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August 1992
Workshop Review Draft

Chapter 5. Reproductive and Developmental Toxicity

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

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Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
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LIST OF ABBREVIATIONS

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

LIST OF ABBREVIATIONS (cont.)

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

LIST OF ABBREVIATIONS (cont.)

HpCDF	Heptachlorinated dibenzofuran
HPLC	High performance liquid chromatography
HRGC/HRMS	High resolution gas chromatography/high resolution mass spectrometry
HxCDD	Hexachlorinated dibenzo-p-dioxin
HxCDF	Hexachlorinated dibenzofuran
ID_{50}	
I-TEF	International TCDD-toxic-equivalency
LD_{50}	Dose lethal to 50% of recipients (and all other subscripter dose levels)
LH	Luteinizing hormone
LDL	Low density liproprotein
LPL	Lipoprotein lipase activity
LOAEL	Lowest-observable-adverse-effect level
LOEL	Lowest-observed-effect level
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
MFO	Mixed function oxidase
mRNA	Messenger RNA
MNNG	*N*-methyl-*N*-nitrosoguanidine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NK	Natural killer
NOAEL	No-observable-adverse-effect level

LIST OF ABBREVIATIONS (cont.)

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

LIST OF ABBREVIATIONS (cont.)

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

AUTHORS AND CONTRIBUTORS

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5. REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

5.1. INTRODUCTION

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, PBBs, and as such serves as the prototype congener for investigating the toxicity elicited by these classes of chemicals. Reproductive and developmental 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 mono*ortho* 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 reproductive and developmental effects of complex halogenated aromatic hydrocarbon mixtures. Such mixtures which contain

both TCDD-like congeners and nonTCDD-like congeners have been implicated in causing reproductive and developmental 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- and nonTCDD-like congeners are not known in some exposure situations.

A mechanism of action which 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, is involved in the antiestrogenic action of TCDD and in its ability to produce structural malformations in mice. However, its role in producing other signs of reproductive and developmental toxicity is less firmly established.

5.2. REPRODUCTIVE TOXICITY

5.2.1. Female

5.2.1.1. REPRODUCTIVE FUNCTION/FERTILITY -- TCDD and its approximate isostereomers have been shown to affect female reproductive end points 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 the Developmental Toxicity section of this chapter.

The study by Murray et al. (1979) employed a multi-generation 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 bw/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. Even so 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 1-2 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 0.001-0.01 μg/kg/day in a 2-year study, Kociba et al. (1978) reported no effects on the female reproductive system.

Allen and his colleagues reported on the effects of TCDD on reproduction in the monkey (Allen et al., 1977; Allen et al., 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 to 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). While 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 50 ppt TCDD there were no overt effects on maternal health, 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 that was fed a normal diet which 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 25-40 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, two of these in animals which demonstrated no maternal toxicity. At a total dose of 0.2 μg/kg, one of four pregnancies ended in abortion. This did not appear 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, there were only three normal births (McNulty, 1984, 1985).

The primary effects 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.

5.2.1.2. **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 six months of exposure to this lower dietary level of TCDD there were was no effect on the serum estradiol and progesterone concentrations of 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 gestation days 4-15. 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 designed to examine alterations in female hormones specifically and carefully.

5.2.1.3. **ANTIESTROGENIC ACTION**

5.2.1.3.1. ***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. While 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 decreased uterine weight, decreased uterine peroxidase activity, and a decrease in the tissue concentration of progesterone receptors (Safe et al., 1991). In addition, when TCDD and 17β-estradiol are co-administered 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) suggest that this apparent lack of an antiestrogenic effect of TCDD may have been due to the young age of the rats used.

5.2.1.3.2. *In Vitro* -- Both TCDD and progesterone can affect a decrease in the nuclear estrogen receptor concentration in rat uterine strips. 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, this result indicates that TCDD and progesterone decrease the nuclear estrogen receptor concentration by different mechanisms (Romkes and Safe, 1988). 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 down regulation 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 a 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 post-confluent 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 the compound.

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-450IA1 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's group has 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. While exposure to 10 pM TCDD resulted in a statistically significant down regulation of estrogen receptors, Israel and Whitlock (1983) reported that this concentration is the approximate EC_{50} for the induction of cytochrome P-450IA1 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's group has used an analog of TCDD, MCDF, that inhibits the 17β-estradiol-induced secretion of the 34, 52 and 160 kDa proteins and down regulates estrogen receptors in MCF-7 cells. These effects occurred at concentrations of MCDF for which there is no detectable induction of EROD activity (Zacharewski et al., 1992). In addition, it has been stated that the down regulation 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 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 aryl hydrocarbon hydroxylase 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. While Gierthy's group pretreated their cultures with serum free medium, this 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's group 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).

5.2.1.3.3. Evidence for an Ah Receptor Mechanism

5.2.1.3.3.1. Ah Receptor Mutants -- While 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 down regulation 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).

5.2.1.3.3.2. Structure Activity Relationships *In Vivo* -- 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%, whereas uterine estrogen receptor concentrations are reduced 53% and 49% 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%, whereas uterine estrogen receptor concentrations are reduced 21% and 24% 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.

5.2.1.3.3.3. **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.

5.2.1.3.3.4. **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 relationship 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.

5.2.2. **Male**

5.2.2.1. **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 testes 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 65 μg/kg administered over 13 weeks (Kociba et al., 1976). At this dose body weights and feed consumption of the rats were also significantly depressed. Thus, suppression of spermatogenesis is not a highly sensitive effect when TCDD is administered to post-weanling animals.

5.2.2.2. **ALTERATIONS IN HORMONE LEVELS** -- 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 is 15 μg/kg, 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).

5.2.2.3. **TARGET ORGAN RESPONSIVENESS**

5.2.2.3.1. **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-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 which 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.

5.2.2.3.2. 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). 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; 1990b). 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 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. Decreased plasma androgen concentrations normally result in compensatory increases in 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.

5.2.2.3.3. 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 in seminal vesicle and ventral prostate weights (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; 1990b). As the 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 these androgens without affecting the responsiveness of the accessory sex organs.

5.2.2.4. SUMMARY -- In conclusion, although the androgenic deficiency is an early-occurring effect following exposure of adult male rats to TCDD, has an ED_{50} in the nonlethal range, and is far more severe in TCDD-treated animals than in pair-fed controls, it is only detected at overtly toxic doses of TCDD that reduce feed intake and body weight. Similarly, effects on male reproductive function and fertility assessed in animals exposed as adults to TCDD are elicited only by overtly toxic doses. Thus, the male reproductive system is relatively insensitive to TCDD toxicity when exposure occurs in adulthood. Male reproductive toxicity induced by perinatal and lactational exposure to lower doses of TCDD will be described in Section 5.3.3.

5.3. DEVELOPMENTAL TOXICITY

The manifestations of developmental toxicity have been divided into three categories for convenience in assessing the data base 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 in mice, provide the most convincing evidence of an Ah receptor-mediated response. However, postnatal functional alterations, some of which may be irreversible, are more sensitive.

5.3.1. Death/Growth/Clinical Signs

5.3.1.1. 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 in depth coverage than will be 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 sacfry 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% 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-450IA1 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., 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 mono*ortho* 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).

5.3.1.2. 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 100-200 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 14-23 times less than the TCDD dose that causes 75% mortality in ring-necked hen pheasants (25 μg/kg body weight) (Nosek et al., 1989, 1991).

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, microophthalmia, beak deformities, cardiovascular malformations, and mortality (Cheung et al., 1981; 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 microopthalmia, 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 (Martin et al., 1989; Nosek et al., 1989). 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 embryomortality.

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-450IA1 induction (Rifkind et al., 1985; Brunstrom and Andersson, 1988; Brunstrom, 1989). However, while induction of cytochrome P-450IA1 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, suppressing 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 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 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 pmol TCDD/egg causes cardiovascular malformations in 50% of treated embryos versus 26-29% of control embryos (Cheung et al., 1981). 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 (Nosek et al., 1989; Martin et al., 1989).

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 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 TCDDs 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 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, microopthalmia, beak deformities, and mortality, and TCDD, 3,3',4,4'-TCB and 3,3',4,4'-TCAOB inhibit lymphoid development (Cheung et al., 1981; 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., 1989).

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 Lund, 1988; Brunstrom and Reutergardh, 1986; Martin et al., 1989; Elliott et al., 1989; Nosek et al., 1989). TCDD is 4-7 times more potent in causing embryo mortality in chicken than pheasant embryos, and 3,3',4,4'-TCB is 20-100 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 embryomortality 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 1000 μg/kg egg had no effect on embryomortality 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 embryomortality among different strains of chickens is quite similar with the LD_{50} in six different strains varying <4-fold (Brunstrom, 1988).

Graded doses of TCDD have been administered to fertilized eastern bluebird and ring-necked pheasant eggs for the purpose of determining a 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 1000 pg TCDD/g egg (Martin et al. 1989). For ring-necked pheasants, the LOAEL was 1000 pg TCDD/g egg and the NOAEL was 100 pg TCDD/g egg (Nosek et al., 1989). In contrast, for chickens. the LD_{50} for embryomortality is 250 pg TCDD/g egg (Allred and Strange, 1977).

5.3.1.3. 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, 1157-5051 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 12 fold 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 susceptibilty 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. This is 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 cumulative doses of TCDD to the pregnant dam on days ~6-15 of gestation. This dosing regimen is presumably, expected to cover the critical period resulting in what might be the maximal possible incidence of prenatal mortality. In nearly all species of adult laboratory mammals however, single lethal doses 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 will illustrate a type of analysis using an index of cumulative maternal dose similar to the type of analysis that might be applied

TABLE 5-1

Relationship Between Maternal Toxicity and Prenatal Mortality in Laboratory Mammals Exposed to TCDD During Gestation[a]

Species/Strain	Daily TCDD Dose (µg/kg/day)	Cumulative TCDD Dose (µg/kg)	Overt Maternal Toxicity[b]	Percent Prenatal Mortality[c]	Reference
Monkey/rhesus		0[d]	-	25	McNulty, 1984
		0.2	-	25	
		1	+	81	
		5	+	100	
Guinea pig/Hartley		0[e]	-	-	Olson and McGarrigle, 1991
		0.15	-	-	
		1.5	+	+	
Rabbit/New Zealand	0[f]	0	-	7	Giavini et al., 1982
	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	1000	-	14	
	200	2000	+	87	
	400	4000	+	97	

[a]Source: Couture et al. 1990
[b]Decreased body weight gain or marked edema compared to vehicle dosed controls. A (+) or (-) indicates the presence or absence of an effect, respectively.
[c]Percentage of absorptions plus late gestational deaths relative to all implantations. A (+) or (-) is given it indicates the presence or absence of an effect, respectively.
[d]TCDD administered in a single or divided doses between gestational days 20 and 40.
[e]Single dose of TCDD administered on gestational day 14.
[f]TCDD administered daily on days 6-15 of gestation.
[g]Significant at $p<0.05$
[h]Single dose of TCDD administered on gestational day 7 or 9.
[i]TCDD administered daily on days 7-16 of gestation.

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 is available in the literature. In this case a difference of one gestational day might be critically important. It turns out that the form of analysis using cumulative maternal dose 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 by the results of this type of an analysis.

Using the cumulative dose data that is given in Table 5-1 there appears to be a 10- to 20-fold difference in the fetolethal potency of TCDD when the monkey/guinea pig is compared to the rabbit/rat/hamster. In the CD-1 mouse administered cumulative doses of TCDD on gestational days 7-16, not including day 6, it appears to require a daily dose of 200 μg TCDD/kg 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 ~1200 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 ~1200 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 about a 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 20 to 30 fold 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 stain when the TCDD was administered to the pregnant dam on gestational days 8, 10, 12 or 14.

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 approximately on gestational day 6. If the embryo/fetus is not exposed to TCDD on 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 day six compared to when the TCDD dose was administered on one of the days 7-15. 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 to 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 does not seem 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% fetal mortality in hamsters is 64-280 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% prenatal mortality is 5-10 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% 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, hematological alterations in the dam (Olson and McGarrigle, 1991), could contribute to prenatal mortality.

In rhesus monkeys, on the other hand, the association between prenatal mortality and maternal toxicity is not as easy to make. Only small numbers of monkeys have been studied to date. However, 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; Allen et al., 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. In four monkeys given a total cumulative dose of TCDD in nine divided doses during the first trimester of pregnancy, McNulty (1984) observed that three animals could not carry their pregnancies to term. Two of these abortions occurred in monkeys that exhibited no overt signs of maternal toxicity, while the third occurred in an overtly affected animal. Given the results of these studies, extrapolation from which is limited by the small number of monkeys

used, it would appear that there is no association between prenatal mortality and maternal toxicity in the monkey even though such an association appears to exist in other mammalian species. Indeed, the studies suggest that prenatal mortality would not be an uncommon occurrence in monkeys at some exposure levels, even when the mother is not overtly affected.

In guinea pigs and monkeys, minimal doses of TCDD that are lethal to the embryo/fetus can in some instances produce no overt toxic effects on the mother. In some cases however, these same doses of TCDD can produce a delayed onset mortality of the dam (Table 5-2). In guinea pigs this is illustrated by the lowest dose of TCDD that significantly increases prenatal mortality, 1.5 μg/kg, being lethal to one of 4 dams (Olson and McGarrigle, 1991). In rhesus monkeys exposed to a total cumulative TCDD dose of 1 μg/kg, 14 of 16 pregnancies were terminated by prenatal mortality, and 20 to 147 days after aborting 8 of 14 females showed signs of maternal toxicity and 3 of these 8 monkeys died (McNulty, 1984; 1985). Nevertheless, in most laboratory mammals, minimal doses of TCDD that produce statistically significant increases in prenatal mortality cause a much higher incidence of mortality to the embryo/fetus than to the dam. In fact, treatment of pregnant rats, rabbits, hamsters and mice with minimal doses of TCDD that result in prenatal mortality does not increase mortality of the dams at all (Table 5-2).

Gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses in most laboratory mammals consisting of thymic hypoplasia, hematologic alterations, subcutaneous edema, decreased fetal growth and prenatal mortality. In addition 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 sign 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

TABLE 5-2

Developmental Toxicity Following Gestational Exposure to 2,3,7,8-TCDD[a]

Species/Strain	Daily Dose[b]	Treatment Days[c]	Sacrifice Day[c]	Maternal Effects[d]	Embryo/Fetal Effects[d]	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 (cont.)

Species/Strain	Daily Dose[b]	Treatment Days[c]	Sacrifice Day[c]	Maternal Effects[d]	Embryo/Fetal Effects[d]	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 (subcutaneous)	6-15	20	none	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
Rhesus monkeys	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[e], 1[e], 1[f] or 5[e] µg/kg	20-40	--	increase in mortality; toxicity	increase in fetal mortality	McNulty, 1985

[a]Source: Couture et al., 1990a
[b]Oral exposure unless otherwise noted
[c]All days adjusted to reflect plug day -- gestation day 0
[d]Effects reported are only those that were statistically significant.
[e]Cummulative dose divided into nine oral doses administered between days 20 and 40 of gestation; two to four monkeys/dose.
[f]Three animals given single oral dose, on either gestation days 25, 30, 35 or 40; 12 monkeys total.
NR = Not reported

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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., 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., 1982), while in the guinea pig and rhesus monkey prenatal mortality is seen (Olson and McGarrigle, 1991; McNulty, 1984).

5.3.1.4 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 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 which stands out as being inconsistent is that 2,2',3,3',4,4'-HCB which has 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).

5.3.1.5. 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

nonTCDD-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 suggested that "exposure to amounts insufficient to produce some effect on the mother probably lessens the chance of fetopathy considerably" (Rogan, 1982). 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 or not there is any association between overt maternal toxicity and embryo/fetal toxicity in 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 incident all of these outcomes were found (Yamashita and Hayashi, 1985; Kuratsune, 1989; Rogan, 1989). Increased prenatal mortality and low birth weight 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 neurobehavioral abnormalities was reported in Yu-Cheng children exposed transplacentally (Rogan et al., 1988; Hsu et al., 1991).

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 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 finger and toe nails, hypersecretion of the meibomian glands, conjunctivitis, gingival hyperplasia, presence of erupted teeth in newborn infants, and 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). Additional effects on human infants that are not related to ectoderm, but resemble effects that have been observed following TCDD exposure in adult monkeys such as subcutaneous edema of the face and eyelids were also reported (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 these effects in human infants with those in adult monkeys exposed to TCDD suggest that the effects in human infants exposed during the Yusho and Yu-Cheng incidents may be caused by exposure to TCDD-like congeners. This possibility is important given the fact that the affected human infants were exposed to a complex mixture of substances that included TCDD-like congeners.

While 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 that the nervous system, like the skin, is derived from embryonic ectoderm (Balinsky, 1970). As will be described in Section 5.3.3.2, 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, some of the Yu-Cheng children that were exposed transplacentally to PCBs, PCDFs and PCQs have affected a clinical impression of developmental or psychomotor delay including impairment of intellectual development (Rogan et al., 1988; Hsu et al., 1991). It is possible to speculate that effects of TCDD-like congeners on the only internal organ derived from ectoderm, the nervous system, are responsible for some of the neurobehavioral effects observed in these children. Additional research is required however, to characterize and elucidate the mechanisms by which TCDD affects the nervous system.

5.3.2. 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 0.0005% of a maternally administered dose reaches the fetal palatal shelves or urinary tract. More specifically, a maternal TCDD dose of 30 μg/kg results in 1.5 pg TCDD/mg in the palatal shelves and 1 pg TCDD/mg in the kidneys 3 days after dosing (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 et al., 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.

Between mammalian species differences exist with respect to susceptibility to the developmental effects of TCDD. While 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).

TABLE 5-3

TCDD Responsiveness of Palatal Shelves From the Mouse, Rat and Human in Organ Culture[a]

Species	Molar Concentration of TCDD		
	Prevention of the Epithelium to Mesenchyme Transformation Process		Cytotoxicity
	LOEL	EC_{100}	
Mouse	$1x10^{-13}$	$5x10^{-11}$	$1x10^{-10}$
Rat[b]	$1x10^{-10}$	$1x10^{-8}$	$1x10^{-7}$
Human[c]	$5x10^{-11}$	$1x10^{-8}$	$1x10^{-7}$

[a]Source: Birnbaum, 1991

[b]At the highest concentration tested, 60% of the palatal shelves failed to undergo programmed cell death.

[c]One of four shelves responded by failing to undergo programmed cell death at $5x10^{-11}$ M.

With respect to the occurrence of similar developmental effects in mammalian species other than the mouse, no other species develops 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).

5.3.2.1. CLEFT PALATE

5.3.2.1.1. 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 ventromedial (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 epithelial cells. The midline seam so formed consists of the two layers of basal cells, all of which remain viable, even though the outer periderm cells die and slough away. As the palatal shelf continues to grow, the bilayer seam, which itself grows at a slower rate, turns into a single layer of cells, and then 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 process involving 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 6-12, 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. Thus, due to the presence of these trapped dead cells, the fusion process was characterized as a programmed cell death (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 not completing the fusion process (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 which prevents the epithelial to mesenchyme transformation process in isolated palatal shelves (LOEL), TCDD concentration that produces a 100% 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 prevent the epithelial to mesenchyme transformation process. 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).

5.3.2.1.2. Evidence for an Ah Receptor Mechanism

5.3.2.1.2.1. 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% incidence of cleft palate in homozygous C57BL/6 (Ah^bAh^b) fetuses, a 13% incidence in heterozygous B6D2F1 (C57BL/6 and DBA/2 hybrid, Ah^bAh^d) fetuses and only a 2% 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 proof positive 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 back crosses between the heterozygous F1 hybrids with each homozygous parental strain.

It has been stated previously (first paragraph of this section), that certain effects of TCDD are well known to segregate with the Ah locus due to the

results of appropriate crosses and back crosses 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 will now be described. It is intended that this information be provided with a considerable degree of detail. This is so that the reader can independently determine whether or not the standard of proof has been satisfied by the evidence available.

In order 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 0-3% incidence of cleft palate formation, whereas four of the five strains with high affinity Ah receptors developed a ≥50% 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). While 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% 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. While 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 or not cleft palate formation segregates with the Ah locus in C57BL/6 and DBA/2 mice by performing simple backcrosses. Instead, they evaluated co-segregation 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%. After similar matings with eight different BXD strains with low affinity Ah receptors, the incidence of TCDF-induced cleft palate formation is <2%. 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 to 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 2-fold 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, 75-100% of all NMRI fetuses develop cleft palates. This is true whether the fetuses remain within the uterus of their natural mother or are transferred into the uterus of a DBA mouse. Under the same conditions, none of the 24 DBA fetuses transferred into an NMRI mother develop a cleft palate, even though 89% of their NMRI litter mates are 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.

5.3.2.1.2.2. Structure Activity -- As TCDD induced cleft palate formation and hydronephrosis in mice appears to be mediated by the Ah receptor, 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 to 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 (Table 5-4), with the notable exception that the apparent binding affinities for the brominated dibenzofurans have not yet been reported.

Other ligands for the Ah receptor that cause cleft palate formation in C57BL/6 mice at nonmaternally toxic doses 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).

Also consistent with the structure-activity relationships for binding to the Ah receptor, 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 di*ortho* and tetra*ortho* 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 mono*ortho* 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 (Table 5-4) (Kannan et al., 1988).

TABLE 5-4

Apparent Ah receptor Binding Affinity and Relative Teratogenic Potency of Halogenated Aromatic Hydrocarbon Congeners[a]

Congener	Apparent Binding Affinity	Relative Potency (ED_{50} TCDD/ED_{50} Congener)	
	EC_{50}[b,c] (mol/L)	Cleft Palate[d]	Hydronephrosis[3]
2,3,7,8-TCDD	1.0×10^{-8}	1.000	1.000
2,3,7,8-TBDD	1.5×10^{-9}	0.235	0.444
2,3,7,8-TBDF		0.100	0.333
2,3,4,7,8-PeCDF	1.5×10^{-8}	0.095	0.057
2,3,7,8-TCDF	4.1×10^{-8}	0.049	0.021
1,2,3,7,8-PeCDF	7.4×10^{-8}	0.026	0.074
1,2,3,4,7,8-HxCDF	2.3×10^{-7}	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	5.0×10^{-6}	0.0000287	0.0000894

[a]Source: Weber et al., 1985; Birnbaum et al., 1987a,b, 1991 and Safe, 1990

[b]Determined for Ah receptor binding in H-4-IIE rat hepatoma cells using ^{3}H-TCDD as the radioactive ligand.

[c]Blank spaces in this column indicate that no EC_{50} value has been reported for the congener in H-4-IIE rat hepatoma cells.

[d]Determined in C57BL/6 mice.

A result that would not be expected according to the structure activity relationships for binding to the Ah receptor is that the di*ortho* chlorine-substituted 2,2',3,3',4,4'-hexachlorobiphenyl causes cleft palate formation and hydronephrosis in mice (Marks and Staples, 1980). However, another di*ortho* chlorine-substituted PCB congener, 2,2',4,4',5,5'-hexachlorobiphenyl, can also 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.

5.3.2.1.3. 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 on days 6-15 of gestation to Sprague-Dawley rats. 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 Fisher 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.

5.3.2.2. HYDRONEPHROSIS

5.3.2.2.1. 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; 1988b). 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 EGF receptor number do not decline. Therefore, in TCDD-treated mice there is a correlation between excessive proliferation of ureteric epithelial cells and increased 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.

5.3.2.2.2. Evidence for an Ah Receptor Mechanism

5.3.2.2.2.1. Genetic -- With respect to involvement of the Ah locus in TCDD-induced hydronephrosis very few genetic studies have been done. Prior to discovery of the Ah locus, however, Courtney and Moore (1971) reported a 62% incidence of hydronephrosis in C57BL/6 mice exposed to a maternal TCDD dose of 3 μg/kg/day on days 6-15 of gestation, whereas the incidence in similarly exposed DBA/2 mice was only 26%. More recently, Silkworth et al. (1989) reported that when TCDD is administered on gestational days 6-15 the incidence of hydronephrosis is dose related. As the maternal dose of TCDD is increased from 0.5-4 μg/kg/day the incidence of hydronephrosis in C57BL/6 mice increases from 31-92%, whereas in DBA/2 mice the incidence varies from 5-37% over the same dose range. In DBA/2 mice the incidence of hydronephrosis increases to 60% when the largest dose of TCDD administered is doubled to 8 μg/kg/day (but does not reach the 92% 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 to that strain (DBA/2) which 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 34-48% in eight strains with high affinity Ah receptors and 3-4% in eight strains with low affinity Ah receptors. These results obtained in the BXD stains 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.

5.3.2.2.2.2. 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 (Table 5-4). This provides further evidence that the Ah receptor mediates the effects of these TCDD-like congeners on the developing mouse kidney.

5.3.2.2.3. 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 >5 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% respectively. This is in contrast to an incidence of <1% in control hamster fetuses. Accordingly, in hamsters hydronephrosis is one of the most sensitive indicators of prenatal toxicity (Olson and McGarrigle, 1991).

5.3.3. Postnatal Effects

5.3.3.1. MALE REPRODUCTIVE SYSTEM OF RATS -- Since TCDD can decrease plasma androgen concentrations and be transferred from mother to young *in utero* and during lactation (Moore et al., 1976; Van den Berg et al., 1987), it is expected to have a great 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 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).

5.3.3.1.1. Perinatal Androgen Deficiency. 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 17-21 of gestation, particularly during the prenatal testosterone surge (days 17-19). On days 18-21 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. Thus, in male rats perinatal exposure to TCDD can produce both prenatal and early postnatal androgenic deficiencies.

5.3.3.1.2. **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% 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% decrease in live birth index (highest dose only), initial 10-15% decreases in body weight (two highest doses) and initial 10-20% 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. No male or female offspring with gross external malformations were found.

5.3.3.1.3. Androgenic Status. Androgenic status of the male offspring which includes such parameters as plasma androgen concentrations and androgen-dependent structures and functions, was reduced by a single maternal TCDD dose as low as 0.16 μg/kg. 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, even when slight decreases in body length were considered. 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 ≤1.7 days.

For accessory sex organs of an adult male rat to grow normally and respond fully to androgens, there is a critical period which starts before birth and lasts until sexual maturity during which adequate concentrations of androgens are necessary (Desjardins and Jones, 1970; Chung and Ferland-Raymond, 1975; Chung and Raymond, 1976; Rajfer and Coffey, 1979; Coffey, 1988). To determine if perinatal TCDD exposure affects postnatal growth of the accessory sex organs, one rat from each litter was sacrificed at 32, 49, 63 and 120 days of age, corresponding to juvenile, pubertal, postpubertal and mature stages of sexual development, respectively. At each developmental stage dose-related decreases in seminal vesicle and ventral prostate weights were found. These decreases could not be explained by decreases in body weight.

There were trends (though not statistically significant) for plasma testosterone and DHT concentrations to be decreased at these times, while plasma LH concentrations were generally unaffected. An exception was a 95% 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. 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). Collectively, these results demonstrate that *in utero* and lactational TCDD exposure decreases androgenic status of male rats from the fetal stage into adulthood. Table 5-5 summarizes these effects (Mably et al., 1991, 1992a).

TABLE 5-5

Effects of *In Utero* and Lactational TCDD Exposure on Indices of Androgenic Status[a]

Index	Lowest Effective Maternal Dose (μg TCDD/kg)[b]	Maximum Effect[c]
Anogenital distance	0.16 (days 1 and 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)
Seminal vesicle weight	0.16 (days 32 and 63)	56% decrease (day 49)
Ventral prostate weight	0.064 (day 32)	60% decrease (day 32)

[a]Source: Mably et al. 1991 and 1992a

[b]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.

[c]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

5.3.3.1.4. 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%. The number of sperm per cauda epididymis was decreased by 75% and 65% 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% 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).

Severe preweaning and/or post-weaning 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). However, reductions in sex organ weights, epididymal sperm reserves and spermatogenesis occurred at the two lowest maternal TCDD doses, neither of which reduced feed intake or body weight of the male offspring. Only at the highest TCDD doses did modest decreases in feed consumption and body weight occur that could contribute to these reproductive system effects (Mably et al., 1992a,c). Thus, undernutrition cannot account for the decreases in spermatogenesis observed at the lower maternal doses of TCDD.

Since 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

TABLE 5-6

Effects of *In Utero* and Lactational TCDD Exposure on Indices of Spermatogenic Function and Reproductive Capability[a]

Index	Lowest Effective Maternal Dose (µg TCDD/kg)[b]	Maximum Effect[c]
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 (day 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]Source: Mably et al. 1991 and 1992c

[b]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.

[c]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

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 49-120 days of age (Mably et al., 1992c).

Plasma testosterone concentrations in the same rats were reduced ≤69% by perinatal TCDD exposure, yet intratesticular testosterone concentrations must be reduced by at least 80% in rats before spermatogenesis is impaired (Zirkin et al., 1989). Based on the magnitude of the reductions in plasma androgen concentrations, it was concluded that corresponding reductions in testicular testosterone production in perinatal TCDD-exposed offspring would probably not be severe enough to impair spermatogenesis (Mably et al., 1992a,c).

In normal rats, daily sperm production does not reach a maximum until 100-125 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, 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, 1 μg/kg, is caused by the same mechanism as that at smaller maternal doses 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 post-leptotene 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.

5.3.3.1.5. 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 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).

5.3.3.1.6. 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). 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 fertility index of the male offspring by 11% and 22%, 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 are 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; number of sperm per ejaculate being close to that required for fertility (Working, 1988). Thus, a percent 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.

5.3.3.1.7. 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 are also involved.

5.3.3.1.7.1. 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 post-ejaculatory interval (Table 5-7). The number of mounts and intromissions (mounts with vaginal penetration) before ejaculation were increased by a maternal TCDD dose of 1.0 μg/kg. The same males exhibited 12- and 11-fold increases in mount and intromission latencies, respectively, and a 2-fold 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% 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. Post-ejaculatory intervals were increased 35% 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.

Since 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. Postpubertal plasma testosterone and DHT concentrations in litter mates of the rats evaluated for masculine sexual behavior were as low as 56% and 62%, respectively, of control (Mably et al., 1991, 1992a). However, plasma testosterone concentrations which were only 33% of control are still sufficient to masculinize sexual behavior of adult male rats (Demassa et al., 1977). Therefore, the modest reductions in adult plasma androgen concentrations

TABLE 5-7

Effects of *In Utero* and Lactational TCDD Exposure on Indices of Sexual Behavior and Regulation of LH Secretion in Adulthood[c]

Index	Lowest Effective Maternal Dose (µg TCDD/kg)[b]	Maximum Effect[c]
MASCULINE SEXUAL BEHAVIOR[d]		
Mount latency	0.16	1200% increase
Intromission latency	0.064	1100% 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
Post-ejaculatory interval	0.40	35% increase
FEMININE SEXUAL BEHAVIOR[e]		
Lordosis quotient[f]	0.16	300% increase
Lordosis intensity score	0.40	50% increase
REGULATION OF LH SECRETION		
LH surge	0.40	460% increase[g]

[a]Source: Mably et al., 1991 and 1992b

[b]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.

[c]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.

[d]Measured when the rats were ~60, 75 and 115 days of age.

[e]Feminine sexual behavior was measured following castration, estrogen priming and progesterone administration. The rats were 170-184 days old.

[f]Number of times lordosis was displayed in reponse to a mount, divided by the number of times each rat was mounted, times 100.

[g]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.

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 post-ejaculatory interval which do not depend on stimulation of the penis for expression (Sachs and Barfeld, 1976). Thus, while 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 12-fold increase in mount latency and increase in post-ejaculatory interval cannot be explained by this mechanism.

5.3.3.1.7.2. **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 6 hours after the progesterone injection on 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% (control) to 54% by the highest maternal TCDD dose, 1.0 μg/kg (Table 5-7). Lordosis intensity scored after Hardy and DeBold (1972) as a (1) for light lordosis, (2) for moderate lordosis and (3) for a full spinal dorsiflexion 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 5-45 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, 0.16 μg/kg, which had no effect on feed intake or body weight. It was concluded that perinatal TCDD exposure feminize sexual behavior in adult male rats independent of undernutrition.

5.3.3.1.7.3. **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).

5.3.3.1.7.4. **Comparison to Other Ah-Receptor Mediated Responses** -- The induction of hepatic 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).

5.3.3.1.7.5. **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.

Treatment of dams on day 15 of gestation with 1.0 μg TCDD/kg significantly decreases plasma testosterone concentrations in male rat fetuses on days 18 and 20 of gestation and in male rat pups 2 hours postpartum (Mably et al., 1992a). Thus, the ability of maternal TCDD exposure to reduce prenatal and early postnatal plasma testosterone concentrations can account, in part, for the impaired sexual differentiation of male rats exposed perinatally to TCDD. Other mechanisms which may potentially contribute to the TCDD-induced impairment in CNS sexual differentiation are: a decrease in the formation of 17ß-estradiol from testosterone within the CNS that is independent of the decrease in plasma testosterone concentrations and/or a reduction in responsiveness of the CNS to estrogen during the critical period of sexual differentiation. The latter mechanism is consistent with the Ah receptor-mediated anti-estrogen action of TCDD described above for rat and mouse uterus and for estrogen responsive MCF-7 and Hepa 1c1c7 cells.

In utero and/or lactational exposure to TCDD may cause similar effects in other animal species, including nonhuman primates (Pomerantz et al., 1986; Thorton and Goy, 1986; Goy et al., 1988), in which sexual differentiation is under androgenic control. In humans social factors account for much of the variation in sexually dimorphic behavior; however, there is 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).

5.3.3.1.8. Relative Sensitivity. The male reproductive system in rats is ~100 times more susceptible to TCDD toxicity when exposure occurs perinatally (ED_{50} 0.16 μg/kg) rather than in adulthood (ED_{50} 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 are also 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).

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. Since 0.064 μg TCDD/kg was the lowest dose tested, a NOAEL for developmental male reproductive toxicity, defined as the lowest dose used that had 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.

5.3.3.2. **NEUROBEHAVIOR** -- Since 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 brain (Carlstedt-Duke et al., 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). No specific information with respect to the presence of Ah receptors at these sites is available. Ah receptors appear to be absent in the human frontal cortex (Silbergeld, 1992).

5.3.3.2.1. **Mice.** CD-1 mice exposed transplacentally to 3,3',4,4'-TCB at a maternal oral dose of 32 mg/kg administered on days 10-16 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 et al. (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 decrease 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 and could potentially involve cholinergeric as well as dopaminergic systems in the brain.

The main problem in applying the above results to TCDD is that the mechanism by which 3,3',4,4'-TCB produces these neurobehavioral effects are not known. The parent compound acting through an Ah receptor might be involved and/or a neurotoxic metabolite of 3,3',4,4'-TCB could be the causative agent. Until the mechanism is resolved, dose-response studies are conducted, and other TCDD-like congeners are evaluated for their ability to produce the effect, the findings cannot be extrapolated with confidence to TCDD. However, 3,3',4,4'-TCB is an Ah receptor agonist and other known developmental effects of this congener are mediated by an interaction of the parent compound with Ah receptors.

5.3.3.2.2. 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).

5.3.3.2.3. 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%) Yu-Cheng children compared with 3 (3%) control children, and of a speech problem in 8 (7%) Yu-Cheng children versus 3 (3%) 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 (Hsu et al., 1991). Also Yu-Cheng children are rated by their parents and teachers to have a higher activity level, more health, habit and behavioral problems, and to have a temperamental clustering closer to that of a "difficult child" (Hsu et al., 1991). It is concluded that in humans transplacental exposure to halogenated aromatic hydrocarbons can affect CNS

function postnatally. However, which congeners, TCDD-like versus nonTCDD-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 nonTCDD-like congeners in causing this toxicity can be understood. Mechanisms that respond uniquely to TCDD-like congeners may not necessarily be involved since 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.

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