine sexual behavior was altered by perinatal TCDD exposure. Male offspring of dams treated on day 15 of gestation with various doses of TCDD up to 1 μg/kg or vehicle were castrated at ~120 days of age, and beginning at ~160 days of age were injected weekly for 3 weeks with 17ß-estradiol benzoate, followed 42 hours later by progesterone. Four to six hours after the progesterone injection at weeks 2 and 3, the male was placed in a cage with a sexually excited control stud male. The

frequency of lordosis in response to being mounted by the stud male was increased from 18 percent (control) to 54 percent by the highest maternal TCDD dose, 1.0 μg/kg (Table 5-7). Lordosis intensity, scored after Hardy and DeBold (1972) as 1 for light lordosis, 2 for moderate lordosis, and 3 for a full spinal dorsoflexion, was increased in male rats by perinatal TCDD exposure. Both effects on lordosis behavior in males were dose related and significant at maternal TCDD doses as low as 0.16 μg/kg (increased lordotic frequency) and 0.40 μg/kg (increased lordotic intensity). Together, they indicate a feminization of sexual behavior in these animals. Although severe undernutrition from 5 to 45 days after birth potentiates the display of lordosis behavior in adult male rats (Forsberg et al., 1985), the increased frequency of lordotic behavior was seen at a maternal TCDD dose of 0.16 μg/kg, which had no effect on feed intake or body weight. It was concluded that perinatal TCDD exposure feminizes sexual behavior in adult male rats independent of undernutrition.

5.2.3.1.6.3. ***Feminization of LH secretion regulation.*** The effect of perinatal TCDD exposure on regulation of LH secretion by ovarian steroids was determined in male offspring at ~270 days of age. There is normally a distinct sexual dimorphism to this response. In rats castrated as adults, estrogen-primed females greatly increase their plasma LH concentrations when injected with progesterone, whereas similarly treated males fail to respond (Taleisnik et al., 1969). Progesterone had little effect on plasma LH concentrations in estrogen-primed control males, but significant increases were seen in males exposed to maternal TCDD doses as low as 0.40 μg/kg. Thus, perinatal TCDD exposure increases pituitary and/or hypothalamic responsiveness of male rats to ovarian steroids in adulthood, indicating that regulation of LH secretion is permanently feminized. Table 5-7 summarizes sexual behavior and LH secretion results (Mably et al., 1991, 1992b).

5.2.3.1.6.4. ***Comparison to other Ah receptor-mediated responses.*** The induction of hepatic cytochrome P-4501A1 and its associated EROD activity are extremely sensitive Ah receptor-mediated responses to TCDD exposure. Yet in 120-day-old male rats that had been exposed to TCDD perinatally, alterations in sexual behavior, LH secretion, and spermatogenesis were observed when induction of hepatic EROD activity could no longer be

detected (Mably et al., 1991, 1992a,b,c). These results suggest that TCDD affects sexual behavior, gonadotrophic function, and spermatogenesis when virtually no TCDD remains in the body and that the demasculinization and feminization of sexual behavior, feminization of LH secretion, and reduced spermatogenesis caused by in utero and lactational exposure to TCDD may be irreversible (Mably et al., 1992b,c).

5.2.3.1.6.5. ***Possible mechanisms and significance.*** The most plausible explanation for the demasculinization of sexual behavior and feminization of sexual behavior and LH secretion is that perinatal exposure to TCDD impairs sexual differentiation of the CNS. Neither undernutrition, altered locomotor activity, reduced sensitivity of the penis to sexual stimulation, nor modest reductions in adult plasma androgen concentrations of the male offspring can account for this effect (Mably et al., 1992b). On the other hand, exposure of the developing brain to testosterone, conversion of testosterone into 17ß-estradiol within the brain, and events initiated by the binding of 17ß-estradiol to its receptor are all critical for sexual differentiation of the CNS and have the potential to be modulated by TCDD. If TCDD interferes with any of these processes during late gestation and/or early neonatal life, it could irreversibly demasculinize and feminize sexual behavior (Hart, 1972; McEwen et al., 1977; Whalen and Olsen, 1981) and feminize the regulation of LH secretion (Gogan et al., 1980, 1981) in male rats in adulthood.

In utero and/or lactational exposure to TCDD may cause similar effects in other animal species, including nonhuman primates (Pomerantz et al., 1986; Thornton and Goy, 1986; Goy et al., 1988), in which sexual differentiation is under androgenic control. In humans, there is evidence that social factors account for much of the variation in sexually dimorphic behavior; there is also evidence that prenatal androgenization influences both the sexual differentiation of such behavior and brain hypothalamic structure (Erhardt and Meyer-Bahlburg, 1981; Hines, 1982; LeVay, 1991).

5.2.3.2. *Female Reproductive System*

Very little investigation on the developing female reproductive system, comparable to that on the male system, has been carried out. Gray et al. (1993) have begun to characterize

the effects of a single maternal exposure (1 ug/kg on gestation day 15) on postnatal development. This exposure was associated with clefting of the phallus/clitoris (with and without hypospadias), incomplete or absent vaginal opening, and a smaller vaginal orifice. There was no effect on estrous cyclicity from puberty to 5 months of age, and small effects on anogenital distance appeared to be associated with body weight changes. Mably et al. (1992a) reported a similar association of anogenital distance and crown-rump length in female offspring.

5.2.3.3. *Neurobehavior*

Because differentiated tissues derived from ectoderm, namely, skin, conjunctiva, nails, and teeth, are sites of action of halogenated aromatic hydrocarbons in transplacentally exposed human infants, another highly differentiated tissue derived from ectoderm, the CNS, should be considered a potential site of TCDD action. In support of this possibility, sexual differentiation of the CNS of adult male rats is irreversibly altered in a dose-related fashion by perinatal exposure to TCDD (Mably et al., 1991, 1992b). As will be shown below, the CNS of mice transplacentally exposed to 3,3′,4,4′-TCB, monkeys perinatally exposed to TCDD, and children transplacentally exposed to a mixture of PCBs, CDFs, and PCQs in the Yu-Cheng incident is also affected. Thus, functional CNS alterations, which may or may not be irreversible, are observed following perinatal exposure to halogenated aromatic hydrocarbons. Ah receptors have been identified in rat brain (Carlstedt-Duke, 1979) but may be associated with glial cells rather than neurons (Silbergeld, 1992). Following administration of ^{14}C-TCDD in the rat, the highest concentrations of TCDD-derived ^{14}C are found in the hypothalamus and pituitary. Much lower concentrations are found in the cerebral cortex and cerebellum (Pohjanvirta et al., 1990). In another study, the Ah receptor was not detected in whole rat or mouse brain but was detected in the cerebrum of the hamster and cerebrum and cerebellum of the guinea pig (Gasiewicz, 1983). Ah receptors appear to be absent in the human frontal cortex (Silbergeld, 1992).

5.2.3.3.1. ***Mice.*** CD-1 mice exposed transplacentally to 3,3′,4,4′-TCB at a maternal oral dose of 32 mg/kg administered on days 10 to 16 of gestation exhibited neurobehavioral,

neuropathological, and neurochemical alterations in adulthood (Tilson et al., 1979; Chou et al., 1979; Agrawal et al., 1981). The neurobehavioral effects consisted of circling, head bobbing, hyperactivity, impaired forelimb grip strength, impaired ability to traverse a wire rod, impaired visual placement responding, and impaired learning of a one-way avoidance task (Tilson et al., 1979). The brain pathology in adult mice exhibiting this syndrome consisted, in part, of alterations in synapses of the nucleus accumbens (Chou et al., 1979). This suggested that in utero exposure to 3,3′,4,4′-TCB may interfere with synaptogenesis of dopaminergic systems. In support of this possibility, Agrawal et al. (1981) found that adult mice transplacentally exposed to 3,3′,4,4′-TCB had decreased dopamine levels and decreased dopamine receptor binding in the corpus striatum, both of which were associated with elevated levels of motor activity. It was concluded that transplacental exposure to 3,3′,4,4′-TCB in mice may permanently alter development of striatal synapses in the brain.

Eriksson (1988) examined the neurobehavioral effects of 3,3′,4,4′-TCB in NMRI mice exposed to a single oral dose of 0.41 or 41 mg/kg on postnatal day 10. Following sacrifice of the mice on day 17, muscarinic receptor concentrations in the brain were significantly decreased at both dose levels. This effect was shown to occur in the hippocampus but not in the cortex. More recently (Eriksson et al., 1991), NMRI mice were exposed to the same two doses of 3,3′,4,4′-TCB similarly administered on postnatal day 10. At 4 months of age, the effects of the PCB on locomotor activity were assessed. At both dose levels, abnormal activity patterns were exhibited in that the treated mice were significantly less active than controls at the onset of testing, but were more active than controls at the end of the test period. This pattern of effects can be interpreted as a failure to habituate to the test apparatus. In contrast to the previous results with CD-1 mice, circling or head bobbing activities were not observed in these animals. Upon sacrifice after the activity testing was complete, a small but statistically significant increase (as opposed to the decrease found after sacrifice on postnatal day 17) in the muscarinic receptor concentration of the hippocampus was found in animals from the high-dose group. These results suggest that the neurochemical effects of 3,3′,4,4′-TCB are complex. Cholinergeric as well as dopaminergic systems in the brain are involved.

Of all the developmental and reproductive endpoints reported in this chapter for laboratory animals, the only ones that have not yet been demonstrated to occur following perinatal exposure to TCDD are the above neurotoxic effects in mice. These have only been studied following perinatal exposure to 3,3'4,4'-TCB. In addition, there is as yet no evidence to show (1) that among inbred mouse strains having low- and high-affinity Ah receptors, susceptibility to 3,3',4,4'-TCB-induced neurotoxicity segregates with the Ah locus or (2) that the rank order binding affinity of congeners for the Ah receptor correlates with their rank order potency for causing these neurotoxic effects in mice. The rapid metabolism of 3,3',4,4'-TCB compared with the relatively slow metabolism of TCDD in mice causes some uncertainty about the potential involvement of the Ah receptor in 3,3',4,4'-TCB-induced neurotoxicity. Contributing to this uncertainty is the hypothesis that 3,3',4,4'-TCB might produce central nervous system effects by being converted to a hydroxylated metabolite that is neurotoxic. Although there is no evidence for or against this hypothesis, there is also no evidence for or against the Ah receptor mechanism hypothesis of 3,3',4,4'-TCB neurotoxicity. Further research is needed to test these hypotheses. In so doing, it should become apparent whether 3,3',4,4'-TCB-induced neurotoxicity effects are relevant to TCDD-induced developmental toxicity.

5.2.3.3.2. ***Monkeys.*** Schantz and Bowman (1989) and Bowman et al. (1989a) have conducted a series of studies on the long-term behavioral effects of perinatal TCDD exposure in monkeys. Because these were the first studies to evaluate the behavioral teratology of TCDD, monkeys exposed to TCDD via the mother during gestation and lactation were screened on a broad selection of behavioral tests at various stages of development (Bowman et al., 1989a). At the doses studied (5 or 25 ppt in the maternal diet), TCDD did not affect reflex development, visual exploration, locomotor activity, or fine motor control in any consistent manner (Bowman et al., 1989b). However, the perinatal TCDD exposure did produce a specific, replicable deficit in cognitive function (Schantz and Bowman, 1989). TCDD-exposed offspring were impaired on object learning, but were unimpaired on spatial learning. TCDD exposure also produced changes in the social interactions of mother-infant dyads (Schantz et al., 1986). TCDD-exposed infants spent more time in close physical

contact with their mothers. The pattern of effects was similar to that seen in lead-exposed infants and suggested that mothers were providing increased care to the TCDD-exposed infants (Schantz et al., 1986).

5.2.3.3.3. ***Humans.*** The intellectual and behavioral development of Yu-Cheng children transplacentally exposed to PCBs, CDFs, and PCQs was studied through 1985 by Rogan et al. (1988). In Yu-Cheng children matched to unexposed children of similar age, area of residence, and socioeconomic status, there was a clinical impression of developmental or psychomotor delay in 12 (10 percent) Yu-Cheng children compared with 3 (3 percent) control children and of a speech problem in 8 (7 percent) Yu-Cheng children versus 3 (3 percent) control children. Also, except for verbal IQ on the Wechsler Intelligence Scale for Children, Yu-Cheng children scored lower than control children on three developmental and cognitive tests (Rogan et al., 1988). Neurobehavioral data on Yu-Cheng children obtained after 1985 shows that the intellectual development of these children continues to lag somewhat behind that of matched control children. In addition, Yu-Cheng children are rated by their parents and teachers as having a higher activity level; more health, habit, and behavioral problems; and a temperamental clustering closer to that of a "difficult child." It is concluded that in humans, transplacental exposure to halogenated aromatic hydrocarbons can affect CNS function postnatally. However, which congeners, TCDD-like versus non-TCDD-like, are responsible for the neurotoxicity is unknown.

Further research on the mechanism of these postnatal neurobehavioral effects, dose-response relationships, and reversibility of the alterations is needed before the role of TCDD-like congeners versus non-TCDD-like congeners in causing this toxicity can be understood. Mechanisms that respond uniquely to TCDD-like congeners may not necessarily be involved, as three lightly chlorinated, ortho-substituted PCB congeners, 2,4,4′-TCB, 2,2′,4,4′-TCB, and 2,2′,5,5′-TCB, have been detected in monkey brain following dietary exposure to Aroclor 1016 and appear to be responsible for decreasing dopamine concentrations in the caudate, putamen, substantia nigra, and hypothalamus of these animals (Seegal et al., 1990). These nonplanar PCB congeners are believed to cause these effects by acting through a mechanism that does not involve the Ah receptor. On the other hand, the results presented

for mice and monkeys suggest that TCDD-like congeners could be involved in producing the observed postnatal neurobehavioral effects in humans.

5.2.4. Cross-Species Comparison of Effect Levels

TCDD exposure levels that cause a variety of developmental effects in different species are summarized for fish in Table 5-8, birds in Table 5-9, and mammals in Table 5-10. Fertilized lake trout eggs and Japanese medaka eggs were exposed to different waterborne concentrations of ^{3}H-TCDD. Estimates of the amount of TCDD in these eggs were then made from measurement of the TCDD-derived radioactivity within them. Fertilized rainbow trout, chicken, ring-necked pheasant, and eastern bluebird eggs were injected directly with the indicated doses of TCDD. Thus, the doses of TCDD given in Tables 5-8 and 5-9 for all fish and bird species represent TCDD egg burdens where a significant portion of the dose may be present within the yolk of the egg rather than the developing embryo.

Mammalian embryo/fetuses, on the other hand, were exposed via administration of TCDD to the pregnant female. Therefore, the doses given in Table 5-10 are maternal TCDD doses, where a significant portion of the dose may be retained by the mother and never actually reach the embryo/fetus. In some studies, pregnant rats and rhesus monkeys were exposed to TCDD on a chronic or subchronic basis, respectively. The doses given in Table 5-10 for these particular studies represent the calculated maternal body burdens at the time of conception. In rats, the duration of chronic exposure was much longer than the whole body elimination half-life for TCDD in rats. Therefore, the body burden of TCDD given for the rat is 92.8 percent of the calculated steady-state body burden. In rhesus monkeys, the half-life for whole body elimination of TCDD is longer than the duration of exposure prior to conception. Therefore, the steady-state body burdens that would be expected for rhesus monkeys exposed to the different levels of dietary TCDD intake are approximately three times greater than the maternal body burdens estimated at the time of conception (Table 5-10).

In both rats and rhesus monkeys, the maternal body burdens are calculated using a one-compartment open model, assuming 86.1 percent bioavailability for TCDD. The

Table 5-8. Cross-Species Comparison of NOAELs and LOAELs for TCDD Developmental Toxicity in Fish

Species	Effect	Exposure	Egg Dose	Effect Level	Reference
Lake trout	Sac fry mortality	Static waterborne	34 ng/kg	NOAEL	Walker et al., 1991
Japanese medaka	Lesions[a]	Static waterborne	<100 ng/kg	NOAEL	Wisk and Cooper, 1990a
Rainbow trout	Sac fry mortality	Single injection	194 ng/kg	NOAEL	Walker et al., 1992
Lake trout	Sac fry mortality Sac fry mortality	Static waterborne Static waterborne	40 ng/kg 55 ng/kg	LOAEL LOAEL	Spitsbergen et al., 1991 Walker et al., 1991
Rainbow trout	Sac fry mortality	Single injection	291 ng/kg	LOAEL	Walker et al., 1992
Japanese medaka	Lesions[a]	Static waterborne	300 ng/kg	LOAEL	Wisk and Cooper, 1990a

[a]Consist of a spectrum of effects including hemorrhage in various areas, pericardial edema, collapse of the yolk sphere, cessation of blood flow throughout the animal, and embryo mortality.

Table 5-9. Cross-Species Comparison of NOAELs and LOAELs for TCDD Developmental Toxicity in Birds

Species	Effect	Exposure	Egg Dose	Effect Level	Reference
Ring-necked pheasant	Embryo mortality	Single injection	100 ng/kg	NOAEL	Nosek et al., 1993
Eastern bluebird	Embryo mortality	Single injection	1,000 ng/kg	NOAEL	Thiel et al., 1988 Martin et al., 1989
Chicken	Cardiac malformations	Single injection	9 ng/kg[a]	LOAEL	Cheung et al., 1981a,b
Chicken	Embryo mortality	Single injection	240 ng/kg	LD_{50}	Allred and Strange, 1977
Ring-necked pheasant	Embryo mortality	Single injection	1,000 ng/kg	LOAEL	Nosek et al., 1993
Ring-necked pheasant	Embryo mortality	Single injection	1,354 ng/kg[b]	LD_{50}	Nosek et al., 1993
Ring-necked pheasant	Embryo mortality	Single injection	2,182 ng/kg[c]	LD_{50}	Nosek et al., 1993
Eastern bluebird	Embryo mortality	Single injection	10,000 ng/kg	LOAEL	Thiel et al., 1988

[a]Chi-square analysis of the data in Table 1 of Cheung et al. (1981b) demonstrated that the incidence of cardiac malformations in all embryos examined at dose levels of 1.6 pmol/egg or greater are significantly ($p < 0.05$) increased compared to the incidence in the control group designated "all examined." Assuming a 55 g egg weight, 1.6 pmol/egg corresponds to a TCDD egg burden of 9 ng/kg.
[b]Injected into the egg albumin.
[c]Injected into the egg yolk.

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Table 5-10. Cross-Species Comparison of NOAELs and LOAELs for TCDD Developmental Toxicity in Mammals

Species	Effect	Exposure	Maternal Dose	Effect Level	Reference
Monkey	Prenatal mortality	Multiple dose	22 ng/kg, 9x, gd 20-40	NOAEL	McNulty, 1984
Rat	Prenatal mortality	1 ng/kg/day	27 ng/kg[a], chronic	NOAEL	Murray et al., 1979
Rat	Prenatal mortality	Multiple dose	30 ng/kg/day, gd 6-15	NOAEL	Sparschu et al., 1971
Mouse	Hydronephrosis	Multiple dose	100 ng/kg/day, gd 6-15	NOAEL	Smith et al., 1976
Mouse	Cleft palate	Multiple dose	300 ng/kg/day, gd 6-15	NOAEL	Neubert and Dillman, 1972
Monkey	Object learning	0.126 ng/kg/day	19 ng/kg[a], subchronic	LOAEL	Schantz and Bowman, 1989
Rat	Male reproductive	Single dose	64 ng/kg, gd 15	LOAEL	Mably et al., 1992a,b,c
Monkey	Prenatal mortality	0.642 ng/kg/day Multiple dose	97 ng/kg[a], subchronic 111 ng/kg, 9x, gd 20-40	LOAEL LOAEL	Schantz and Bowman, 1989 McNulty, 1984
Rabbit	Extra ribs	Multiple dose	100 ng/kg/day, gd 6-15	LOAEL	Giavini et al., 1982b
Rat	Fetal growth	Multiple dose	125 ng/kg/day, gd 6-15	LOAEL	Sparschu et al., 1971
Rabbit	Prenatal mortality	Multiple dose	250 ng/kg/day, gd 6-15	LOAEL	Giavini et al., 1982b
Rat	Prenatal mortality	10 ng/kg/day Multiple dose	270 ng/kg[a], chronic 500 ng/kg/day, gd 6-15	LOAEL LOAEL	Murray et al., 1979 Sparschu et al., 1971
Mouse	Hydronephrosis	Multiple dose	500 ng/kg/day, gd 6-15	LOAEL	Silkworth et al., 1989
Guinea pig	Prenatal mortality	Single dose	1,500 ng/kg, gd 14	LOAEL	Olson and McGarrigle, 1992
Hamster	Thymic hypoplasia	Single dose	1,500 ng/kg, gd 7 or 9	LOAEL	Olson and McGarrigle, 1992
Mouse	Cleft palate	Multiple dose	3,000 ng/kg/day, gd 6-15	LOAEL	Courtney and Moore, 1971
Hamster	Prenatal mortality	Single dose	18,000 ng/kg/day, gd 7 or 9	LOAEL	Olson et al., 1990
Mouse	Prenatal mortality	Single dose	24,000 ng/kg/day, gd 6	LOAEL	Couture et al., 1990b

[a]Maternal body burdens of TCDD at the time of conception were calculated by assuming a one-compartment open model and half-life for whole body TCDD elimination of 400 days in the monkey (McNulty et al., 1982) and 23.7 days in the rat (Rose et al., 1976). A bioavailabilty of 86.1 percent was used in the monkey and rat (Rose et al., 1976). The daily dietary exposure levels in rhesus monkeys were approxiately 5 and 25 ppt at the NOAEL and LOAEL doses, respectively. Rhesus monkeys were exposed to these levels of TCDD for 7 months prior to conception. At this time (0.525 half-lives) the cumulative amount of TCDD in rhesus monkeys was 30.5 percent of the calculated steady-state level. Rats were exposed to the indicated daily doses of TCDD for a period of 90 days (3.8 half-lives) prior to conception. At this time the cumulative amount of TCDD in rats was 92.8 percent of the calculated steady-state level.
gd=gestational day.

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bioavailability used for TCDD was determined in rats (Rose et al., 1976). As no estimate for TCDD bioavailability has been reported in rhesus monkeys, the same 86.1 percent value was used. The whole body elimination half-life used for TCDD in the rat is 23.7 days (Rose et al., 1976). McNulty et al. (1982) estimated a half-life of approximately 1 year for TCDD elimination from adipose tissue in the rhesus monkey, and for calculation of the body burdens estimated in Table 5-10, this half-life was rounded to 400 days for whole body elimination. The maternal body burden given for chronic exposure in the rat was calculated from the data of Murray et al. (1979). The maternal body burden given for subchronic exposure in the rhesus monkey was calculated from data obtained from Dr. R. E. Bowman (personal communication), which included the daily dietary TCDD exposure level for each pregnant female used in the studies reported by Bowman et al. (1989a,b) and Schantz and Bowman (1989). Dr. Bowman's results indicate that the range of TCDD half-lives in these monkeys was 200 to 600 days, which is consistent with the results of McNulty et al. (1982). The body burdens estimated for rhesus monkeys used in these studies are averages based on the average daily TCDD consumption of all pregnant females used at a particular level of maternal TCDD exposure.

As summarized in Table 5-8, lake trout and rainbow trout sac fry and Japanese medaka embryos are similarly affected by a spectrum of lesions that includes hemorrhage, edema, collapse of the yolk sac, cessation of blood flow, and embryo mortality. Estimates of the NOAEL and LOAEL are given in Table 5-8 for the appearance of these lesions in Japanese medaka embryos and for embryo mortality in the two trout species. Although fertilized lake trout eggs and Japanese medaka eggs were exposed to various TCDD concentrations dissolved in static water, and fertilized rainbow trout eggs were injected directly with TCDD, the egg doses given in Table 5-8 represent the concentration of TCDD within the eggs themselves. Therefore, the different NOAELs and LOAELs for developmental toxicity in different fish species probably represent species differences in susceptibility to TCDD-induced developmental toxicity rather than differences in method of TCDD exposure. Of the three fish species, lake trout sac fry are the most sensitive to TCDD-induced mortality. However, based on the LOAELs shown in Table 5-8, the difference in susceptibility between fish species may be less than tenfold.

Based on the LOAELs shown in Table 5-9, the sensitivity of different bird species to TCDD-induced embryo mortality varies by more than fortyfold. The chicken embryo is more susceptible to TCDD-induced mortality than are embryos of the ring-necked pheasant and eastern bluebird. In addition, chicken embryos are highly sensitive to the formation of TCDD-induced structural defects in the heart and aortic arch. The incidence of cardiac malformations in the chicken embryo is increased at an egg exposure level as low as 9 ng TCDD/kg egg. However, such cardiac malformations have not been found in any other bird species that has been examined.

Table 5-10 summarizes the levels of TCDD exposure that cause certain structural malformations, functional alterations, and prenatal mortality in the embryo/fetus of different mammalian species. Based on the LOAELs given in Table 5-10, functional alterations in learning behavior and the male reproductive system occur at lower TCDD doses than those required to produce structural malformations. Although TCDD-induced developmental toxicity has been extensively studied in mice and rats, the LOAELs in Table 5-10 indicate that the embryo/fetus of rodent species is generally not as sensitive to TCDD-induced prenatal mortality as is the embryo/fetus of the rhesus monkey. The sensitivity of the embryo/fetus to TCDD-induced prenatal mortality in different mammalian species varies approximately 240-fold. This is in contrast to the 1,000- to 5,000-fold variation in the LD_{50} of TCDD when adult animals of these same species are exposed. The agreement between studies with respect to the LOAEL in Table 5-10 for prenatal mortality in rats and monkeys is particularly striking. The 500 ng/kg dose of TCDD on gestational days 6 to 15 that caused prenatal mortality in rats (Sparschu et al., 1971) agrees with the maternal TCDD body burden of 270 ng/kg calculated from the chronic exposure of rats by Murray et al. (1979) to within a factor of 2. Similarly, the TCDD dose of 111 ng/kg that was given to rhesus monkeys nine times during the first trimester of pregnancy (McNulty, 1984) agrees with the maternal body burden of 97 ng/kg that increased prenatal mortality in rhesus monkeys following subchronic dietary exposure (Schantz and Bowman, 1989).

5.3. REPRODUCTIVE TOXICITY

5.3.1. Female

5.3.1.1. *Reproductive Function/Fertility*

TCDD and its approximate isostereomers have been shown to affect female reproductive endpoints in a variety of animal studies. Among the effects reported are reduced fertility, reduced litter size, and effects on the female gonads and menstrual/estrous cycle. These studies are reviewed below. Other TCDD effects on pregnancy maintenance, embryo/fetotoxicity, and postnatal development are covered in Section 5.2 of this chapter.

The study by Murray et al. (1979) employed a multigenerational approach, examining the reproductive effects of exposure of male and female rats over three generations to relatively low levels of TCDD (0, 0.001, 0.01, and 0.1 μg/kg/day). There was variation in the fertility index in both the control and the exposed groups, and a lower than desirable number of impregnated animals in the exposed groups. Nevertheless, the results showed exposure-related effects on fertility, an increased time between first cohabitation and delivery, and a decrease in litter size. The effects on fertility and litter size were observed at 0.1 μg/kg/day in the F_0 generation and at 0.01 μg/kg/day in the F_1 and F_2 generations. Additionally, in a 13-week exposure to 1 to 2 μg/kg/day of TCDD in nonpregnant female rats, Kociba et al. (1976) reported anovulation and signs of ovarian dysfunction, as well as suppression of the estrous cycle. However, at exposures of 0.001 to 0.01 μg/kg/day in a 2-year study, Kociba et al. (1978) reported no effects on the female reproductive system.

Allen and colleagues reported on the effects of TCDD on reproduction in the monkey (Allen et al., 1977, 1979; Barsotti et al., 1979; Schantz et al., 1979). In a series of studies, female rhesus monkeys were fed 50 or 500 ppt TCDD for ≤9 months. Females exposed to 500 ppt showed obvious clinical signs of TCDD toxicity and lost weight throughout the study. Five of the eight monkeys died within 1 year after exposure was initiated. Following 7 months of exposure to 500 ppt TCDD, seven of the eight females were bred to unexposed males. The remaining monkey showed such severe signs of TCDD toxicity that she was not bred due to her debilitated state. Of the seven females that were evaluated for their reproductive capabilities, only three were able to conceive and, of these, only one was able to carry her infant to term (Barsotti et al., 1979). When females exposed to 50 ppt TCDD in

the diet were bred at 7 months, two of eight females did not conceive and four of six that did conceive could not carry their pregnancies to term. As one monkey delivered a stillborn infant, only one conception resulted in a live birth (Schantz et al., 1979). As described in an abstracted summary, these results at 50 and 500 ppt TCDD are compared with a group of monkeys given a dietary exposure to PBB (0.3 ppm, Firemaster FF-1) in which seven of seven exposed females were able to conceive, five gave birth to live, normal infants, and one gave birth to a stillborn infant (Allen et al., 1979). Although the effects at 500 ppt TCDD may be associated with significant maternal toxicity, this would not appear to be the case at the lower dose. After administration of 50 ppt TCDD, no overt effects on maternal health were observed, but the ability to conceive and maintain pregnancy was reduced (Allen et al., 1979).

In a similar series of experiments, female rhesus monkeys were fed diets that contained 0, 5, and 25 ppt TCDD (Bowman et al., 1989b; Schantz and Bowman, 1989). Reproductive function was not altered in the 5 ppt group, as seven of eight females mated to unexposed males after 7 months of dietary exposure to TCDD were able to conceive. Six of these females gave birth to viable infants at term and one gave birth to a stillborn infant. This was not significantly different from the results of the control group, which was fed a normal diet that contained no TCDD. All seven of the monkeys in this control group were able to conceive and give birth to viable infants. The 25 ppt dietary exposure level, however, did affect reproductive function. Only one of the eight females in this group that was mated gave birth to a viable infant. As in the 50 ppt group from earlier studies, there were no serious health problems exhibited by any females exposed to 0, 5, or 25 ppt TCDD. Therefore, the results in the 25 and 50 ppt groups suggest that maternal exposure to TCDD before and during pregnancy can result in fetomortality without producing overt toxic effects in the mother.

McNulty (1984) examined the effect of a TCDD exposure during the first trimester of pregnancy (gestational age 25 to 40 days) in the rhesus monkey. At a total dose of 1 μg/kg given in nine divided doses, three of four pregnancies ended in abortion and two of these abortions occurred in animals that displayed no maternal toxicity. At a total dose of 0.2 μg/kg, one of four pregnancies ended in abortion. This did not appear to be different from

the control population, but the low number of animals per group did not permit statistical analysis. McNulty (1984) also administered single 1 μg/kg doses of TCDD on gestational days 25, 30, 35, or 40. The number of animals per group was limited to three, but it appeared that the most sensitive periods were the earlier periods, days 25 and 30, and that both maternal toxicity and fetotoxicity were reduced when TCDD was given on later gestational days. For all days at which a single 1 μg TCDD/kg dose was given (gestational day 25, 30, 35, or 40), 10 of 12 pregnancies terminated in abortion. Thus, of 16 monkeys given 1 μg TCDD/kg in single or divided doses between days 25 and 40 of pregnancy, only three normal births occurred (McNulty, 1984, 1985).

Of increasing interest is the recent report that TCDD exposure is associated with the appearance of endometriosis (Rier et al., 1993). Endometriosis is characterized by endometrial cell growth outside the uterus and can be associated with infertility and pain. Rier et al. (1993) determined the incidence of endometriosis in a colony of rhesus monkeys that had been chronically exposed to TCDD 10 years earlier. They reported a 43-percent and a 71-percent incidence at 5 ppt and 25 ppt, respectively, compared with a control incidence of 33 percent. Moreover, the severity of endometriosis was dose dependent. Other studies are now under way to further examine the relationship of endometriosis following exposure to dioxin or dioxin-like compounds.

In conclusion, the primary effects of TCDD on female reproduction appear to be decreased fertility, inability to maintain pregnancy for the full gestational period, and in the rat, decreased litter size. In some studies, signs of ovarian dysfunction such as anovulation and suppression of the estrous cycle have been reported (Kociba et al., 1976; Barsotti et al., 1979; Allen et al., 1979). Unfortunately, the amount of attention that has been given to the female reproductive system, especially in the nonpregnant state, has been limited. In addition, there is little information on how TCDD toxicity involving the mother and/or placenta might affect fetal development.

5.3.1.2. *Alterations in Hormone Levels*

The potential for TCDD to alter circulating female hormone levels has been examined, but only to a very limited extent. In monkeys fed a diet that contained 500 ppt

TCDD for ≤9 months, the length of the menstrual cycle, as well as the intensity and duration of menstruation, were not appreciably affected by TCDD exposure (Barsotti et al., 1979). However, there was a decrease in serum estradiol and progesterone concentration in five of the eight exposed monkeys, and in two of these animals the reduced steroid concentrations were consistent with anovulatory menstrual cycles. In summary form, Allen et al. (1979) described the effects of dietary exposure of female monkeys to 50 ppt TCDD. After 6 months of exposure to this lower dietary level of TCDD, there was no effect on serum estradiol and progesterone concentrations in these monkeys. Thus, the presence of these hormonal alterations is dependent on the level of dietary TCDD exposure. Shiverick and Muther (1983) reported that there was no change in circulating levels of estradiol in the rat after exposure to 1 μg/kg/day on gestational days 4 to 15. Taking all of these results together, the effect of TCDD exposure on circulating female hormone levels may depend both on species and level of exposure. It appears that any significant effect is only seen at relatively high exposure levels, but very little research has been done and the studies to date have not been specifically designed to carefully examine alterations in female hormones.

5.3.1.3. *Antiestrogenic Action*

5.3.1.3.1. ***In vivo.*** Estrogens are necessary for normal uterine development and for maintenance of the adult uterus. The cyclic production of estrogens partially regulates the cyclic production of FSH and LH that results in the estrous cycling of female mammals. In addition, estrogens are necessary for the maintenance of pregnancy. Any effect that causes a decrease in circulating or target cell estrogen levels can alter normal hormonal balance and action.

Early experimental results in rats and monkeys indicated that TCDD may have an antiestrogenic action. Following administration of 1 μg TCDD/kg/day to rats for 13 weeks, Kociba et al. (1976) reported morphologic changes in the ovaries and uterus that were interpreted as being due to a suppression or inhibition of the estrous cycle. Rhesus monkeys exposed to 500 ppt of TCDD in the diet for 6 months developed hormonal irregularities in their estrous cycles that were associated with reduced conception rates as well as a high incidence of early spontaneous abortions (Allen et al., 1977; Barsotti et al., 1979).

In rhesus monkeys, the severity of the TCDD-associated reproductive alterations was correlated with decreased plasma levels of estrogen and progesterone (Barsotti et al., 1979). Thus, one possible mechanism for these effects would be increased metabolism of estrogen and progesterone due to induction by TCDD of hepatic microsomal enzymes and/or a decrease in the rate at which these steroids are synthesized. On the other hand, serum concentrations of 17β-estradiol are not significantly affected when TCDD is administered to pregnant rats (Shiverick and Muther, 1983). Thus, an alternative mechanism for TCDD-associated reproductive dysfunction could involve effects of TCDD on gonadal tissue itself, such as a decrease in its responsiveness to estrogen. In support of this latter mechanism, the administration of TCDD to CD-1 mice results in a decreased number of cytosolic and nuclear estrogen receptors in hepatocytes and uterine cells. Although TCDD treatment induces hepatic cytochrome P-450 levels in these animals, it has no effect on serum concentrations of 17β-estradiol (DeVito et al., 1992). This indicates that the antiestrogenic effect of TCDD in CD-1 mice is not caused by a decrease in circulating levels of estrogen.

Effects of estrogen on the uterus include a cyclic increase in uterine weight, increased activity of the enzyme peroxidase, and an increase in the tissue concentration of progesterone receptors. Antiestrogenic effects of TCDD administration to female rats include a decrease in uterine weight, decrease in uterine peroxidase activity, and a decrease in the concentration of progesterone receptors in the uterus (Safe et al., 1991). In addition, when TCDD and 17β-estradiol are coadministered to the same female rat, the antiestrogenic action of TCDD diminishes or prevents 17β-estradiol-induced increases in uterine weight, peroxidase activity, progesterone receptor concentration, and expression of EGF receptor mRNA (Astroff et al., 1990; Safe et al., 1991). Similarly, in mice TCDD administration decreases uterine weight and antagonizes the ability of 17β-estradiol to increase uterine weight (Gallo et al., 1986).

The ability of TCDD to antagonize the effects of exogenously administered estrogen in the rat is dependent on the age of the animal. In 21-day-old rats, TCDD does not affect 17β-estradiol-induced increases in uterine weight or progesterone receptor concentration. On the other hand, in 28-day-old intact rats and 70-day-old ovariectomized rats, both of these 17β-estradiol-mediated responses are attenuated by TCDD (Safe et al., 1991). Previously, it had been reported that TCDD administration does not alter the dose-dependent increase in

uterine weight due to exogenously administered estrone in sexually immature rats (Shiverick and Muther, 1982). The later work by Safe et al. (1991) suggests that this apparent lack of an antiestrogenic effect of TCDD may have been due to the young age of the rats used.

5.3.1.3.2. ***In vitro.*** Both TCDD and progesterone can affect a decrease in the nuclear estrogen receptor concentration in rat uterine strips (Romkes and Safe, 1988). However, the effect of progesterone is inhibited by actinomycin D, cycloheximide, and puromycin, whereas the effect of TCDD is inhibited only by actinomycin D. The reasons that the TCDD-induced decrease in nuclear estrogen receptors is blocked by a transcription inhibitor, but not by protein synthesis inhibitors, are not understood. However, these results indicate that TCDD and progesterone decrease the nuclear estrogen receptor concentration by different mechanisms. In addition, the antiestrogenic actions of TCDD can be demonstrated in cell culture, and two prominent mechanisms could potentially be involved. They are (1) increased metabolism of estrogen due to Ah receptor-mediated enzyme induction and (2) a downregulation of estrogen receptors within the target cell.

In MCF-7 cells, which are estrogen-responsive cells derived from a human breast adenocarcinoma, antiestrogenic effects caused by the addition of TCDD to the culture medium include a reduction of the 17β-estradiol-induced secretion of a 160 kDa protein, 52 kDa protein, and 34 kDa protein (Biegel and Safe, 1990). These last two proteins are believed to be procathepsin D and cathepsin D, respectively. In addition, treatment of MCF-7 cells with TCDD suppresses the 17β-estradiol-enhanced secretion of tPA and inhibits estrogen-dependent postconfluent cell proliferation (Gierthy et al., 1987; Gierthy and Lincoln, 1988). Thus, cultured MCF-7 cells have several estrogen-dependent responses that are inhibited by TCDD; this characteristic makes them a useful model system for studying the antiestrogenic actions of dioxin.

In cultured MCF-7 cells, TCDD treatment induces aryl hydrocarbon hydroxylase (AHH) activity, the hallmark response of Ah receptor binding, and increases hydroxylation of 17β-estradiol at the C-2, C-4, C-6α, and C-15α, positions (Spink et al., 1990). It turns out that the particular cytochrome P-450 that catalyzes the C-2, C-15α, and C-6α hydroxylations of 17β-estradiol is cytochrome P-4501A1, which is identical to AHH (Spink

et al., 1992). TCDD treatment also results in reduced levels of occupied nuclear estrogen receptors (Harris et al., 1990). These results indicate, in MCF-7 cells, that the antiestrogenic effect of TCDD could result from (1) an increased metabolism of estrogens due to Ah receptor-mediated enzyme induction and/or (2) a decreased number of estrogen receptors in the nucleus. Safe and his colleagues have published TCDD-concentration-response information for both the TCDD-induced decrease in occupied nuclear estrogen receptors (Harris et al., 1989) and the induction of AHH and EROD activities in MCF-7 cells (Harris et al., 1990). In addition, they have reported that TCDD causes a decreased number of cytosolic and nuclear estrogen receptors in Hepa 1c1c7 cells, which are a mouse hepatoma cell line (Zacharewski et al., 1991).

Independent analysis of the data suggests that the EC_{50} values for these effects are not dissimilar enough to distinguish between the proposed mechanisms. Instead, it appears as though TCDD induces the enzymes AHH and EROD over the same concentration range that it causes a decreased concentration of occupied nuclear estrogen receptors in MCF-7 cells. In Hepa 1c1c7 cells, the lowest concentration used was 10 pM. Although exposure to 10 pM TCDD resulted in a statistically significant downregulation of estrogen receptors, Israel and Whitlock (1983) reported that this concentration is the approximate EC_{50} for the induction of cytochrome P-4501A1 mRNA and enzyme activity in these cells. Therefore, in Hepa 1c1c7 cells as well as in MCF-7 cells, it would appear that the TCDD concentrations required to produce enzyme induction and reduction in occupied nuclear estrogen receptor levels are not dissimilar enough to distinguish between the two proposed mechanisms.

More recently, Safe and his colleagues have used an analog of TCDD, MCDF, which inhibits the 17β-estradiol-induced secretion of the 34, 52, and 160 kDa proteins and downregulates estrogen receptors in MCF-7 cells. These effects occurred at concentrations of MCDF at which there is no detectable induction of EROD activity (Zacharewski et al., 1992). In addition, it has been stated that the downregulation of estrogen receptors in Hepa 1c1c7 cells can be detected as early as 1 hour after exposure of the cell cultures to 10 nM TCDD (Zacharewski et al., 1991). This time is slightly less than the 2 hours that was required for Israel and Whitlock (1983) to detect an increase in cytochrome P-450IA1 mRNA levels after exposure of Hepa 1c1c7 cells to 10 pM TCDD. After exposure of Hepa 1c1c7

cells to a maximally inducing concentration of 1 nM TCDD, however, there are significant increases in the cellular concentration of cytochrome P-450IA1 mRNA after 1 hour, whereas the induction of AHH activity takes slightly longer (Israel and Whitlock, 1983).

Gierthy et al. (1987) reported that exposure of MCF-7 cells to 1 nM TCDD caused suppression of the 17β-estradiol-induced secretion of tPA. This effect of TCDD, however, occurred in the absence of any measurable decrease in the whole cell concentration of estrogen receptors, even though the cultures were pretreated with serum-free medium to reduce cell proliferation and maximize the cellular content of estrogen receptors. Gierthy's group pretreated their cultures with serum-free medium, which was done to reduce cell proliferation and maximize the cellular content of estrogen receptors. The disparity between this result of Gierthy et al. (1987), which suggests no effect of TCDD on the estrogen receptor content of MCF-7 cells, and the results of Safe and his colleagues to the contrary in this same cell line remains largely unexplained. Overall, it appears as though no obvious distinction between the two proposed mechanisms can be made at the present time. Therefore, it seems that the antiestrogenic effect of TCDD results from both an increased metabolism of estrogen and a decreased number of estrogen receptors. It is important to note that TCDD does not compete with radiolabeled estrogens or progesterone for binding to estrogen or progesterone receptors and that these steroids do not bind to the Ah receptor or compete with radiolabeled TCDD for binding (Romkes et al., 1987; Romkes and Safe, 1988).

5.3.1.3.3. ***Evidence for an Ah receptor mechanism.***

5.3.1.3.3.1. ***Ah receptor mutants.*** Although the precise cellular mechanism by which TCDD produces its antiestrogenic effect is subject to a discordance between two primary schools of thought, there is agreement that the response is mediated by the Ah receptor. Thus, the antiestrogenic effects of TCDD in cultured cells appear to involve an Ah receptor-mediated alteration in the transcription of genes. This is indicated by studies using wild-type Hepa 1c1c7 cells and mutant Hepa 1c1c7 cells in culture (Zacharewski et al., 1991). In wild-type cells, TCDD reduces the number of nuclear estrogen receptors, and this response can be inhibited by cycloheximide and actinomycin D. However, in class 1 mutants, which

have relatively low Ah receptor levels, TCDD has only a small effect. Similarly, in class 2 mutants, which have a defect in the accumulation of transcriptionally active nuclear Ah receptors, there was no effect of TCDD on the number of nuclear estrogen receptors. Taken together, these results indicate that the downregulation of estrogen receptors in Hepa 1c1c7 cells involves an Ah receptor-mediated effect on gene transcription. As previously noted, TCDD induces cytochrome P-4501A1 mRNA transcription and enzyme activity in Hepa 1c1c7 cells (Israel and Whitlock, 1983). This effect is also Ah receptor mediated (Nebert and Gielen, 1972).

5.3.1.3.3.2. ***Structure-activity relationships in vivo.*** The relative potencies of halogenated aromatic hydrocarbon congeners as inhibitors of uterine peroxidase activity in the rat are similar to their relative Ah receptor-binding affinities (Astroff and Safe, 1990). Only limited relative potency information is available for the reduction of hepatic and uterine estrogen receptor concentrations per se by these substances in rats. TCDD and 1,2,3,7,8-PeCDD both exhibit high affinity for the Ah receptor. At an 80 μg/kg dose of either of these two substances, hepatic estrogen receptor concentrations are reduced 42 and 41 percent, whereas uterine estrogen receptor concentrations are reduced 53 and 49 percent by TCDD and 1,2,3,7,8-PeCDD, respectively. On the other hand, 1,3,7,8-TCDD and 1,2,4,7,8-PeCDD bind less avidly to the Ah receptor. At a 400 μg/kg dose of either of these two substances, hepatic estrogen receptor concentrations are reduced 36 and 40 percent, whereas uterine estrogen receptor concentrations are reduced 21 and 24 percent by 1,3,7,8-TCDD and 1,2,4,7,8-PeCDD, respectively (Romkes et al., 1987). As the potency of these congeners for reducing estrogen receptor concentrations correlates with their Ah receptor-binding affinities, these in vivo results provide evidence that the antiestrogenic effect of TCDD is mediated by the Ah receptor.

5.3.1.3.3.3. ***Genetic evidence.*** Consistent with the interpretation based on structure-activity relationships, there is a greater reduction in the number of hepatic estrogen receptors when Ah^bAh^b C57BL/6 mice are exposed to TCDD than when Ah^dAh^d DBA/2 mice are similarly exposed (Lin et al., 1991). To date, however, the antiestrogenic effects have not been

studied in the progeny of test crosses between Ah^bAh^b and Ah^dAh^d mouse strains that respectively produce Ah receptors with high- or low-binding affinity for TCDD. Therefore, the potential segregation of the antiestrogenic effects of TCDD with the Ah locus has not been verified by the results of genetic crosses.

5.3.1.3.3.4. ***Structure-activity relationships in vitro.*** The Ah receptor is detectable in MCF-7 cells, and AHH as well as EROD activities are both inducible in these cells (Harris et al., 1989). The relative abilities of TCDD and other CDD, CDF, and PCB congeners to suppress 17β-estradiol-induced secretion of tPA by MCF-7 cells are consistent with the structure-activity relationships for other Ah receptor-mediated responses (Gierthy et al., 1987). In addition, the rank order of potency for several Ah receptor agonists in reducing nuclear estrogen receptors in MCF-7 cells is TCDD > 2,3,4,7,8-PeCDD > 2,3,7,8-TCDF > 1,2,3,7,9-PeCDD > 1,3,6,8-TCDF (Harris et al., 1990). The rank order of potency for these substances is consistent with their relative activities as Ah receptor agonists. These results in vitro support a role for the Ah receptor in the antiestrogenic actions of TCDD.

5.3.2. Male

5.3.2.1. *Reproductive Function/Fertility*

TCDD and related compounds decrease testis and accessory sex organ weights, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility when given to adult animals in doses sufficient to reduce feed intake and/or body weight. Certain of these effects have been reported in chickens, rhesus monkeys, rats, guinea pigs, and mice treated with overtly toxic doses of TCDD, TCDD-like congeners, or toxic fat that was discovered later to contain TCDD (Allen and Lalich, 1962; Allen and Carstens, 1967; Khera and Ruddick, 1973; Kociba et al., 1976; Van Miller et al., 1977; McConnell et al., 1978; Moore et al., 1985; Chahoud et al., 1989; Morrisey and Schwetz, 1989). In testis of these different species, TCDD effects on spermatogenesis are characterized by loss of germ cells, the appearance of degenerating spermatocytes and mature spermatozoa within the lumens of seminiferous tubules, and a reduction in the number of tubules containing mature spermatozoa (Allen and Lalich, 1962; Allen and Carstens, 1967; McConnell et al., 1978;

Chahoud et al., 1989). The lowest cumulative dose of TCDD to decrease spermatogenesis in the rat was 1 μg/kg/day administered 5 days a week for 13 weeks (Kociba et al., 1976). With this dosage regimen, which resulted in a TCDD body burden of approximately 20 μg/kg at the end of the dosing period (Rose et al., 1976), body weights and feed consumption of the rats also were significantly depressed. Thus, suppression of spermatogenesis is not a highly sensitive effect when TCDD is administered to postweanling animals.

5.3.2.2. *Alterations in Hormone Levels*

The effects of TCDD on the male reproductive system are believed to be due in part to an androgenic deficiency. This deficiency is characterized in adult rats by decreased plasma testosterone and DHT concentrations, unaltered plasma LH concentrations, and unchanged plasma clearance of androgens and LH (Moore et al., 1985, 1989; Mebus et al., 1987; Moore and Peterson, 1988; Bookstaff et al., 1990a). The ED_{50} of TCDD for producing this effect in adult male rats on day 7 after dosing is 15 μg/kg (Moore et al., 1985), and it can be detected within 1 day of treatment. As described in the following sections, the cause of the androgenic deficiency is decreased testicular responsiveness to LH and increased pituitary responsiveness to feedback inhibition by androgens and estrogens (Moore et al., 1989, 1991; Bookstaff et al., 1990a,b; Kleeman et al., 1990).

5.3.2.3. *Target Organ Responsiveness*

5.3.2.3.1. ***Inhibition of testicular steroidogenesis.*** Testicular steroidogenesis occurs within Leydig cells and is regulated primarily by plasma LH concentrations (Payne et al., 1985; Hall, 1988). Binding of LH to the LH receptor causes cAMP and possibly other second messengers to be formed (Cooke et al., 1989). In response, cholesterol is rapidly transported to the initial enzyme in the testosterone biosynthetic pathway, a cholesterol side chain cleavage enzyme, which is a cytochrome P-450 (cytochrome $P\text{-}450_{scc}$) located on the inner side of the inner mitochondrial membrane that converts cholesterol to pregnenolone. The mobilization of free cholesterol rather than its conversion to pregnenolone and other metabolites is generally considered to be the rate-limiting step in testicular steroidogenesis.

TCDD inhibits testosterone biosynthesis, predominantly if not exclusively by inhibiting the mobilization of free cholesterol that acts as a substrate for cytochrome P-450$_{scc}$ (Moore et al., 1991). Thus, in the testes of TCDD-treated rats, cholesterol is provided to the cytochrome P-450$_{scc}$ enzyme at too slow a rate to maintain androgenic homeostasis, even when the plasma LH concentration characteristic of "normal" androgen levels is present.

5.3.2.3.2. ***Altered regulation of pituitary LH secretion.*** In TCDD-treated male rats, the expected increase in plasma LH concentration that would facilitate testicular compensation for the decreased plasma androgens does not occur (Moore et al., 1989; Ruangwises et al., 1991). The failure of the plasma LH concentration to rise appropriately is not caused by an increase in the plasma clearance of LH or by a decrease in the maximal rate of pituitary LH synthesis or secretion (Bookstaff et al., 1990a,b). Rather, TCDD alters the feedback regulation of LH secretion in male rats by increasing the potency of testosterone and its metabolites (DHT and 17β-estradiol) as inhibitors of LH secretion. The ED$_{50}$ of TCDD for enhancing the testosterone-mediated inhibition of LH secretion 7 days after treatment is the same as its ED$_{50}$ for causing the androgenic deficiency (15 μg/kg). Also, both responses are detected within 1 day of TCDD dosing and are fully developed after 7 days when the ED$_{50}$s were determined.

Decreased plasma androgen concentrations normally result in compensatory increases in both the number of pituitary GnRH receptors and the responsiveness of the pituitary to GnRH. TCDD treatment prevents the increases in GnRH receptor number and responsiveness that would be expected in the light of the decreased plasma androgen concentrations (Bookstaff et al., 1990b). The pituitary is thus a target organ for TCDD because its responsiveness to hormones secreted by the testis (testosterone) and hypothalamus (GnRH) is altered by TCDD.

If the plasma LH concentrations in TCDD-treated rats did increase appropriately in response to decreased plasma androgens, it is expected that plasma androgens would return to normal levels (Kleeman et al., 1990). This is because the testes of TCDD-treated rats are capable of synthesizing more testosterone than is needed to maintain androgen concentrations in the physiological range, although this would require significantly elevated levels of LH in

TCDD-treated rats. The fact that there is a testicular reserve capacity to provide for sufficient amounts of androgen synthesis, even when compromised, underscores the importance of the effects of TCDD on pituitary LH secretion in producing the effects of TCDD on plasma androgen concentrations.

5.3.2.3.3. ***Differential responsiveness of androgen target organs.*** The dose-related reductions in plasma testosterone and DHT concentrations in intact adult rats are accompanied by similar dose-related reductions (ED_{50} 15 μg TCDD/kg) in seminal vesicle and ventral prostate weights measured 7 days after dosing (Moore et al., 1985). In contrast, TCDD has no effect on accessory sex organ weights (or plasma androgen concentrations) in castrated adult rats implanted with either testosterone- or DHT-containing capsules (Moore and Peterson, 1988; Bookstaff et al., 1990a,b). As trophic responsiveness of the seminal vesicles and ventral prostate to testosterone and DHT are unaffected by postpubertal TCDD treatment, it follows that TCDD can increase responsiveness of the pituitary to androgens without affecting responsiveness of the accessory sex organs to androgens.

5.3.2.3.4. ***Relative sensitivity.*** The male reproductive system in rats is ~100 times more susceptible to TCDD toxicity when exposure occurs perinatally (ED_{50} for the most sensitive effects, 0.16 μg/kg) rather than in adulthood (ED_{50} for the most sensitive effects, 15 μg/kg). To illustrate this sensitivity, a single maternal TCDD dose as low as 0.064 μg/kg given on day 15 of gestation significantly decreases epididymis and cauda epididymis weights, cauda epididymal sperm numbers, and daily sperm production in male offspring at various stages of sexual development. Decreases in ventral prostate weights in 32-day-old male offspring and in older males, increases in the number of mounts preceding ejaculation, and increases in intromission latency also are produced by maternal TCDD doses as low as 0.064 μg/kg. The 0.064 μg TCDD/kg dose is not maternally toxic and produces no signs of overt toxicity in male or female offspring. Other effects of perinatal exposure on the male reproductive system were detected at a maternal TCDD dose of 0.16 μg/kg or higher (Mably et al., 1991, 1992a,b,c). On the other hand, when exposure occurs in adulthood, relatively large doses in the overtly toxic range are required to cause decreases in spermatogenesis and in ventral

prostate and caput epididymis weight (Kociba et al., 1976; Moore et al., 1985). Kociba et al. (1976) reported that accessory sex organ weights and spermatogenesis are decreased in rats following exposure to 1 μg TCDD/kg/day, 5 days per week for 13 weeks. Using the parameters for TCDD half-life and bioavailability in the rat determined by Rose et al. (1976), this dosage regimen results in a TCDD body burden of approximately 20 μg/kg at the end of the dosing period.

In adult rats, the most sensitive toxic responses to TCDD have been observed following long-term, low-level exposure. In a 3-generation reproduction study, Murray et al. (1979) reported that dietary administration of TCDD at doses as low as 0.01 μg/kg/day significantly affected reproductive capacity in female rats, with no effects seen at 0.001 μg/kg/day (NOAEL). The same NOAEL was found in a 2-year chronic toxicity and oncogenicity study in which an increased incidence of certain types of neoplasms was altered among rats given TCDD doses of 0.01 or 0.1 μg/kg/day (Kociba et al., 1978). Based on the pharmacokinetics of TCDD in the rat (Rose et al., 1976), the steady-state body burden of TCDD in these rats that were chronically dosed (>90 days) with either 0.01 or 0.001 μg TCDD/kg/day is approximately 0.29 μg/kg (LOAEL) and 0.029 μg/kg (NOAEL), respectively. Yet, Mably et al. (1991, 1992a,b,c) found that a single TCDD dose of 0.064 μg/kg given on day 15 of gestation produces a number of statistically significant effects on the reproductive system of male rat offspring. Because 0.064 μg TCDD/kg was the lowest dose tested, a NOAEL for developmental male reproductive toxicity, which is defined as the lowest dose used that has no statistically significant effect, could not be determined by Mably et al. (1991, 1992a,b,c). It is concluded that developmental effects on spermatogenesis occur at a maternal TCDD dose that is lower than any previously shown to produce toxicity in rats.

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6. CARCINOGENICITY OF TCDD IN ANIMALS*

6.1. INTRODUCTION

Additional scientific information on the use of animal cancer data for estimating human risks from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has become available since 1988. Much of the data on tumor incidence in experimental animals available in 1988 demonstrated that TCDD is a carcinogen at multiple sites in both sexes of rats and mice. Some of the cancers occurred following particularly low doses. Since 1988, TCDD has been shown to be a carcinogen in hamsters, and during the past 3 years, some of the tumor incidence data in rat liver have been reevaluated.

In the past few years, there have been several studies that have impact on the evaluation of cancer studies in experimental animals. For example, the evidence is now considerably stronger that TCDD does not damage DNA directly through the formation of DNA adducts. However, mechanisms have been proposed supporting the possibility that TCDD might alter the DNA-damaging potential of some endogenous compounds, including estrogens. In addition, there have been numerous reports on TCDD-mediated modifications of growth factor pathways and cytokines in experimental animals and cell systems. Some of the altered systems include those for epidermal growth factor, transforming growth factor α, estrogen, glucocorticoids, tumor necrosis factor-α, interleukin 1β, plasminogen inactivating factor, and gastrin. Many of these pathways are involved in cell proliferation and differentiation and provide plausible avenues for researching the mechanisms responsible for the carcinogenic actions of TCDD. These effects are consistent with the generally accepted conclusion that TCDD acts as a "tumor promoter" in multistage models for chemical carcinogenesis and is virtually devoid of initiating activity in these models. It is important to note that "tumor promotion" is an operational and not a mechanistic term and that multiple mechanisms of

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tumor promotion are likely. Each of these mechanisms may be fundamentally different from the other.

Over the past few years, there has been growing consensus that most, if not all, of TCDD's biochemical and toxic effects require interaction with the aryl hydrocarbon (Ah) receptor. The properties of the Ah receptor and the mechanisms whereby this receptor regulates gene expression will be evaluated in other chapters. However, formation of the Ah receptor-TCDD complex is only the first of many steps involved in the production of a biochemical and toxic effect. Although we are gaining knowledge of details of the subsequent steps, we know very little about some components of the Ah receptor-mediated responses. It is clear, however, that cell-specific factors other than the Ah receptor must be involved in determining tissue responses once TCDD binds the Ah receptor.

Evaluation of dose response is one of the more important issues that affect dioxin risk assessments. The focus of this controversy centers on whether the effects of dioxin exhibit thresholds. It now appears that for some responses there is a proportional relationship between receptor occupancy and response, which is evidenced by a linear relationship between target dose and effect over a wide dose range. However, different dose-response relationships are seen for different responses so it is probably inappropriate to use a single surrogate marker to estimate dioxin's risks. Furthermore, these data reveal there is no unifying dose-response relationship for all Ah receptor-mediated events.

Another controversial area in risk assessment is whether experimental animal models are appropriate for estimating human risks. During the past few years, there has been increasing evidence that biochemical and toxic responses resulting from human exposure to TCDD and its structural analogs appear to be similar to responses in experimental animals. However, it may be possible that humans are sensitive or resistant for some responses. There also is increasing awareness that interindividual variations in human responses to dioxin are a complicating factor in risk assessment; it appears there are individuals who are responsive and nonresponsive to numerous environmental chemicals, including TCDD.

Much of the controversy surrounding dioxin risk assessment reflects the selection of mathematical models: threshold, linear multistage, or others. We now know considerably more about the mechanisms of actions of dioxin, and this knowledge may permit the

construction of biologically based models that reduce some of the uncertainty in current risk estimates. These approaches and recent advances on mechanisms of tumor promotion and dose-response relationships for biochemical and biological events relevant to the carcinogenic actions of dioxin will be discussed in more detail in Chapter 8, Dose-Response Modeling for 2,3,7,8-TCDD.

6.2. ANIMAL BIOASSAYS FOR CANCER

Several long-term bioassays for carcinogenicity of TCDD have been conducted in several species. All studies have produced positive results. It is clear that TCDD is a multisite carcinogen in both sexes of rats and mice (U.S. EPA, 1985; Huff et al., 1991; Zeise et al., 1990). It also is a carcinogen in the hamster, which is considered the most resistant species to the acute toxic effects of TCDD. The important studies are summarized in Table 6-1, including information on species, sex, and tumor site. The Kociba and National Toxicology Program (NTP) studies are the most comprehensive and relevant to risk characterization and are described in the following paragraphs.

6.2.1. Kociba Study

The most cited cancer bioassay for TCDD was published by Kociba et al. (1978). It was a lifetime feeding study of male and female Sprague-Dawley rats using doses of 0, 1, 10, and 100 ng/kg/day. There were 50 males and 50 females in each group. Data derived from these studies have been used as the basis for many risk assessments for TCDD. The most significant finding was an increase in hepatocellular hyperplastic nodules and hepatocellular carcinomas in female rats. The carcinomas were significantly elevated above the control incidence at the 100 ng/kg/day dose, whereas increased incidences of hyperplastic nodules were evident in the 10 ng/kg/day dose group. There have been two reevaluations of the Kociba slides of liver sections (Squire, 1980; Sauer, 1990). The Squire review was requested by EPA as an independent review of the slides. The Sauer review used diagnostic criteria for liver tumors described by Maronpot et al. (1986). Liver tumor incidences for the three evaluations are compared in Table 6-2. Although there are some quantitative differences in the evaluations, the lowest detectable effect is consistently at 10 ng/kg/day for

Table 6-1. Sites for Increased Cancer in Animal Bioassays

Species/Strain/Study	Sex	Site
Rats/Sprague-Dawley Kociba et al., 1978	male	tongue nasal turbinates/hard palate
	female	lung nasal turbinates/hard palate liver
Rats/Osborne-Mendel NTP, 1982a	male	thyroid adrenal cortex
	female	liver adrenal cortex subcutaneous fibrosarcoma
Mice/B6C3F1 NTP, 1982a	male	liver
	female	subcutaneous fibrosarcoma liver thyroid
Mice/B6C3 and B6C Della Porta et al., 1987	male	thymic lymphomas
	female	liver
Hamsters/Syrian Golden Rao et al., 1988	male	facial skin carcinoma

Table 6-2. Different Evaluations of Kociba Liver Tumor Data in Female Rats[a,b]

Study	Tumor type[c]	Control	Dose (ng/kg/day)		
			1	10	100
Kociba et al., 1978	hyperplastic nodule	8/86 p<0.0001	3/50 p=0.8	18/50 p<0.001	23/50 p<0.001
	hepatocellular carcinoma	1/86 p<0.0001	0/50 --	2/50 p=0.3	11/50 p<0.001
	hyperplastic nodule; hepatocellular carcinoma	9/86 p<0.0001	3/50 p=0.7	20/50 p<0.001	34/50 p<0.001
Squire, 1980	neoplastic nodule; hepatocellular carcinoma	16/68 p<0.0001	8/50 p=0.7	27/50 p<0.001	33/47 p<0.001
Sauer, 1990	hepatocellular adenoma	2/86 p<0.0001	1/50	9/50 p<0.01	14/50 p<0.001
	hepatocellular carcinoma	0/86 p<0.01	0/50	0/50	4/50 p<0.05
	hepatocellular adenoma; hepatocellular carcinoma	2/86 p<0.0001	1/50 --	9/50 p<0.01	18/50 p<0.001

[a]Source: Kociba et al., 1978.

[b]p-Values for Fisher's exact test are given below the incidence data for TCDD-treated animals; Cochran-Armitage trend test p-values are given below the control incidences.

[c]Hyperplastic nodule, neoplastic nodule, and hepatocellular adenoma are equivalent and interchangeable lesions.

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liver tumor incidence. In the 10 ng/kg/day dose group, hyperplastic nodules of the liver were observed in female rats (18 in Kociba, 27 in Squire). Two females had carcinomas of the liver. In the recent reevaluation of liver lesions by Sauer (1990), nine females were identified with hepatocellular adenomas and none with carcinomas; thus only one-third of the previously observed tumors were confirmed. There was no detectable increase in liver tumor incidences in male rats (Table 6-1) in any of the dose groups. The mechanism responsible for dioxin-mediated sex specificity for hepatocarcinogenesis in rats is not clear but may involve estrogens. This is discussed in the section on tumor promotion.

Kociba et al. (1978) had reported that chemically related preneoplastic or neoplastic lesions were not found in the 1 ng/kg/day dose group. However, Squire identified two male rats in the 1 ng/kg/day dose group with squamous cell carcinoma of the nasal turbinates/hard palate, and one of these male rats had a squamous cell carcinoma of the tongue. These are both rare tumors for Sprague-Dawley rats, and these sites are targets for TCDD, implying that the 1 ng/kg/day may not represent a no-observed-effect level (NOEL). However, no dose-response relationships were evident for tumors at these sites (Huff et al., 1991).

In addition to carcinoma of the liver, tongue, nasal turbinates, and hard palate, increased lung tumor incidences were observed in female rats (seven in Kociba, nine in Squire). The increase at the high dose (100 ng/kg/day) was statistically significant for keratinizing squamous cell carcinomas.

One of the more interesting findings in the Kociba bioassay was reduced tumor incidences of the pituitary, uterus, mammary gland, pancreas, and adrenals. For example, carcinomas of the mammary gland occurred in 8 of 86 control female rats, whereas the incidence was 0/49 in the 1 ng/kg/day dose group. However, the incidence of mammary gland carcinomas in the medium- and high-dose groups was similar to that of control rats, suggesting that protection against breast cancer might be a low-dose effect. These findings, coupled with the sex specificity of TCDD-induced liver tumors, emphasize that the carcinogenic actions of TCDD involve a complex interaction of hormonal factors. Moreover, it appears likely that cell-specific factors modulate TCDD/hormone actions relevant to cancer. There is considerable controversy concerning the possibility that TCDD-induced liver tumors are a consequence of cytotoxicity. Goodman and Sauer (1992) have

extended the reevaluation of the Kociba slides to include liver toxicity data and have reported a correlation between the presence of overt hepatotoxicity and the development of hepatocellular neoplasms in female rats. With the exception of two tumors in controls and one each in the low- and mid-dose groups, all liver tumors occurred in livers showing clear signs of toxicity. However, male rat livers exhibit cytotoxicity in response to high TCDD doses, yet they do not develop liver tumors. Moreover, both intact and ovariectomized female rats exhibit liver toxicity in response to TCDD, yet TCDD is a promoter in intact but not ovariectomized rats (Lucier et al., 1991). Therefore, it appears that if cytotoxicity is playing a role in liver tumorigenesis, other factors must also be involved. Also, there is little information on the role of cytotoxicity in TCDD-mediated cancer at other sites such as the lung and thyroid.

6.2.2. NTP Study

The NTP study was conducted using Osborne-Mendel rats and B6C3F1 mice (NTP, 1982a). Groups of 50 male rats, 50 female rats, and 50 male mice received doses of 0, 10, 50, or 500 ng/kg/week TCDD by gavage in two administrations each week for 2 years; groups of 50 female mice were given 0, 40, 200, or 2000 ng/kg/week. These exposures correspond to average daily doses of 1.4, 7.1, or 71 ng/kg/day for rats and male mice and to doses of 5.7, 28.6, or 286 ng/kg/day for female mice, so the doses were roughly similar to those used in the Kociba dietary study. There were no statistically significant dose-related decreases in survival in any sex-species group.

Tumor data in the NTP bioassay are summarized in Tables 6-3 and 6-4. TCDD-induced malignant liver tumors occurred in the high-dose female rats and in male and female mice. These can be considered to result from TCDD exposure because they are relatively uncommon lesions in control Osborne-Mendel rats (male, 1/208; female, 3/208), are seen in female rats and mice of both sexes, and their increasing incidence with increasing dose is statistically significant (Cochran-Armitage trend test, $p=0.004$). Because liver tumors were increased in both sexes of mice, this effect is not female specific as observed in rats. Interestingly, liver tumor incidences were decreased in female rats in both the NTP and Kociba low doses (not statistically significant compared with controls). For example, the

Table 6-3. Tumor Incidences in Male and Female Osborne-Mendel Rats Given TCDD by Gavage for 2 Years[a,b]

Target organ/tumor type	Sex	Dose (ng/kg/day)			
		0	1.4	7.1	71
Thyroid follicular cell adenoma	males	1/69 p=0.006	5/48 p=0.042	6/50 p=0.021	10/50 p=0.001
Liver neoplastic nodule		0/74 p=0.005	0/50 --	0/50 --	3/50 p=0.06
Adrenal cortex adenoma		6/72 p=0.26	9/50 p=0.09	12/49 p=0.015	9/49 p=0.09
Liver neoplastic nodule	females	5/75 p<0.001	1/49 --	3/50 --	12/49 p=0.006
Adrenal cortex adenoma or carcinoma		11/73 p=0.014	9/49 p=0.4	5/49 --	14/46 p=0.039
Subcutaneous fibrosarcoma		0/75 --	2/50 p=0.16	3/50 p=0.06	4/49 p=0.023

[a]Source: NTP, 1982a.

[b]p-Values under the tumor incidence data of controls are from Cochran-Armitage test for dose-related trend, and p-values under TCDD-treated groups are from Fisher's exact test.

Table 6-4. Tumor Incidences in Male and Female B6C3F1 Mice Given TCDD by Gavage for 2 Years[a,b]

Target organ/tumor type	Sex	Dose (ng/kg/day)			
		0	1.4	7.1	71
Liver	male				
carcinoma		8/73 p=0.002	9/49 p=0.19	8/49 p=0.28	17/50 p=0.002
adenoma		7/73 p=0.024	3/49 --	5/49 p=0.6	10/50 p=0.09
Lung adenoma or carcinoma		10/71 p=0.004	2/48 --	4/48 --	13/50 p=0.08
Subcutaneous fibrosarcoma	female	1/74 p=0.007	1/50 p=0.6	1/48 p=0.6	5/47 p=0.032
Liver					
carcinoma		1/73 p=0.008	2/50 p=0.4	2/48 p=0.4	6/47 p=0.014
adenoma		2/73 p=0.11	4/50 p=0.2	4/48 p=0.2	5/47 p=0.08
Thyroid follicular cell adenoma		0/69 p=0.016	3/50 p=0.07	1/47 p=0.4	5/46 p=0.009
Lymphoma		18/74 p=0.011	11/50 --	13/48 p=0.4	20/47 p=0.029

[a]Source: NTP, 1982a.

[b]p-Values for controls represent Cochran-Armitage trend test, and p-values for TCDD-treated groups are derived from Fisher's exact test.

combined control incidence data were 11/161 (7%) compared with 4/99 (4%) in the low-dose group.

The incidences of thyroid gland (follicular cell) tumors were increased in all three dose groups in male rats. Because the responses in the two highest dose groups are highly significant, the statistically significant elevation of incidence in the lowest dose group (Fisher exact p-value=0.042) is considered to be caused by exposure to TCDD. Thus, for this study the lowest-observed-effect level (LOEL) is 1.4 ng/kg/day and a NOEL was not achieved within the specified dose range, suggesting that thyroid tumor incidence may be the most sensitive site for TCDD-mediated carcinogenesis. Because 70 ng/kg/day is above the maximum-tolerated dose (MTD) (Huff et al., 1991), thyroid tumors occur at doses more than 50 times lower than the MTD.

TCDD-induced neoplasms of the adrenal gland were observed in the 7.1 ng/kg/day/dose group in male rats and in high-dose female rats. Fibrosarcomas of the subcutaneous tissue were significantly elevated in high-dose female mice and female rats. One additional tumor type, lymphoma, was seen in high-dose female mice. Lung tumors were elevated in high-dose female mice; the increase was not statistically significant when compared with concurrent controls, but the increase was dose related (Cochran-Armitage trend test, p=0.004).

Therefore, TCDD is a multisite complete carcinogen (Huff, 1992) and induced neoplasms in rats and mice of both sexes. As in Kociba et al. (1978), liver tumors were observed with greater frequency in treated female rats, but in male rats, the thyroid appears to be the most sensitive (increased tumor incidence at doses as low as 1.4 ng/kg/day).

6.2.3. Syrian Golden Hamster

Groups of 10 to 24 male Syrian Golden hamsters were given two to six intraperitoneal or subcutaneous injections of TCDD over a 4-week period at doses of 0, 50, or 100 μg/kg TCDD in dioxane (Rao et al., 1988). The experiments were terminated after 12 to 13 months. The 100 μg/kg groups (total dose of 600 μg/kg) from both injection routes developed squamous cell carcinomas of the skin in the facial region: 4/18 (22%) from the intraperitoneal injection and 3/14 (21%) from the subcutaneous injection. The lesions were large (1.5 to 3 cm) with extensive necrosis, and some metastasized to the lung. The earliest

neoplasms were detectable 8 months after the initial injection. Similar lesions were not seen in hamsters receiving two intraperitoneal injections of 100 μg/kg TCDD or six subcutaneous injections of dioxane vehicle, and none have been reported over the past 10 years in this laboratory. An extensive study by Pour et al. (1976) identified only one skin papilloma in 533 control Syrian hamsters. This report demonstrates that the hamster, a nonresponsive species (for acute toxic effects), is susceptible to the carcinogenic actions of TCDD at doses well below the maximum-tolerated dose.

6.2.4. B6C3 and B6C Mice

In a study by Della Porta et al. (1987), TCDD was administered intraperitoneally in corn oil at doses of 0, 1, 30, and 60 μg/kg to groups of 89 to 186 B6C3 and B6C mice of both sexes once weekly for 5 weeks starting at day 10 of life, and the animals were observed until 78 weeks of age. Histopathological observations were limited to the liver, kidney, and organs with apparent or suspected pathological changes. Thymic lymphomas were induced at the 60 μg/kg level in both sexes of both hybrids and at 30 μg/kg in all but female B6C3 mice. Neoplasms of the liver occurred in male B6C3 mice at 30 μg/kg and female B6C3 mice at 60 μg/kg. In a separate experiment, groups of 42 to 50 B6C3 mice were exposed to 0, 2.5, and 5.0 μg/kg TCDD in corn oil by gavage once weekly for 52 weeks starting at 6 weeks of age. The study was stopped at 110 weeks. Increased incidences of liver tumors were related to TCDD exposure at both dose levels.

In summary, there is convincing evidence in the scientific literature that TCDD is a potent multisite carcinogen in both sexes of several species, and carcinogenic effects have been observed at doses that are orders of magnitude less than the maximum-tolerated dose.

6.2.5. Carcinogenicity of Related Compounds

A mixture of two isomers of hexachlorodibenzo-*p*-dioxin (HCDD) (1,2,3,6,7,8 and 1,2,3,7,8,9) was given by gavage twice weekly for 2 years to Osborne-Mendel rats and B6C3F1 mice (NTP, 1980). The doses of HCDD were 0, 1.25, 2.5, or 5 μg/kg/week in rats and male mice. Doses for female mice were 0, 2.5, 5, and 10 μg/kg/week. There was no effect of administration of HCDD on survival of either sex of rats or mice (NTP, 1980).

Results revealed that HCDD increased liver tumors in both sexes of rats and mice although female rats seemed to be more sensitive than male rats (significant increases detected in female rats in the 1.25 μg/kg/week dose group, equivalent to 180 ng/kg/day). Therefore, HCDD is approximately 1/20 as potent a liver carcinogen as TCDD.

Dermal applications of the same HCDD mixture as described above (NTP, 1982b) were given to Swiss Webster mice for 104 weeks (three weekly). For the first 16 weeks, doses of 5 ng/application were used. Thereafter, doses of 10 ng/application were used. No HCDD-exposure-related carcinogenic responses were noted.

Dibenzo-*p*-dioxin given in the diet for 2 years at concentrations of 0, 5,000, and 10,000 ppm did not increase carcinogenic responses in Osborne-Mendel rats or B6C3F1 mice (NCI, 1979a). 2,7-Dichlorodibenzo-*p*-dioxin (DCDD) in the diet of Osborne-Mendel rats for 110 weeks or B6C3F1 mice for 90 weeks at levels of 0, 5,000, or 10,000 ppm did not increase neoplasms in male or female rats or in female mice. In male mice, increased incidences of lymphoma or hemangiosarcoma were observed in the low-dose group and neoplasms of the liver were observed in both dose groups (NCI, 1979b). The more highly chlorinated dibenzo-*p*-dioxins (CDDs) and dibenzofurans (CDFs) have not been studied in long-term animal cancer bioassays. Many of the CDDs and CDFs bioaccumulate and exhibit toxicities similar to those of TCDD and are considered to be carcinogens (EPA Science Advisory Board, 1989).

6.3. MECHANISMS OF TCDD CARCINOGENICITY

There is substantial evidence that TCDD is not a direct genotoxic agent. Because "genotoxic" and "nongenotoxic" are controversial and often misused terms, it is prudent to describe accurately the scientific criteria used to call a chemical "genotoxic" or "nongenotoxic" (IARC, 1992). Some of the criteria for designating TCDD a nongenotoxic agent are that it does not bind covalently to DNA (does not form DNA adducts). Although one study detected radioactivity associated with crude DNA preparations after in vivo exposure, no study that has rigorously looked for TCDD-DNA adducts has been positive. TCDD is negative in short-term tests for genotoxicity and is a potent promoter and weak initiator in multistage models for chemical carcinogenesis. In a recent study (Turteltaub et

al., 1990) using accelerator mass spectrometry, DNA adducts were not detected in rodent tissue following exposure to TCDD. This method is extraordinarily sensitive, being capable of detecting one adduct in 10^{12} normal nucleotides. Randerath et al. (1988) were unable to detect TCDD-related DNA adducts by the sensitive ^{32}P postlabeling method (limit of detection of one adduct in 10^9 normal nucleotides). For comparison, approximately one adduct in 10^6 normal nucleotides is found in rodent tissues following carcinogenic doses of benzo(a)pyrene (7,8-diol-9,10 epoxide deoxyguanosine DNA adduct) or methylnitrosourea (0^6 methylguanine).

Another criterion for designating TCDD a "nongenotoxic carcinogen" is that numerous studies have demonstrated that TCDD is negative in the *Salmonella*/Ames test in the presence or absence of a mixed-function oxidase (MFO) activating system. These negative studies have encompassed 13 different bacterial strains with tests performed in 9 laboratories (Wassom et al., 1977; Kociba, 1984; IARC, 1982; Giri, 1987; Shu et al., 1987). Using its battery of tests for genetic toxicity, the NTP (1984) concluded that TCDD was nonmutagenic. Additionally, several scientific panels have stated that false negatives for TCDD genetic toxicity are highly unlikely (EPA Science Advisory Board, 1984). TCDD has been found to promote the transformation of C3H/10T1/2 cells; it was concluded that this response did not reflect TCDD's ability to directly damage DNA (Abernethy et al., 1985). In human populations accidentally or occupationally exposed to TCDD, there is no consistent evidence for increased frequencies of chromosomal aberrations in workers exposed to TCDD (Shu et al., 1987).

Although TCDD is negative in genetic toxicity tests, recent reports have demonstrated that high doses of TCDD (50 to 100 μg/kg) induce single-strand breaks in Sprague-Dawley rats, presumably as a consequence of increased lipid peroxidation (Wahba et al., 1988, 1989). In another set of studies, increased frequency of sister chromatid exchanges was observed in lymphocytes of people exposed to pentachlorinated dibenzo-*p*-dioxins (PCDFs) in Taiwan when those lymphocytes were challenged with α-naphthoflavone (Lundgren et al., 1986, 1988). The possible mechanism responsible for this effect is that the PCDFs cause increased rates of metabolic activation of α-naphthoflavone to DNA reactive metabolites (Lundgren et al., 1987). These findings are consistent with the idea that TCDD's ability to

induce drug-metabolizing enzymes (CYP1A1 and 1A2) may lead to an increased rate of formation of DNA reactive metabolites of some carcinogens, most notably the polycyclic aromatic hydrocarbons (PAHs) and aromatic amines. However, there is evidence that the opposite effect occurs in some cases because in vivo exposure to CYP1A1 inducers actually leads to a decrease in DNA adducts in target tissue following in vivo exposure to PAHs such as benzo(a)pyrene (Cohen et al., 1979; Parkinson and Hurwitz, 1991). It can reasonably be concluded that TCDD exposure may increase the rate of DNA adduct formation for some carcinogens but decreases the rate for others and that predictions should not be made without experimental data on DNA adduct concentrations in control and TCDD-treated animals.

A final criterion for designating TCDD a nongenotoxic carcinogen is that it is a potent tumor promoter and a weak initiator or noninitiator in two-stage models for liver (Pitot et al., 1980; Graham et al., 1988; Lucier et al., 1991; Clark et al., 1991a; Flodstrom and Ahlborg, 1991) and skin (Poland et al., 1982). These findings are discussed in more detail in the section on tumor promotion, including plausible mechanisms for the tumor-promoting actions of TCDD such as TCDD-mediated increases in cell proliferation rates of genetically altered cells. However, a recent report by Yang et al. (1992) has demonstrated that immortalized human keratinocytes cultured with TCDD were neoplastically transformed, as evidenced by tumorigenic activity of those cells in nude mice. This response is characteristic of genotoxic carcinogens and occurred at a low TCDD concentration (0.1 nm). For comparison, induction of CYP1A2 in these same cells was not detected until a dose of 3 nm was used (Yang et al., 1992).

It is now accepted by the scientific community that most if not all of TCDD's toxic and biochemical effects, including tumor promotion, are Ah receptor dependent and that TCDD provides an example for evaluating the issues relevant to risk assessment for receptor-mediated carcinogens. The steps involved in Ah receptor-mediated events are reviewed in Chapter 2, Mechanism(s) of Action.

6.4. INITIATION/PROMOTION STUDIES

The multistage nature of chemical carcinogenesis is being defined by an increasing understanding of the discrete steps required to produce a genetically altered cell that is

clonally expanded and ultimately progresses to a tumor (IARC, 1992; Barrett and Wiseman, 1987; Swenberg et al., 1987; Barrett, 1992) (Figure 6-1). Briefly, the process involves damage to a specific site on DNA, a round of cell replication to fix that damage into the genome, clonal expansion of the genetically altered cells (tumor promotion), followed by additional genetic damage and rounds of cell replication (tumor progression). Figure 6-1 schematizes the multistage nature of cancer. The birth and death rates of genetically altered cells compared with normal cells are the centerpiece of risk assessment models that recognize the multistage nature of chemical carcinogenesis (Moolgavkar and Knudson, 1981; Portier, 1987). The roles of protooncogene activation and tumor suppression gene inactivation have provided clues in attempts to discern discrete steps in carcinogenesis. It is also clear that cell proliferation is an essential component of chemical carcinogenesis, for without it DNA damage would not be fixed into the genome and clonal expansion of genetically altered cells would not occur.

Concurrent with our increased understanding of the mechanistic underpinnings of chemical carcinogenesis, multistage models have been developed to identify the particular stage or stages in which carcinogens act to increase tumor incidence. There is a wealth of information on liver initiation/promotion protocols in the scientific literature (Pitot and Sirica, 1980; Farber, 1984; Pitot and Campbell, 1987). These protocols frequently employ a single initiating dose of a chemical that damages DNA, followed by enhancement of cell replication (partial hepatectomy or cytotoxicity) to fix that damage into the genome (initiation), and then chronic exposure to a chemical that produces clonal expansion of the genetically altered cells (promotion). Increased tumor incidence is produced by chemicals that act at either stage. It is important to note that "initiation" and "promotion" are operational and not mechanistic terms because both stages are likely to be composed of multiple steps, and the mechanisms are not mutually exclusive. Nevertheless, the protocols have provided valuable information in our attempts to understand chemical carcinogenesis. Detailed descriptions of initiation/promotion protocols in liver and skin are provided elsewhere (Pitot and Campbell, 1987; Dragan et al., 1991; Pitot et al., 1987; Farber, 1984; Slaga et al., 1982; Peraino et al., 1981; Ito et al., 1980).

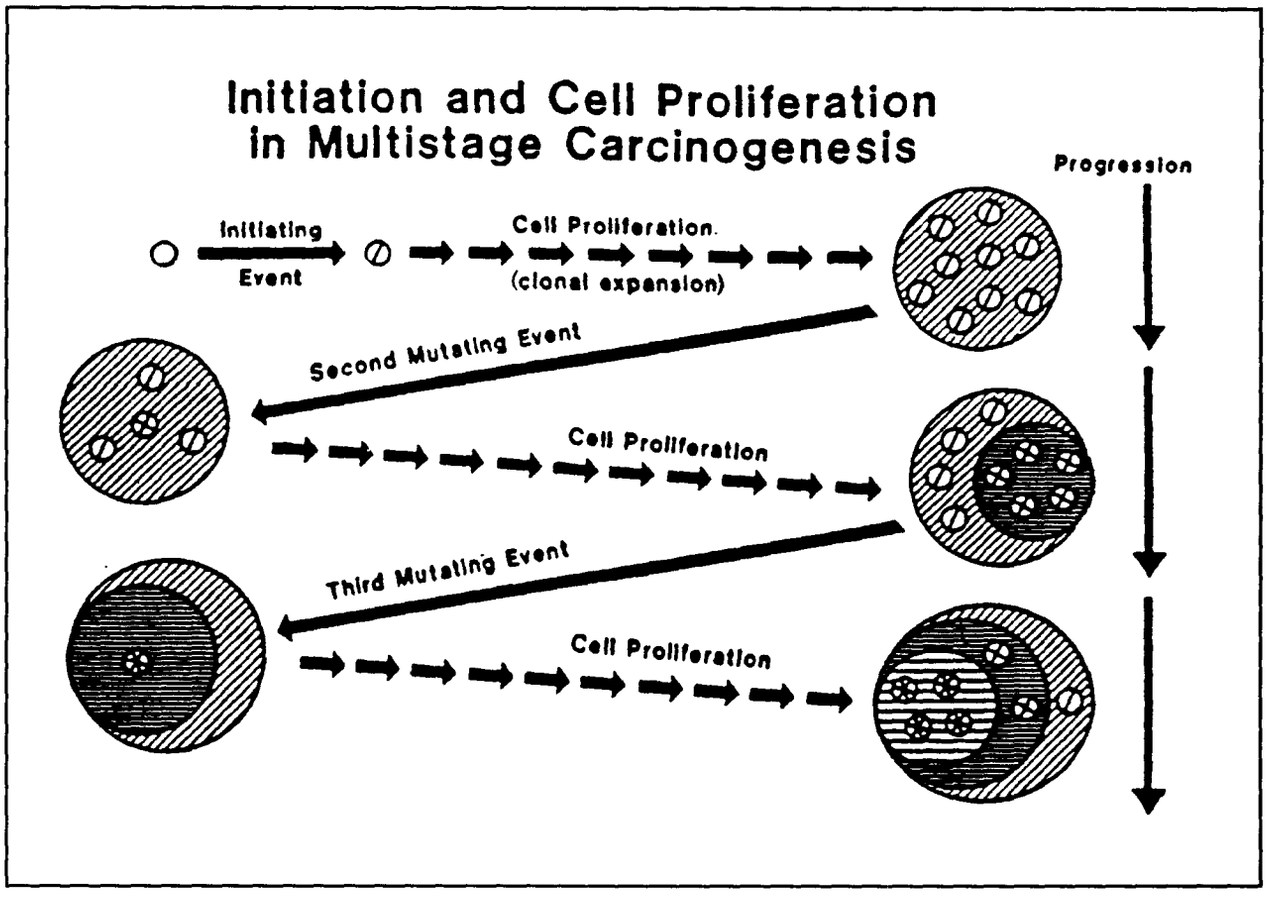

Figure 6-1. Schematic representation of multistep carcinogenesis including the roles of genetic damage and cell proliferation. It is important to note that several DNA-damaging steps and several cell proliferation steps are likely to be involved during the complete process of chemical carcinogenesis.

Source: Swenberg et al., 1987.

6.4.1. Two-Stage Models in Rat Liver

Pitot et al. (1980) reported that TCDD was a potent liver tumor promoter when rats were initiated with a single dose of diethylnitrosamine (DEN) followed by chronic TCDD exposure (0.14 and 1.4 μg/kg subcutaneously once every 2 weeks for 7 months). These doses are equivalent to 10 and 100 ng TCDD/kg/day (the medium and high dose in the Kociba bioassay). Histological evaluation revealed that five of seven animals that had received DEN and the high TCDD dose had hepatocellular carcinomas. No liver tumors were evident in rats receiving DEN only, DEN/low-dose TCDD, or TCDD only (high or low dose). Enzyme-altered foci (EOF) in liver also were evaluated in this study, and these are considered to represent preneoplastic lesions because increases in EOF are associated with liver cancer in rodents (Maronpot et al., 1989; Popp and Goldsworthy, 1989; Pitot et al., 1989; Williams, 1989). The EOF data were consistent with the tumor data in that a large proportion of the liver was occupied by preneoplastic lesions (43%) in animals receiving DEN and the high dose of TCDD. A much smaller proportion of the liver was occupied by EOF in the other groups. This work provides strong evidence that TCDD is a potent tumor promoter in liver.

A second set of studies (Graham et al., 1988; Lucier et al., 1991; Clark et al., 1991a; Dragan et al., 1992) have confirmed and extended Pitot's findings, including data on the mechanistic basis for TCDD's tumor-promoting effects in rat liver. These studies also used DEN as the initiator and have demonstrated that TCDD's liver tumor-promoting actions are ovarian dependent. This finding is consistent with 2-year bioassays showing that TCDD is a hepatocarcinogen in female rats but not in male rats. In the tumor-promoting studies (Graham et al., 1988; Lucier et al., 1991), DEN was used as the initiating agent and TCDD (biweekly doses of 1.4 μg TCDD/kg, equivalent to 100 ng/kg/day for 30 weeks) was used as the promoter. There were four groups of intact female rats (controls, TCDD only, DEN only, and DEN/TCDD). The same four groups were used following ovariectomy. Data revealed that TCDD was a much weaker liver tumor promoter in ovariectomized rats (Table 6-5). For example, there were 387 gamma glutamyl transpeptidase (GGT) foci/cm^3 in intact rats compared with 80 in ovariectomized rats in the DEN/TCDD groups. Corresponding differences were evident in the proportion of liver occupied by GGT foci; 0.37% in

Table 6-5. Preneoplastic Foci and Cell Proliferation After 30 Weeks of TCDD as Tumor Promoter[a]

	S/C[b]	S/TCDD	DEN/C	DEN/TCDD
GGT + foci/cm^3				
intact	6	5	44	387[c]
ovariectomized	0	0	30	80
GGT + foci (vol. fraction)[c]				
intact	0.01	0.01	0.03	0.37[c]
ovariectomized	0	0	0.03	0.08
BrdU-labeling index[d]				
intact	0.3[c]	6.0[c]	0.8	7.3[c]
ovariectomized	1.1	1.0	1.1	0.7

[a]Source: Clark et al., 1991a.
[b]S/C = Controls; S/TCDD = TCDD only; DEN/C = DEN only, no TCDD; DEN/TCDD = DEN initiated and TCDD promoted.
[c]Significantly different from ovariectomized.
[d]Percentage of hepatocytes undergoing replicative DNA synthesis in 1 week following 30 weeks of TCDD exposure.

DEN/TCDD intact rats compared with 0.08% in DEN/TCDD ovariectomized rats. Few or no foci were found in the control or TCDD-only groups. Placental glutathione transferase (PGT) is being used increasingly as a phenotypic marker of enzyme-altered foci (Ito et al., 1989), and results with this marker of preneoplasia were similar to those for GGT in that ovariectomy protected against the liver tumor-promoting actions of TCDD.

The influence of ovariectomy on liver tumor incidence was evaluated in a parallel experiment using the same treatment groups in which TCDD was administered for 60 weeks. In the intact DEN/TCDD rats, liver tumor incidence was 13/37, with a total of 32 tumors compared with 7/39 (11 total tumors) in DEN/TCDD ovariectomized rats. Both hepatocellular adenomas and carcinomas were evident, along with a smaller incidence of hepatocholangiomas and hepatocholangiocarcinomas.

The mechanisms responsible for the protective effect of ovariectomy are not clear, but ovarian influences on liver TCDD retention do not seem to be involved; liver TCDD concentrations were ~20 ppb in both intact and ovariectomized rats (Lucier et al., 1991), which is similar to liver concentrations reported by Kociba et al. (1978) using the same dose of TCDD (100 ng/kg/day) but for 2 years rather than 60 weeks. One plausible mechanism may be related to cell proliferation: TCDD did not stimulate cell proliferation rates in ovariectomized rats whereas a mean increase of tenfold was apparent in intact rats receiving 100 ng TCDD/kg for 30 weeks (Table 6-5) (Lucier et al., 1991). There was considerable interindividual variation in both cell proliferation rates and enzyme-altered foci in the DEN/TCDD groups. Comparisons of the two data sets revealed a strong positive correlation between enzyme-altered foci and cell proliferation, although the importance of this finding is diminished by the fact that cell proliferation was quantified in nonlesioned hepatocytes. The mechanism whereby ovarian hormones and TCDD interact to produce cell proliferation in hepatocytes may involve growth factor pathways. Consistent with this idea, TCDD produced a loss of plasma membrane epidermal growth factor (EGF) receptor in intact rats but not ovariectomized rats (Clark et al., 1991a). EGF is thought to provide a mitogenic stimulus in hepatocytes and play a key role in hepatocarcinogenesis (Vickers and Lucier, 1991; Velu, 1990; Shi and Yager, 1989; Eckl et al., 1988). A schematic representation of a plausible

mechanism for the role of estrogen in TCDD-mediated liver cancer in rats is given in Figure 6-2.

Another possible mechanism for the influence of the ovaries is that TCDD induces cytochrome P-4501A2, which could lead to DNA reactive metabolites of 17β-estradiol, the naturally occurring estrogen. P-4501A2 catalyzes the formation of catechol estrogens that are considered by some to be DNA reactive precursors (Metzler, 1984; Li and Li, 1990).

The chlorinated dibenzofurans and other chlorinated dibenzo-*p*-dioxins are also liver tumor promoters. In a recent study (Flodstrom and Ahlborg, 1992), enzyme-altered foci were increased in female Sprague-Dawley rat livers by an initiating dose of DEN followed by TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin, or 2,3,4,7,8-pentachlorodibenzofuran used as the promoting agent. Comparative potencies indicated that the two CDDs were nearly equipotent and the PCDF was about 1/10th as potent as TCDD. These results are consistent with the idea that the hepatocarcinogenic actions of TCDD and its structural analogs are Ah receptor dependent.

6.4.2. Rat Lung

Because the lung and respiratory tract seem to be target sites for TCDD carcinogenesis in humans (Fingerhut et al., 1991), it is of interest to evaluate whether TCDD is a tumor promoter in rodent lung. The only published report on lung tumors used DEN as the initiating agent and TCDD (100 ng/kg/day for 60 weeks) as the promoting agent (Clark et al., 1991a). Both intact and ovariectomized rats were used, and the results were surprising. In contrast to liver tumor promotion, lung tumors were seen only in DEN/TCDD ovariectomized rats (4/37). No lung tumors were present in DEN/TCDD intact rats, in DEN only/TCDD only, or in control rats with or without ovariectomy. The background incidence of lung tumors in rats is very low so the lack of tumors in controls was not unexpected (Haseman et al., 1984). The four tumors in DEN/TCDD intact rats were composed of two squamous cell carcinomas and two adenocarcinomas.

The rodent tumorigenicity data provide clues to the complex hormonal interactions that produce site-specific carcinogenic actions of TCDD. Liver tumors are ovarian dependent, whereas the ovaries appear to protect against TCDD-mediated tumor promotion in lung.

POSSIBLE SEQUENCE OF EVENTS INVOLVED IN ESTROGEN-DEPENDENT TCDD PROMOTION OF LIVER TUMORS

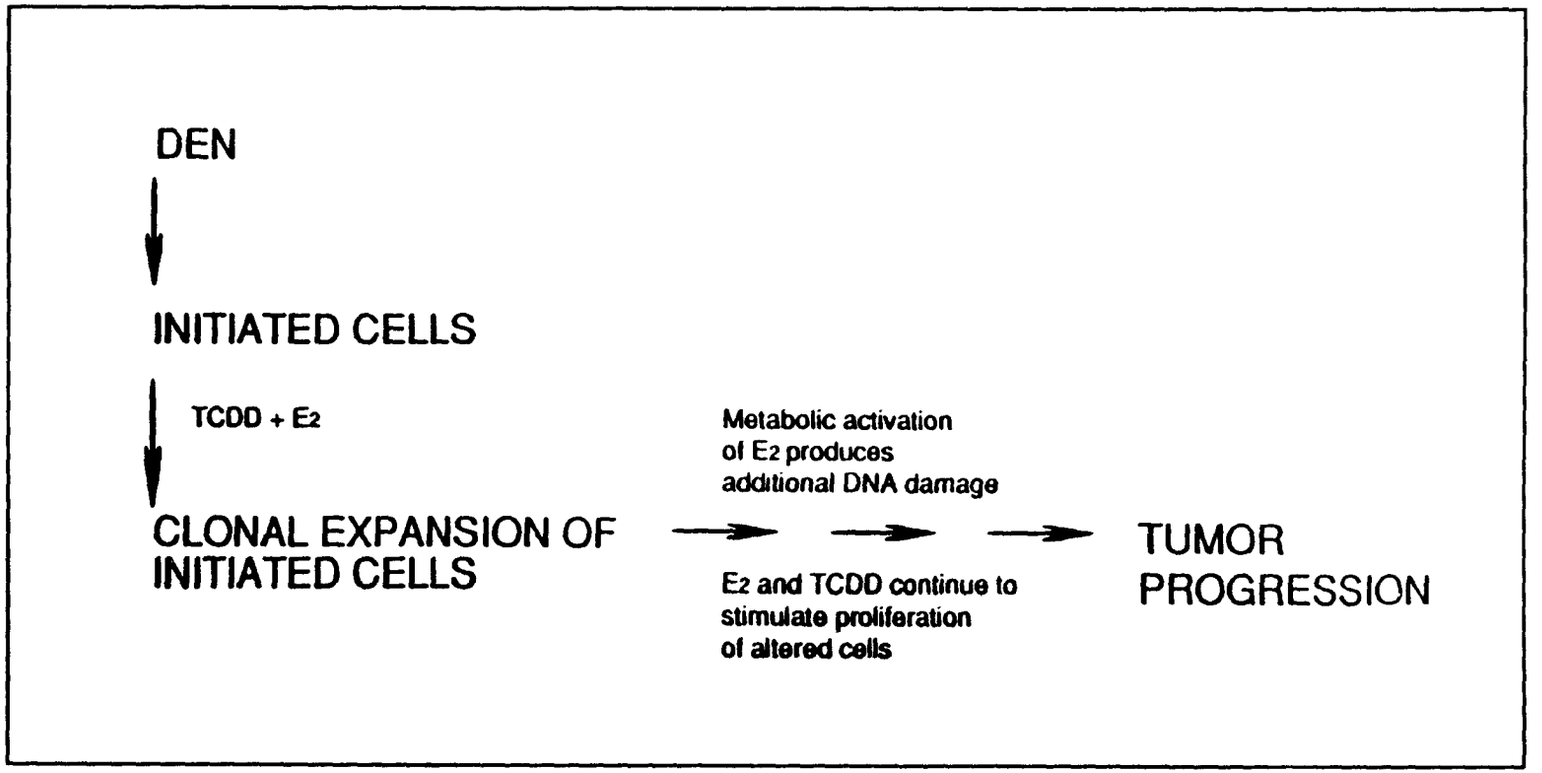

Figure 6-2. Operational model of TCDD/estrogen interactions relative to tumor promotion in a two-stage model of hepatocarcinogenesis. Clonal expansion of initiated cells may reflect stimulation of mitogenesis through receptor-mediated events involving epidermal growth factor receptor, estrogen receptor, and the Ah receptor.

Source: Vickers and Lucier, 1991.

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Therefore, the rat tumor data are of interest because recent epidemiologic studies (Chapter 7) have shown that TCDD exposure is associated with an increase in respiratory tract tumors.

6.4.3. Mouse Skin

Initiation/promotion studies on skin have demonstrated that TCDD is a potent tumor promoter in mouse skin as well as rat liver. Poland et al. (1982) administered a single dermal initiating dose of *N*-methyl-*N*-nitrosoguanidine (MNNG) to hairless mice followed by twice weekly doses of TCDD (3.75, 7.5, 15, or 30 ng) or TPA (1 or 3 μg) for 20 weeks. TCDD promoted the development of papillomas at all doses, and the response was dose dependent (100% of the animals had tumors in the high-dose TCDD group). Control animals or animals receiving MNNG or TCDD only exhibited only a low incidence of tumors. These studies demonstrate that TCDD is at least two orders of magnitude more potent an agent than tetradecanoyl phorbol acetate (TPA) in mouse skin (Poland et al., 1982). Based on structure activity and genetic studies, it appears that the skin tumor-promoting actions of TCDD are Ah receptor dependent. Moreover, tumorigenic responses segregate with the hr locus, and biochemical responses such as CYP1A1 induction can occur without carcinogenesis (Poland and Knutson, 1982; Poland et al., 1982).

Other studies have tested TCDD as an initiator and TPA as a promoter in CD-1 mice (DiGiovanni et al., 1977). Results revealed that TCDD had weak or no initiating activity in this system. To better understand the possible influence of TCDD-mediated induction of cytochrome P-450 on the carcinogenicity of PAHs, TCDD was coadministered with benzo(a)pyrene or dimethylbenzanthracene to mice followed by promotion with TPA (Cohen et al., 1979). Results revealed that TCDD decreased tumor incidence of both PAHs compared with controls. However, coadministration of TCDD with 3-methylcholanthrene to mice produced tumor incidences similar to those produced by 3-methylcholanthrene alone (Kouri et al., 1978). These results are consistent with the findings that TCDD induction of drug-metabolizing enzymes is associated with both metabolic activation as well as deactivation of PAHs (Lucier et al., 1979).

The relative toxicity and tumor-promoting capacity of two CDFs (2,3,4,7,8-CDF and 1,2,3,4,7,8-CDF) have been investigated in hairless mice (Hebert et al., 1990). These

studies used a treatment protocol similar to that of Poland et al. (1982), including the use of MNNG as the initiating agent and varying doses of TCDD, 2,3,4,7,8-CDF, or 1,2,3,4,7,8-CDF for 20 weeks. Proliferative lesions (squamous cell papilloma, squamous cell carcinoma, or hyperproliferative nodules) were quantified. Results demonstrated that 2,3,4,7,8-CDF was 0.2 to 0.4 times as potent as TCDD and that 1,2,3,4,7,8-CDF was 0.08 to 0.16 times as potent as TCDD. These data suggest that the tumor-promoting potencies of structural analogs of TCDD, like the promotion of liver tumors, reflect relative binding properties to the Ah receptor. However, this is an effect of chronic exposure; therefore, rates of metabolism/clearance would obviously affect correlations between Ah receptor binding and tumor promotion.

Taken together, results on initiation/promotion protocols indicate that TCDD is an extraordinarily potent promoter of liver and skin tumors (Pitot et al., 1987), and the results provide strong evidence that the carcinogenic actions are Ah receptor mediated. A summary of studies on tumor promotion by TCDD or the polychlorinated dibenzofurans is given in Table 6-6. Plausible mechanisms of actions responsible for the tumor-promoting actions of TCDD and the impact of these mechanisms on dose-response relationships are presented in the next section.

6.5. BIOCHEMICAL RESPONSES

The list of biochemical effects produced by TCDD in humans, experimental animals, and cell systems is expanding. These effects include those that may alter normal cell regulatory processes such as cell proliferation and differentiation, metabolic capacity, and hormonal pathways. This section on biochemical responses will summarize some of the changes produced by TCDD, including discussion of (a) possible relevance of the response to TCDD-mediated cancer, (b) whether the response is Ah receptor mediated, (c) whether information is available on the role of transcriptional activation, (d) dose-response relationships, and (e) whether animal models are consistent with human responses. This chapter will not attempt to evaluate all of the biochemical and molecular responses to TCDD but will focus on the ones that are either the most relevant to carcinogenic responses or have received the most study. The responses selected for evaluation are cytochrome P-4501A1

Table 6-6. Summary of Positive Tumor-Promoting Studies on TCDD and CDFs

Species/Sex	Initiator	Promoter	Site	Reference
Rat/female	DEN	TCDD	Liver	Pitot et al., 1980
Rat/female	DEN	TCDD	Liver	Graham et al., 1988
Rat/female	DEN	TCDD	Liver	Lucier et al., 1991
Rat/female	DEN	TCDD	Liver	Clark et al., 1991a
Rat/female	DEN	TCDD	Liver	Flodstrom and Ahlborg, 1991
Rat/female	DEN	TCDD	Liver	Flodstrom and Ahlborg, 1992
Rat/female	DEN	PCDFs	Liver	Flodstrom and Ahlborg, 1992
Rat/female	DEN	TCDD	Liver	Dragan et al., 1992
Rat/female	DEN	TCDD	Liver	Lucier et al., 1992
Mice/female hairless	MNNG	TCDD	Skin	Poland et al., 1982
Mice/female hairless	MNNG	PCDFs	Skin	Hebert et al., 1990
Rat/female (ovariectomized)	DEN	TCDD	Lung	Clark et al., 1991a

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(CYP1A1), cytochrome P-4501A2 (CYP1A2), EGFR, estrogen receptor (ER), and UDP-glucuronosyltransferase (UDPGT). Table 6-7 lists many of the biochemical changes produced by TCDD in in vivo and/or in vitro systems and some information on mechanisms of action.

6.5.1. CYP1A1 and 1A2

The most studied response to TCDD has been induction of cytochrome P-450 isozymes (Whitlock, 1990; Silbergeld and Gasiewicz, 1989; Poland and Knutson, 1982). The first reports of P-450 induction in vivo and in vitro appeared in 1973 (Lucier et al., 1973; Greig and DeMatteis, 1973; Poland and Glover, 1973), and hundreds of papers have been published on the subject since that time. These papers have dealt with various aspects of TCDD-mediated induction of P-450, such as isozyme specificity, time course, structure-activity relationships, molecular mechanisms of transcriptional activation of the CYP1A1 gene, identification of transcriptional activating factors, tissue and cell specificity, and dose-response relationships. The molecular mechanisms responsible for enzyme induction are described in the chapter by Whitlock in this volume.

The mechanistic relationship of CYP1A1 and 1A2 induction to cancer or any other toxic end point following dioxin exposure has not been demonstrated, yet considerable controversy exists on this subject (Roberts, 1991). Since CYP1A1 functions to catalyze the metabolic activation of many chemicals such as the polycyclic aromatic hydrocarbons to DNA reactive metabolites, it has been postulated that induction of CYP1A1 might enhance the carcinogenic actions from a given exposure level to many PAHs. Usually, however, preinduction of CYP1A1 diminishes the carcinogenic potency of PAHs such as 3-methylcholanthrene, benzo(a)pyrene, and 7,2-dimethylbenzanthracene if exposure to the inducing agent is short term (Parkinson and Hurwitz, 1991; Wattenberg, 1985; Cohen et al., 1979; Wattenberg, 1978; Miller et al., 1958). Induction also protects against the carcinogenic actions of aflatoxin, diethylnitrosamine, arylamines, and urethane. Protection occurs at numerous cancer sites including liver and lung. Several lines of evidence support the idea that enzyme induction is the mechanism responsible for the protective effect. First, treatment of mice, deficient in the Ah receptor, with inducers does not protect against PAH-mediated cancer

Table 6-7. Classification of Members of the Ah Gene Battery[a]

Class	Gene/Product	Secreted protein
Activation of gene transcription; Ah receptor-mediated	CYP1A1, Cytochrome P1450	-
	Gst-Ya, glutathione S-transferase	-
	Nmo-1, menadione oxidoreductase	-
Activation of gene transcription; AhR agonist-mediated	Clone 1, unknown gene	?
	CYP1A2, cytochrome P2450	-
	PAI-2, plasminogen activator inhibitor-2	+
	T-ALDH, aldehyde deydrogenase	-
Induction of mRNA levels; AhR agonist-mediated	Clone 141, unknown gene	?
	c-erb A related, hormone receptor	-
	GST-Yb, glutathione S-transferase	-
	GST-Yc	-
	αhCG, human chorionic gonadotropin	+
	IL-β, interleukin-1β	+
	MDR-1, multidrug resistance	-
	Testosterone 7 α-hydroxylase	-
	TGF-α, transforming growth factor-α	+
Induction of enzyme activity; Ah receptor-mediated	ODC, ornithine decarboxylase	-
	Ugt-1, UDP-glucuronyl transferase	-
	EGFR, epidermal growth factor receptor	-
	ER, estrogen receptor	-
	Gastrin	+
	TNF-α, tumor necrosis factor-α	+
Induction of enzyme activity; AhR agonist-mediated	ALAS, δ-aminolevulinic acid synthetase	-
	Aryl hydrocarbon-binding protein	-
	Choline kinase	-
	60-kd microsomal esterase	-
	Malic enzyme	-
	Phospholipase A_2	-
	Protein kinase C	-
	Enzyme $pp60^{c\text{-}src}$, tyrosine kinase	-

[a]Source: Sutter and Greenlee, 1992.

(Kouri et al., 1978). Second, the ability of inducing agents to protect against cancer is positively correlated with their potency as inducing agents (Wattenberg and Leong, 1970; Arcos et al., 1961). Third, the inducing agent must be administered at least 1 day prior to treatment, which allows sufficient time for the inducer to produce elevated levels of CYP1A1 (Parkinson et al., 1983; Wheatley, 1968).

The most probable mechanism responsible for the protective effect of enzyme induction is that it leads to decreased concentrations of promutagenic DNA adducts in target tissues. These findings appear to contradict the knowledge that CYP1A1 is required for the metabolism of PAHs, aflatoxin, and several other carcinogens to DNA reactive arene oxides (Guengerich, 1988; Levin et al., 1982; Conney, 1982). For example, the promutagenic DNA adduct of benzo(a)pyrene appears to be a 7,8-diol-9,10 epoxide metabolite adducted to deoxyguanosine, and formation of this metabolite requires two separate actions of CYP1A1. The contradiction can be resolved by analysis of the entire metabolic pathways for chemical carcinogens whose potencies are decreased by pretreatment with inducing agents. In addition to CYP1A1-mediated increases in metabolic activation, this cytochrome also converts PAHs to inactive metabolites (Thakker et al., 1985; Pelkonnen and Nebert, 1982). Moreover, induction of uridine diphosphoglucuronyltransferase also occurs coordinately with CYP1A1 induction (Lucier et al., 1986). This enzyme also detoxifies metabolites of PAHs and other carcinogens and facilitates their excretion from the body (Thakker et al., 1985; Nemoto and Gelboin, 1976). Therefore, it appears that TCDD-mediated enzyme induction increases the rate of detoxification of some carcinogens to a greater extent than it increases the rate of formation of DNA-damaging metabolites.

Although there is no clear mechanistic link between CYP1A1 induction and cancer, it is important to note that many CYP1A1 inducers are themselves carcinogens when encountered in chronic dosing regimens; therefore, the protective effect of inducing agents appears to be limited to short-term exposure. For example, benzo(a)pyrene, 3-methylcholanthrene, and TCDD are CYP1A1 inducers and multisite carcinogens (Vanden Heuvel and Lucier, 1993; Levin et al., 1982; Slaga et al., 1979; Sims and Glover, 1974).

The relationship of CYP1A2 induction to the carcinogenic actions of other compounds is less clear than it is for CYP1A1. For example, CYP1A2 catalyzes the formation of catechol

estrogens from 17β-estradiol (Graham et al., 1988). The catechol estrogens are considered to be possible toxic metabolites in that they could lead to increased free radical damage to cellular macromolecules such as DNA (Li and Li, 1990; Metzler, 1984). This mechanism could be responsible, in part, for the findings that TCDD is a hepatocarcinogen in female rats but not male rats and that ovariectomy protects against the hepatocarcinogenic actions of TCDD. Also consistent with the hepatocarcinogenicity data is the observation that CYP1A2 is induced in liver but not in extrahepatic organs, with the possible exception of the nasal mucosa (Goldstein and Linko, 1984). In contrast, CYP1A1 induction occurs in virtually every tissue of the body, which is consistent with the observation that the Ah receptor is found in a wide variety of cell types.

There are a number of studies described in the scientific literature on dose-response relationships for TCDD's effects on CYP1A1 and 1A2 (DeVito et al., 1991; Lin et al., 1991a; Kedderis et al., 1991; Harris et al., 1990a; Goldstein and Safe, 1989; Abraham et al., 1988; Lucier et al., 1986, 1973; Vecchi et al., 1983; Kitchin and Woods, 1979; Poland and Glover, 1973). These studies include single and chronic dosing schedules (Tritscher et al., 1992; Graham et al., 1988; Sloop and Lucier, 1987), time-course evaluations, and species comparisons. Dose-response relationships have been evaluated by quantitation of CYP1A1- and 1A2-dependent enzyme activities, quantitation of mRNA levels by Northern blot analysis, and quantitation of CYP1A1 and 1A2 protein by radioimmunoassay and also by immunolocalization in tissue sections. All of the above methods have yielded consistent results. The single-dose ED_{50} for CYP1A1 or 1A2 induction is approximately 0.5 to 1.5 μg TCDD/kg in both rats and mice. In a chronic exposure situation, the ED_{50} is in the range of 5 to 10 ng/kg/day (Tritscher et al., 1992). The limit of detection for enzyme induction varies depending on the method used for quantitation; that is, P-450-dependent enzyme activities, mRNA, or protein. Recently, it was shown (Kohn et al., 1993) that TCDD-mediated increases in CYP1 in mRNA were detectable following a single dose of 0.1 ng/kg, which produces a TCDD liver concentration equivalent to a chronic dose of 2 to 5 pg/kg/day.

Evaluations of various data sets for TCDD-mediated dose-response relationships have revealed some interesting information. One way of analyzing data for linearity or

nonlinearity of dose response for receptor-mediated events is the Hill equation (Hayashi and Sakamoto, 1986). A Hill coefficient of 1 suggests a linear relationship between exposure and dose throughout the experimental dose range and would predict a proportional relationship between target tissue concentration of TCDD and biological response at all dose levels. This would imply that the response had no practical threshold or "no effect level." Hill coefficients greater than 1 would indicate sublinearity in dose response, whereas a Hill coefficient of less than 1 would indicate supralinearity for response in the low-dose region. Analyses of both single exposure as well as chronic exposure data for CYP1A1 and CYP1A2 induction in rat or mouse liver indicate a Hill coefficient of slightly greater than 1 for CYP1A1 and slightly less than 1 for CYP1A2 (Portier et al., 1993; Kohn et al., 1993). Although these analyses involve an extrapolation beyond the range of experimental data, they are consistent with the hypothesis that there is no threshold for TCDD-mediated induction of CYP1A1 and 1A2.

Immunological detection of induced CYP1A1 and 1A2 in liver sections obtained from rats exposed chronically to TCDD suggests hepatocyte heterogeneity in response to TCDD (Tritscher et al., 1992; Bars and Elcombe, 1991). For example, relatively low doses of TCDD (1 ng/kg/day) appear to maximally induce some cells around the centrilobular region. Increasing doses of TCDD increase the number of cells responding rather than the amount of induction in responding cells. These data, which document cell differences in sensitivity to induction, complicate evaluation of dose-response relationships. For example, some hepatocytes appear to be maximally induced by low doses of TCDD, whereas other hepatocytes exhibit no detectable P-450 induction response at these same doses. As discussed earlier, a mechanistic link between P-450 induction and cancer has not been established. Evaluations of P-450 induction and TCDD-mediated cell proliferation by immunocytochemical methods in rat liver reveal that cells expressing CYP1A1 and 1A2 are different from those exhibiting TCDD-mediated increases in DNA replication (Lucier et al., 1992).

Placentas from Taiwanese women exposed to rice oil contaminated with polychlorinated dibenzofurans have markedly elevated levels of CYP1A1 (Lucier et al., 1987; Wong et al., 1986). Comparison of these data with induction data in rat liver suggests that humans are at

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

6.5.2. Epidermal Growth Factor Receptor

EGF is a potent mitogen and stimulates the generation of mitotic signals in both normal and neoplastic cells (Stoscheck and King, 1986; Carpenter and Cohen, 1979). Several lines of evidence suggest that the EGF receptor and its ligands, including transforming growth factor-α, possess diverse functions relevant to cell transformation and tumorigenesis (Velu, 1990; Marti et al., 1989; Mukku and Stancel, 1985). In fact, the mechanism of action for several tumor promoters such as phenobarbital and the phorbol esters is thought to involve the EGF receptor pathway (Stoscheck and King, 1986). A schematic representation of the proposed mechanism for EGF-stimulated mitogenesis is given in Figure 6-3.

Several studies have shown that TCDD decreases the binding capacity of the plasma membrane EGF receptor for its ligand without a change in K_d (Clark et al., 1991a; Lin et al., 1991a; Abbott and Birnbaum, 1990; Astroff et al., 1990; Sunahara et al., 1989; Hudson et al., 1985; Madhukar et al., 1984). One study used a range of TCDD doses (3.5 to 125 ng/kg/day) for 30 weeks to evaluate the effects of TCDD exposure on EGF receptor in rat liver plasma membranes. There was a clear dose-response relationship for TCDD's effects on the total binding capacity of the EGF receptor although TCDD did not produce a change in binding affinity of the receptor. The maximal effect was a threefold decrease in the concentration of plasma membrane EGF receptor and the ED_{50} was $\sim$10 ng/kg/day based on

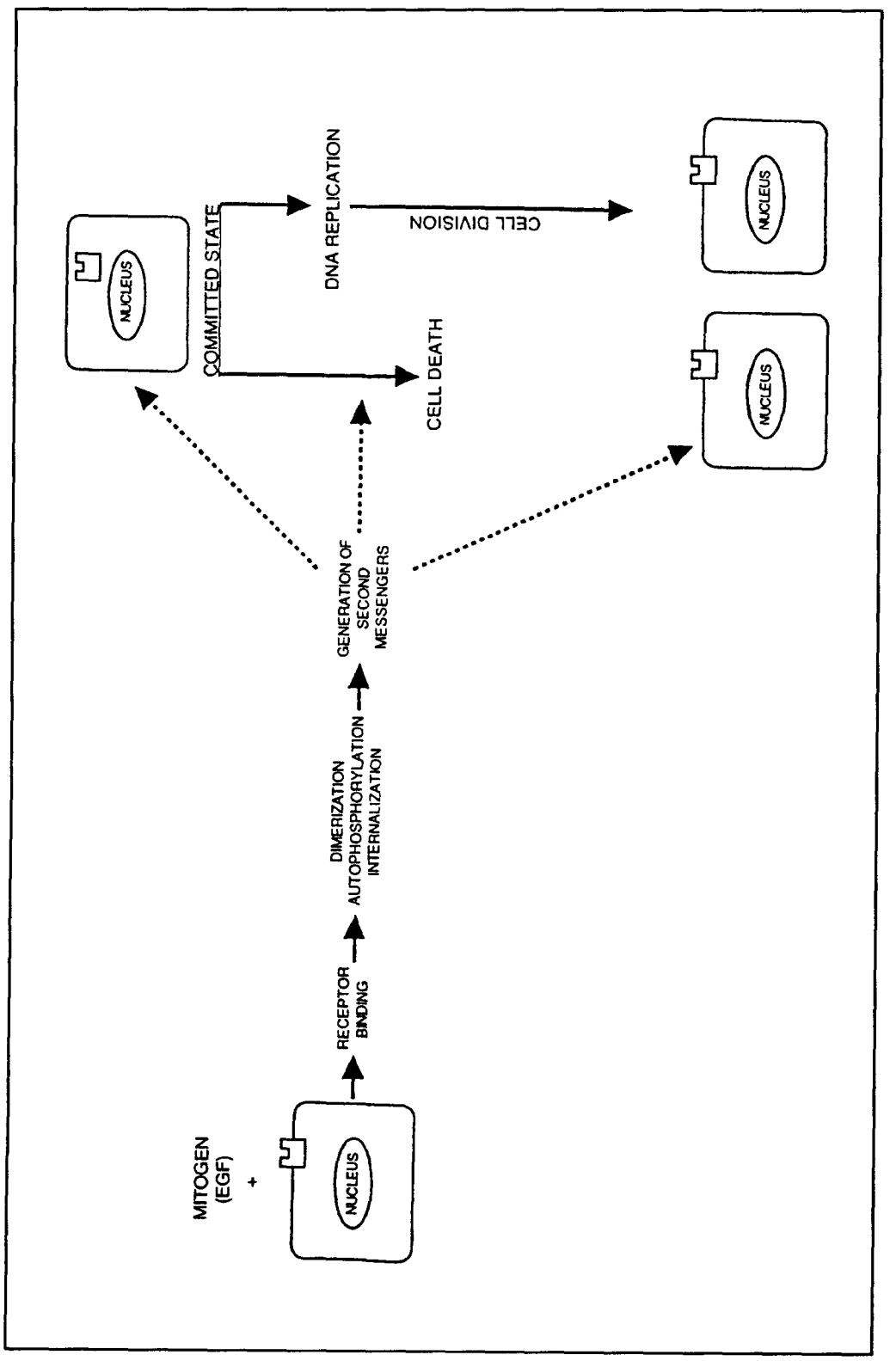

Figure 6-3. Plausible mechanism for the role of EGF-mediated stimulation of mitotic activity.

Source: Stoscheck and King, 1986.

administered dose and ~2 ppb TCDD based on liver TCDD concentration. These values are similar to the ED_{50} for induction of CYP1A1 and CYP1A2 for 30-week exposures. The dose-response data, like the data for CYP1A1 and CYP1A2 induction, were subjected to curve-fitting analyses using the Hill equation (Portier et al., 1992). This analysis indicated that a Hill coefficient of 1 provided the best fit, suggesting that there is a linear relationship between target tissue dose and the magnitude of response for effects on the EGF receptor. Although Hill analyses of dose-response data for TCDD's effects on the EGF receptor, CYP1A1 induction, and CYP1A2 induction are inconsistent with the idea of a threshold, the lowest dose used in these experiments was 100 pg/kg/day so the possibility exists that dose-response relationships are different in the very low-dose region (1 to 10 pg/kg/day) encountered as background human exposures.

Dose-response data on EGFR were compared with dose-response relationships for TCDD-mediated increases in cell proliferation and growth of preneoplastic lesions within the framework of a two-stage model for hepatocarcinogenesis in rats (Lucier et al., 1992). Results indicate that cell proliferation and the growth of preneoplastic lesions are less sensitive responses to TCDD than is loss of plasma membrane EGF receptor. Therefore, the EGF receptor may be involved in the hepatocarcinogenic actions of TCDD, but dose-response relationships for this effect may be different from dose-response relationships for liver cancer in rats. These data reflect the knowledge that several steps and/or several genes are involved in the modulation of coordinated biological responses.

The mechanism by which TCDD alters EGF receptor-binding capacity is not fully understood although TCDD does not appear to decrease EGF receptor mRNA (Lin et al., 1991a; Osborne et al., 1988). By using congenic mice deficient in the high-affinity Ah receptor, TCDD's effects on the EGF receptor were shown to require the Ah receptor (Lin et al., 1991a). In control animals, the EGF receptor is distributed on the surface of the plasma membrane and is composed of an external ligand-binding domain, a transmembrane domain, and an intercellular domain (Velu, 1990; Carpenter, 1987). Ligands for the EGF receptor (EGF or TGF-α) in the intracellular space bind the EGF receptor, producing a conformational change that stimulates the intercellular region to catalyze phosphorylation of the receptor itself as well as other proteins involved in cell regulation. The process results in

internalization of the receptor characterized by an increase in cytosolic EGFR coupled with a decrease in membrane-bound receptor. The effects of TCDD and CDFs on the number of binding sites for the plasma membrane EGF receptor are correlated with a concomitant decrease in EGF-stimulated autophosphorylation of the EGF receptor, indicating that TCDD produces a true functional change in the EGF receptor (Clark et al., 1991a; Sunahara et al., 1989; Nelson et al., 1988; Sunahara et al., 1988). Importantly, the addition of EGF to hepatocytes or several cell lines in culture produces a loss of plasma membrane EGF receptor coupled with a loss of EGF-stimulated autophosphorylation (Velu, 1990; Carpenter, 1987). Therefore, TCDD produces an EGF receptor-like response consistent with the idea that TCDD enhances the generation of cellular mitotic signals.

Although TCDD exposure mimics EGF actions in hepatocytes, TCDD itself does not appear to bind to the EGF receptor. The most plausible mechanism for effects on the EGF receptor involves the finding that TCDD induces production of TGF-α in hepatocytes as well as human keratinocytes (Choi et al., 1991). This response could alter control of normal growth patterns since TGF-α binds the EGF receptor with high affinity, leading to enhanced production of mitogenic signals. Alternatively, TCDD may affect EGF receptor transcription. In fact, TCDD has been shown to decrease uterine EGF receptor mRNA levels (Astroff et al., 1990). Receptor concentrations may also be altered by other events such as posttranslational glycosolation, increased lysosomal degradation, or alterations in signal transduction pathways such as protein kinases (Madhukar et al., 1988). It is also possible that TCDD alters phosphorylation of the EGF receptor by activation of protein kinase C, resulting in decreased binding capacity of the plasma membrane EGF receptor. This effect occurs following exposure to the tumor promoter TPA and is associated with decreased autophosphorylation rates and EGF receptor internalization (Beguinot et al., 1985; Cochet et al., 1984). In any event, TCDD-mediated alterations in EGF receptor pathways may, in part, be responsible for the tumor-promoting actions of TCDD by enhancement of mitotic signals.

The effects on the EGF receptor system may be mediated by estrogen action, and it has been postulated that the estrogen and EGF receptor pathways are integrated by "cross talk" mechanisms (Ignar-Trowbridge et al., 1992; Astroff et al., 1990). In vivo and in vitro

studies have demonstrated that TCDD alters the estrogen receptor (DeVito et al., 1992; Lin et al., 1991a; Clark et al., 1991a; Umbreit and Gallo, 1988; Romkes et al., 1987) and estrogens can, in turn, alter EGF receptor binding and cellular distribution (Vickers and Lucier, 1991; Vickers et al., 1989; Mukku and Stancel, 1985). Moreover, studies conducted within the framework of a two-stage model for hepatocarcinogenesis have demonstrated that TCDD-mediated decreases in plasma membrane EGF receptor are ovarian dependent (Clark et al., 1991a). These studies concluded that ovarian hormones are essential to the tumor-promoting actions of TCDD in that TCDD does not induce hepatocyte proliferation or stimulate the growth of preneoplastic lesions in ovariectomized rats (see Section 6.4, Initiation/Promotion Studies).

Evidence indicates that TCDD and its structural analogs produce the same effects on the EGF receptor in human cells and tissues as observed in experimental animals. First, incubation of human keratinocytes with TCDD decreases plasma membrane EGF receptor, and this effect is associated with increased synthesis of TGF-α (Choi et al., 1991; Hudson et al., 1985). Second, placentas from humans exposed to rice oil contaminated with polychlorinated dibenzofurans exhibit markedly reduced EGF-stimulated autophosphorylation of the EGF receptor, and this effect occurred with similar sensitivity to that observed in rats (Lucier, 1991; Sunahara et al., 1989). The magnitude of the effect on autophosphorylation was positively correlated with decreased birth weight of the offspring.

6.5.3. UDP-Glucuronosyltransferases

Several studies have shown that TCDD induces synthesis of at least one isozyme of UDPGT (Lucier et al., 1973, 1974, 1986) by a mechanism that requires the Ah receptor (Bock, 1991). The gene UGT-1 regulates synthesis of the UDPGT isozyme, which conjugates numerous substrates including 1-naphthol, p-nitrophenol, and thyroxine (Burchell et al., 1991). This gene contains a TCDD-responsive element that permits transcriptional activation following binding of the TCDD-Ah receptor complex. Other chemicals that bind the Ah receptor, such as 3-methylcholanthrene and benzo(a)pyrene, also induce UGT-1 (Bock, 1991). UDPGTs are considered a deactivation pathway for numerous environmental chemicals and endogenous compounds such as steroid hormones by rendering them water

soluble and excretable as a consequence of the catalytic addition of a glucuronide moiety (Tephly and Burchell, 1990). Therefore, induction of UDPGT may be responsible, in part, for the finding that pretreatment with TCDD leads to diminished DNA adducts for PAHs and decreased concentrations of some steroid hormones.

Conjugation of thyroxine by UGT-1 leads to deactivation and elimination of this thyroid hormone (Henry and Gasiewicz, 1987; Bastomsky, 1977). The decreased levels of thyroxine, associated with UDPGT induction, produces decreased feedback inhibition of the pituitary gland, which responds by secreting increased amounts of TSH (Sanders et al., 1988; Barter and Klaassen, 1992). Several studies have provided evidence that prolonged stimulation by TSH produces an oncogenic effect on the thyroid (Hill et al., 1989). Interestingly, rat liver EGF receptor may, in part, be regulated by thyroid hormones (Mukku, 1984). Increased incidence of thyroid tumors is the most sensitive end point in cancer bioassays, as evidenced by a statistically significant increase at a dose of 1.4 ng/kg/day. Consistent with this hypothesis, rodent studies have shown that TCDD and other inducers of hepatic UDPGT decrease thyroxine concentration in blood, which is associated with increased levels of thyroid-stimulating hormone (Barter and Klaassen, 1992; Henry and Gasiewicz, 1987).

Dose-response studies for TCDD's inductive effects on hepatic UDPGT in rats have demonstrated that the single dose ED_{50} is approximately 0.7 μg/kg, which is similar to the ED_{50} for CYP1A1 induction (Lucier et al., 1986). Furthermore, the shape of the dose-response curve for both responses is similar. There are no data on UDPGT induction in long-term studies. Because humans have the dioxin-responsive UDPGT (UGT-1) (Burchell et al., 1991) and TCDD induces UDPGT in human hepatocyte cell cultures, it is reasonable to assume that TCDD and its structural analogs would induce UDPGT in humans although laboratory data are needed to validate this assumption.

6.5.4. Estrogen Receptor

Several lines of evidence have demonstrated that interactions of TCDD and estrogens are critical to some of the carcinogenic responses to TCDD. Although the precise mechanisms of those interactions have not been established, recent data indicate that TCDD

effects on the ER and on estrogen metabolism are involved. The mechanisms for TCDD/estrogen interactions appear to be tissue specific. Of particular interest is the finding that TCDD increases liver tumor incidence in rats and at the same time decreases tumor incidence in organs such as the mammary gland, uterus, and pituitary (Kociba et al., 1978). Therefore, TCDD/estrogen interactions will be examined separately for liver and other endocrine organs.

The liver contains a fully functional ER that possesses characteristics similar to those identified for ER in the mammary gland and uterus (Mastri and Lucier, 1983; Powell-Jones et al., 1981; Eisenfeld et al., 1976). For example, the liver exhibits high-affinity binding for 17β-estradiol and other potent estrogens, liver ER binding is specific for estrogens, the ligand receptor complex interacts reversibly with DNA, and this interaction leads to transcriptional activation of estrogen-responsive genes. Synthesis of hepatic ER, unlike ER in other target tissues, is under pituitary control (Lucier et al., 1981). Treatment of rats with a single dose of TCDD decreases binding capacity of the hepatic ER, and this effect is correlated with a decrease in ER protein (Zacharewski et al., 1991, 1992; Harris et al., 1990b; Romkes and Safe, 1988; Romkes et al., 1987). TCDD also decreases rat hepatic ER in chronic exposure experiments with a threefold decrease evident following a dose of 100 ng/kg/day for 30 weeks (Clark et al., 1991b). TCDD also decreases hepatic ER binding in C57Bl6 mice, but a much higher dose is needed to produce this effect in congenic mice deficient in the high-affinity Ah receptor, indicating that TCDD-mediated decreases in ER are dependent on the Ah receptor (Lin et al., 1991b). Dose-response studies in mice demonstrate that the single-dose ED_{50} is ~ 0.7 μg TCDD/kg, similar to the ED_{50} for other biochemical end points such as CYP1A1 induction, loss of plasma membrane EGF receptor, and induction of UDPGT. The observation that TCDD decreases hepatic ER is in apparent contradiction to the finding that TCDD increases hepatocyte proliferation because the ER is thought to produce mitogenic signals. However, quantitation of ER in control and TCDD-treated rats was done using preparations from liver homogenates. Immunolocalization studies are needed so that the relationship of ER concentrations to cell proliferation in normal and preneoplastic cells can be more carefully evaluated.

In addition to effects on hepatic ER, TCDD may influence estrogen action in another way. CYP1A2 efficiently catalyzes the conversion of estrogens to catechol estrogens in liver (Graham et al., 1988; Dannan et al., 1986). CYP1A2 is not found in extrahepatic tissues, with the possible exception of the nasal cavity, so catechol estrogen formation would be expected to occur only in liver. Catechol estrogens have been postulated to possess macromolecule-damaging properties as a consequence of free radical generation (Li and Li, 1990; Metzler, 1984). Therefore, TCDD may increase the DNA-damaging capacity of estrogens in liver as a function of CYP1A2 induction. This effect may, in part, explain the carcinogenic actions of TCDD in female rat liver and is consistent with the knowledge that ovariectomy protects against the hepatocarcinogenic actions of TCDD and that male rats do not appear to be susceptible to TCDD-induced liver tumors (Lucier et al., 1991; Kociba et al., 1978). It is important to note that cancer is more than a two-stage process and the stage-specific actions of TCDD in multistage cancer models are not known, although TCDD-mediated cell proliferation and possible indirect genotoxic effects may be critical at more than one stage. A hypothetical mechanistic scheme for TCDD-mediated liver cancer is shown in Figure 6-2.

The finding that chronic TCDD exposure decreases tumor incidences in the pituitary, mammary gland, and uterus may also reflect TCDD's effects on ER and estrogen metabolism. As discussed above, TCDD decreases uterine ER concentrations in cytosolic and nuclear fractions of rats and mice, and these changes are associated with diminished estrogen action in in vivo as well as in vitro studies. TCDD also increases estrogen metabolism, presumably as a consequence of CYP1A2 in liver and UDPGT induction in liver and extrahepatic tissues (Shiverick and Muther, 1982). Likewise, the addition of TCDD to a breast cancer cell line (MCF-7) results in increased estrogen degradation (Gierthy et al., 1988). However, there are only small effects on serum 17-β estradiol levels following administration of TCDD to either rats or mice (Shiverick and Muther, 1983). Therefore, the effect on serum estradiol is considerably less sensitive than the effects on the uterine receptor. This comparison has led investigators to conclude that the antiestrogenic actions of dioxins are primarily caused by effects on ER levels in reproductive tract tissues. Consistent with this hypothesis, Fernandez and Safe (1992) have shown that TCDD is antimitogenic in

human breast cancer cells. Final evaluation of the role of estrogen metabolism awaits data on concentrations of estrogens in responsive cells of control and TCDD-treated rats, which may be different from serum estradiol levels. In any event, it appears clear that TCDD does possess antiestrogenic properties that are likely to be important to decreased tumor incidences in some reproductive tract and endocrine organs. Numerous studies have documented that the estrogen receptor is found in virtually every tissue of the body although the effects of TCDD on human estrogen receptor in vivo have not been studied.

6.5.5. Other Biochemical End Points

TCDD alters a number of other pathways involved in the regulation of cell differentiation and proliferation. The specific relationships of these effects to multistage carcinogenesis are not known, but the broad array of effects on hormone systems, growth factor pathways, cytokines, and signal transduction components is consistent with the notion that TCDD is a powerful growth dysregulator. It is also consistent with the findings that TCDD alters cancer risks at a large number of sites, possibly reflecting multiple mechanisms of carcinogenicity. Biochemical/molecular/endocrine changes produced by TCDD include the glucocorticoid receptor (Sunahara et al., 1989), tyrosine kinase (Madhukar et al., 1988), gastrin (Mabley et al., 1990), interleukin 1β (Sutter et al., 1991), plasminogen activator inhibitor (Sutter et al., 1991), tumor necrosis factor-α (Clark et al., 1991b), gonadotropin-releasing hormone (Moore et al., 1989), testosterone (Moore et al., 1985), and luteinizing hormone (Mabley et al., 1992). The importance of these responses to the carcinogenic process should not be diminished by the lack of detail presented here. In every case studied, these responses have been shown to be dependent on the Ah receptor.

6.6. SUMMARY AND WEIGHT OF EVIDENCE FROM ANIMAL STUDIES

There have been several long-term studies designed to determine if TCDD is a carcinogen in experimental animals. All of these studies have been positive and demonstrate that TCDD is a multisite carcinogen, is a carcinogen in both sexes and in several species including the Syrian hamster, is a carcinogen in sites remote from the site of treatment, and increases cancer incidence at doses well below the MTD. In two-stage models for liver and

skin cancer, it is clear that TCDD is a potent promoting agent with weak or no initiating activity. This finding is not surprising because TCDD does not form DNA adducts and is negative in short-term tests for genetic toxicity. The general consensus is that TCDD is an example of receptor-mediated carcinogenesis in that (1) interaction with the Ah receptor appears to be a necessary early step, (2) TCDD modifies a number of receptor and hormone systems involved in cell growth and differentiation such as the epidermal growth factor receptor and the estrogen receptor, and (3) hormones exert a profound influence on the carcinogenic actions of TCDD. For example, ovarian hormones are essential for the hepatocarcinogenic actions of TCDD in rats, whereas TCDD promotion of lung tumors in rats appears to occur only in the absence of ovarian hormones. Although tumor promotion data for the polychlorinated dibenzofurans and coplanar polychlorinated biphenyls are limited, it appears that these compounds are liver tumor promoters with potencies dependent on their binding affinity to the Ah receptor.

Some of the central issues in the risk assessment of TCDD and its structural analogs are (1) characterization of the shape of the dose-response curve for receptor-mediated events, (2) evaluation of the relevance of animal data in estimating human risks, and (3) the health consequences of background exposures (1 to 10 pg TEQ/kg/day) of dioxin and its structural analogs. With regard to the shape of the dose-response curve, it is clear from animal studies that there are different dose-response curves for different TCDD effects, which is consistent with the generally accepted dogma for steroid receptor-mediated responses. In general, the biochemical/molecular responses such as cytochrome P-450 induction do not show evidence for a threshold although unequivocal conclusions cannot be made about the mechanistic link, if any, between biochemical responses and toxic effects. In fact, coordinated biological responses such as TCDD-mediated cell proliferation and growth of preneoplastic lesions (foci of cellular alterations in liver) appear to be less sensitive end points, although evaluation of these responses is complicated by a high degree of interindividual variation: some animals do not exhibit any increase in cell proliferation in response to TCDD exposure.

The mechanistic basis for interindividual variation is unclear, and this lack of knowledge complicates approaches to estimate human risks from experimental animal data. However, several studies indicate that, for the most part, humans appear to respond like experimental

animals for biochemical and carcinogenic effects. However, data from epidemiology studies are difficult to evaluate because the carcinogenic effects, if any, resulting from background TCDD exposures are not known, although biochemical effects such as cytochrome P-450 induction may be produced by background exposures.

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