4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

The kinetic data on PCDDs have been reviewed (Olson, 1994).

In all vertebrate species studied so far, the 2,3,7,8-substituted PCDDs are almost exclusively retained in all tissue types, particularly fat and liver (Van den Berg *et al.*, 1994).

The penetration of 2,3,7,8-TCDD into human skin *in vitro* has been studied (Weber *et al.*, 1991a). At a dose level of 6.5 ng/cm^2 and using acetone as the vehicle, the absorption rate was about 5 pg/cm^2 per hour. When mineral oil was used as the vehicle, the absorption rate was about 1 pg/cm^2. These values represent a low rate of skin penetration. The stratum corneum appears to act as a reservoir.

The absorption and elimination kinetics of 2,3,7,8-TCDD were investigated in a 42-year-old male volunteer weighing 92 kg who ingested 105 ng [1,6-^{3}H]2,3,7,8-TCDD (13.0 μCi) (Poiger & Schlatter, 1986); > 87% was absorbed and 11.5% of the dose was excreted in the faeces within three days, representing non-absorbed 2,3,7,8-TCDD. Thereafter, the daily faecal excretion amounted to 0.03% of the dose. Based on analytical results in adipose tissue obtained by biopsy and faecal samples from up to 125 days after

dosing, an elimination half-life of 5.8 years was calculated. The data were compatible with first-order elimination kinetics. The maximal excretion of unmetabolized 2,3,7,8-TCDD in the faeces was 50% (Wendling *et al.*, 1990b). In a follow-up of this experiment up to five years after dosage, an elimination half-life of 9.7 years was determined (Schlatter, 1991).

In a study of 36 Viet Nam veterans of Operation Ranch Hand (see Section 1.3.1(*a*)(i), (*a*)(ii)), the decline in serum levels of 2,3,7,8-TCDD indicated a half-life of 7.1 years (Pirkle *et al.*, 1989). In a follow-up examination of 337 Ranch Hand veterans in 1994, a half-life of 11.3 years was found (Wolfe *et al.*, 1994). In a 10-year follow-up study of these Ranch Hand veterans, a half-life estimate of 8.7 years with a 95% CI of 8.0–9.5 years was determined. Half-life increased significantly with increasing body fat, but not with age or relative changes in the percentage of body fat (Michalek *et al.*, 1996).

In a group of 48 workers who were exposed to various PCDDs and PCDFs in a herbicide-producing plant, Boehringer Ingelheim, AG, in Hamburg, Germany (see Section 1.3.1(*a*)(i)), a median half-life of 7.2 years was calculated for 2,3,7,8-TCDD (Flesch-Janys *et al.*, 1996).

In another study with 243 workers exposed to 2,3,7,8-TCDD in a reactor accident in a plant of BASF, AG, Ludwigshafen, Germany, estimated half-lives of 5.1 and 8.9 years were determined for individuals with 20% or 30% body fat, respectively (Ott & Zober, 1996).

In infants, less than 10% of ingested 2,3,7,8-TCDD is excreted in the faeces (Körner *et al.*, 1993; Abraham *et al.*, 1994; Dahl *et al.*, 1995).

When expressed on a total tissue lipid basis, 2,3,7,8-TCDD is distributed equally in the liver and adipose tissue or in the blood and adipose tissue (Leung *et al.*, 1990).

Mammalian (including human) data have been used to formulate kinetic models that have a broad applicability for any pattern of exposure from background to highly toxic levels. Relationships between 2,3,7,8-PCDD congener concentrations in adipose tissue and variations in the proportion of such tissue were established that indicate a need for caution in clearance rate measurements based solely on tissue lipids (Carrier *et al.*, 1995a,b; Van der Molen *et al.*, 1996).

There is a paucity of data on other PCDD congeners. Preliminary kinetic data, based on questionable analytical methods applied to fat samples from one individual exposed to technical pentachlorophenol, were derived for hexa-, hepta- and octa-CDDs (Górski *et al.*, 1984). Half-lives of 3.2–5.7 years were calculated. Flesch-Janys *et al.* (1996) reported median half-lives for six PCDD congeners from 3.7 years (1,2,3,4,6,7,8-HpCDD) to 15.7 years (1,2,3,7,8-PeCDD) in a group of 48 occupationally exposed individuals (see above). Comparison of daily intakes and body burdens of individual congeners with the corresponding data for 2,3,7,8-TCDD yielded estimated elimination half-lives of about five years for 1,2,3,7,8-PeCCD, 15 years for 1,2,3,4,7,8-HxCDD, 25 years for 1,2,3,4,6,7,8-HpCDD and 50 years for OCDD (Schlatter, 1991).

4.1.2 *Experimental systems*

(*a*) *Absorption*

Absorption across the gastrointestinal mucosa depends on the vehicle and on the molecular size and solubility of the congener. These factors seem to be most significant for the hepta- and octa-CDDs. There appear to be only minor differences in absorption between rodent species (Van den Berg *et al.*, 1994). Single-dose exposure studies with 2,3,7,8-TCDD showed 70–90% absorption from the gastrointestinal tract in rats, hamsters and guinea-pigs (Piper *et al.*, 1973; Allen *et al.*, 1975; Rose *et al.*, 1976; Olson *et al.*, 1985; Decad *et al.*, 1990), whereas less than 50% gastrointestinal absorption was found in female C57BL/6J and male ICR/Ha Swiss mouse strains (Koshakji *et al.*, 1984; Curtis *et al.*, 1990). When 2,3,7,8-TCDD was administered in the diet to rats for up to six weeks, absorption was only 50–60% (Fries & Marrow, 1975) compared with 70% following a single dose in corn oil (Piper *et al.*, 1973). No significant influence of age was found for gastrointestinal absorption of 2,3,7,8-TCDD in rats (Hébert & Birnbaum, 1987). Absorption of 1,2,3,7,8-PeCDD is in the same range as that of 2,3,7,8-TCDD (Yoshimura *et al.*, 1986; Brewster & Birnbaum, 1987). The only other PCDD congener of which the gastrointestinal absorption has been studied in some detail is OCDD; only 2–15% of a single oral dose was taken up by rats (Birnbaum & Couture, 1988). For metabolites of 2,3,7,8-TCDD, it has been shown that enterohepatic circulation is not significant in the rat (Ramsey *et al.*, 1982).

The uptake of PCDDs by dermal permeation and pulmonary absorption is much more limited than uptake after oral ingestion (Nessel *et al.*, 1992; Diliberto *et al.*, 1996). For 2,3,7,8-TCDD, dermal permeation is strongly dose-dependent in rats (Banks *et al.*, 1990). Uptake from the application site is slow but also depends on the vehicle used and shows a good inverse correlation with the octanol–water partition coefficient (Brewster *et al.*, 1989; Banks & Birnbaum, 1991; Jackson *et al.*, 1993). The bioavailability after dermal exposure is probably less than 1% for all congeners (Van den Berg *et al.*, 1994).

The adsorption of PCDDs on various environmental matrices can lead to a significant reduction in bioavailability, which depends strongly on the properties of the particles (Nessel *et al.*, 1992; Van den Berg *et al.*, 1994). On the basis of a number of studies with rodents, a bioavailability for PCDDs from soil of 25–50% was suggested for the Cl_4–Cl_6 congeners, while 10% was proposed for the Cl_7 and Cl_8 congeners. For combustion particles (fly ash), a bioavailability of 5–20% was proposed as a realistic estimate for PCDDs, this being high for the hepta- and octa-chlorinated PCDDs (Van den Berg *et al.*, 1994).

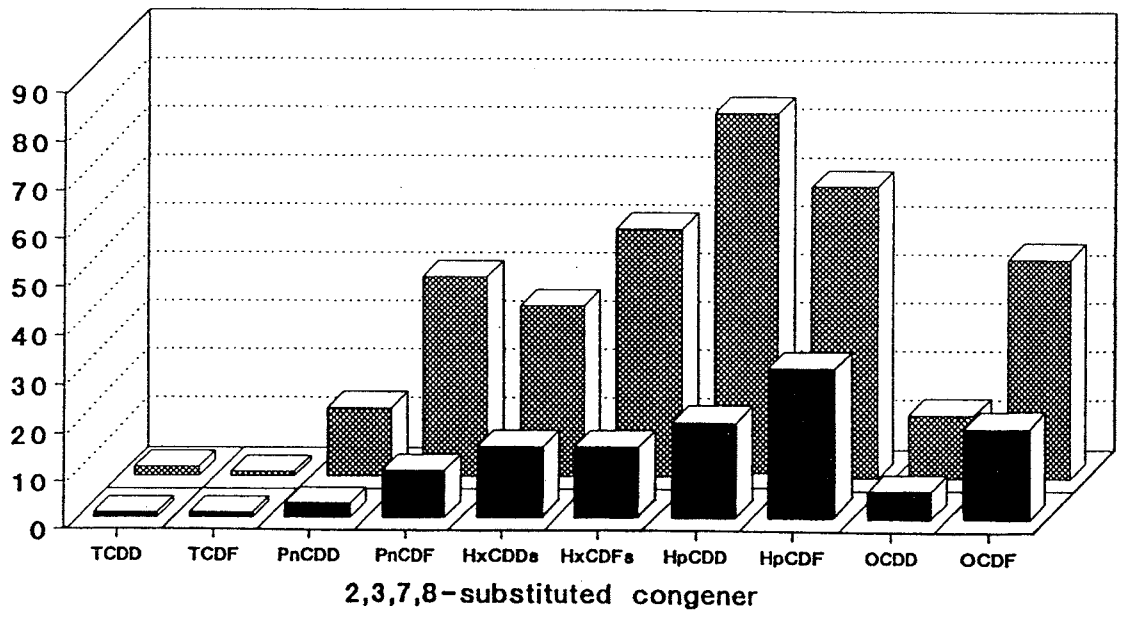
(*b*) *Body distribution*

In all rodent species, the liver and adipose tissue are the major storage sites for PCDDs. However, in certain species, the skin can also act as an important storage site and high concentrations can be found in adrenals. Most detailed information is available for 2,3,7,8-TCDD, but reasonable quantities of data are available to support this primary tissue distribution in rodents for the higher chlorinated congeners. Long retention in tissues is caused primarily by the steric hindrance towards cytochrome P450 activity

caused by chlorine atoms on the 2,3,7,8 positions, resulting in limited metabolism (Van den Berg *et al.*, 1994). Some tissue retention has been observed for certain non-2,3,7,8-substituted PCDDs in rodents and monkeys (Abraham *et al.*, 1989; Neubert *et al.*, 1990a), but the levels should not be considered as toxicologically relevant. Transport in the blood occurs through binding to plasma lipids and lipoproteins, with increasing affinity found for the higher chlorinated congeners in the plasma proteins (Patterson *et al.*, 1989; Schecter *et al.*, 1990h). The majority of 2,3,7,8-TCDD is bound to very low-density lipoprotein, followed by low-density lipoprotein and high-density lipoprotein (Marinovich *et al.*, 1983).

Depending on the experimental conditions, 25–70% of the administered dose of 2,3,7,8-TCDD is stored in the liver of rats, mice, hamsters and guinea-pigs approximately one day after exposure. Studies with mixtures of PCDDs showed that in rodents the Cl_5 and Cl_6 congeners have higher hepatic retention than 2,3,7,8-TCDD (Van den Berg *et al.*, 1994). As a result, the liver to adipose tissue concentration ratio strongly increases with increasing chlorination. Measurement of tritiated 2,3,7,8-TCDD administered in corn oil by gastric instillation to adult female rhesus monkeys, infant male rhesus monkeys and young adult male Sprague-Dawley rats indicated that liver retention of the dose was > 40% in rats, but < 10% in the rhesus monkeys. A large percentage of the dose was located in monkey tissues with a high lipid content, especially the skin, muscle and adipose tissue. In the rats, these tissues had much lower levels of 2,3,7,8-TCDD (Van Miller *et al.*, 1976). Differences in liver to adipose tissue ratio between rats and marmosets (*Callithrix jacchus*) have been observed (see **Figure 2**) (Olson, 1994).

Figure 2. The liver-to-adipose tissue ratios for 2,3,7,8-substituted PCDDs and PCDFs in marmosets and rats



From Olson (1994)
Grey bars: rat; black bars: marmoset

Hepatic disposition of 2,3,7,8-TCDD is dose-dependent (Kociba *et al.*, 1978; Abraham *et al.*, 1988); this has been suggested to be caused by the presence in the liver of inducible protein binding sites, at least in mice (Poland *et al.*, 1989). As a possible mechanism, binding to CYP1A2 in the liver has been proposed (Voorman & Aust, 1987; Poland *et al.*, 1989; Voorman & Aust, 1989). In rats, 2,3,7,8-TCDD was equally distributed between hepatic P9 (mitochondrial, lysosomal, nuclear) and S9 (cytosol and microsomal) fractions, localization in the S9 being in microsomes. In extrahepatic tissues (lung/kidneys), 2,3,7,8-TCDD was concentrated in the P9 fraction, while in the S9 fraction localization was in the cytosol. Use of CYP1A1 and CYP1A2 immunoreactive proteins and measurement of marker enzyme activities, ethoxyresorufin *O*-deethylase and methoxyresorufin *O*-demethylase, respectively, showed that CYP1A1 was induced in microsomes of all three organs, while CYP1A2 was induced only in liver (Santostefano *et al.*, 1996). However, the role of other dioxin (inducible) hepatic binding proteins cannot be excluded (Landers *et al.*, 1990; Buckley-Kedderis *et al.*, 1992; Tritscher *et al.*, 1992; Van den Berg *et al.*, 1994).

Pre- and postnatal transfer of PCDDs has been studied in rats and mice. Lactational transfer was found to be one or two orders a magnitude higher than placental transfer in these rodents (Nau & Bass, 1981; Weber & Birnbaum, 1985; Nau *et al.*, 1986; Van den Berg *et al.*, 1987; Li *et al.*, 1995a). The level of placental transfer depends on the molecular size, with the highest fetal retention observed for 2,3,7,8-TCDD (Van den Berg *et al.*, 1987). In the mouse embryo, the liver is the major storage site, with two to five times higher concentrations than those found in other tissues (Nau & Bass, 1981; Krowke & Neubert, 1990). In contrast, there is no evidence for hepatic concentration in the fetal rat liver. From a quantitative point of view, lactational transfer in marmosets and rhesus monkeys is also more important than placental transport, but placental transport in marmosets can result in significant accumulation in fetal tissues (Hagenmaier *et al.*, 1990b; Krowke *et al.*, 1990). On the basis of studies with complex mixtures of PCDDs and PCDFs, it appears that these compounds are mobilized primarily from adipose tissue and not from the liver in rats (Van den Berg *et al.*, 1987). In marmosets, lactational transfer can lead to concentrations in the tissues of offspring that are equal to or higher than those found in maternal tissues. In both rats and marmosets, lactational transfer decreases with increasing number of chlorine atoms in the PCDD molecule (Van den Berg *et al.*, 1987; Krowke *et al.*, 1990).

(*c*) *Metabolism*

The metabolism of PCDDs has been studied primarily in rodents. In rats, oxidation by P450 occurs preferentially at the lateral, 2, 3, 7 and 8 positions, yielding primarily mono- and dihydroxylated metabolites. Furthermore, sulfur-containing metabolites have been identified, which probably arose through glutathione conjugation (Tulp & Hutzinger, 1978). Studies with 2,3,7,8-TCDF in rats suggest that CYP1A1 is directly involved in phase I metabolism of these compounds and not CYP1A2 (Tai *et al.*, 1993). If all of the 2, 3, 7 and 8-positions are substituted with chlorine atoms, metabolic conversion of the molecule is strongly hindered. Recent quantum-mechanical calculations determining highest occupied molecular orbital (HOMO) energy levels for the dibenzo-*para*-dioxin

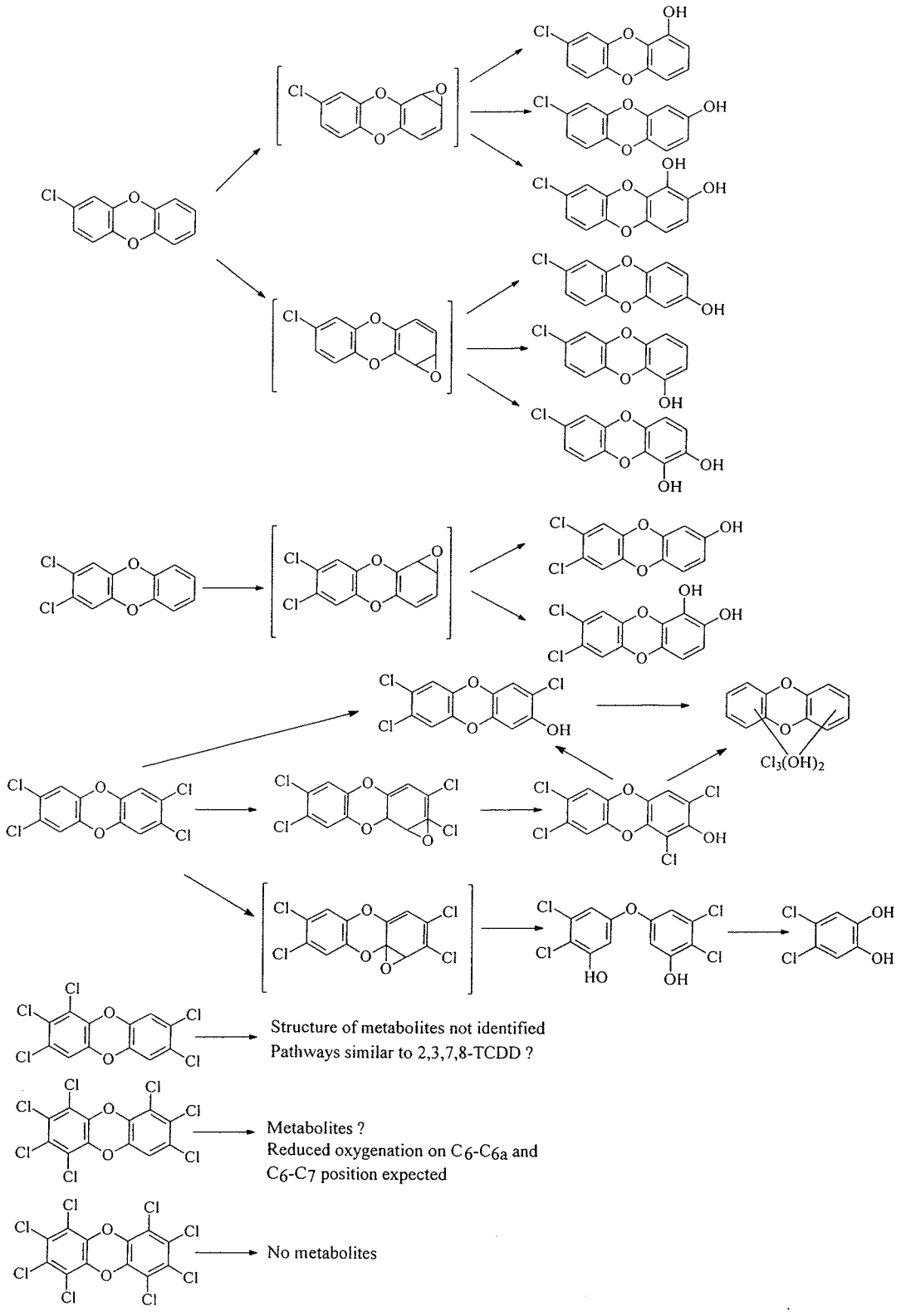
molecule have confirmed the higher susceptibility for epoxidation of the 2–3 or 7–8 positions in the molecule (Weber *et al.*, 1996). Although 2,3,7,8-TCDD is highly resistant towards biotransformation, small amounts of metabolites have been identified in rats and dogs. Metabolic transformation included oxidative and reductive dechlorination, including NIH shifts (shift of a chlorine substituent). In addition, oxygen bridge cleavage was found to be another important pathway (Poiger & Schlatter, 1979; Ramsey *et al.*, 1982; Poiger & Buser, 1984; Huwe *et al.*, 1996). Conjugation appears to be important for the elimination of PCDDs from the body, as many 2,3,7,8-TCDD metabolites were present as glucuronide conjugates in the bile of the rat (Poiger & Buser, 1984; Wroblewski & Olson, 1985; Huwe *et al.*, 1996). From a qualitative point of view, metabolic pathways are fairly similar among rodent species, but large quantitative differences can be observed (Van den Berg *et al.*, 1994; Larsen *et al.*, 1996). Guinea-pigs show a lower metabolic capacity than rats, Syrian hamsters and mice (Wroblewski & Olson, 1985). Limited information is available about the metabolic conversion of higher chlorinated 2,3,7,8-substituted PCDDs, but it can be expected that pathways will be similar to those for 2,3,7,8-TCDD, provided that susceptible positions are not sterically hindered by additional chlorines. In **Figure 3**, a generalized scheme of metabolic pathways for PCDDs is given (Van den Berg *et al.*, 1994).

(d) Excretion

The elimination of PCDDs has been studied in several laboratory species under a variety of experimental conditions (Van den Berg *et al.*, 1994). After exposure, the compounds are usually more rapidly eliminated from blood and muscle tissue than from liver and adipose tissue. In some species, such as primates, elimination from the skin is similar to that from the adipose tissue (Birnbaum *et al.*, 1980; Brewster & Birnbaum, 1987; Brewster *et al.*, 1988, 1989). For most body compartments, the elimination of 2,3,7,8-substituted PCDDs can be described by a one-compartment open model (Hiles & Bruce, 1976; Rose *et al.*, 1976), but models with two- or three-phase elimination have also been applied. In view of the non-linear distribution of PCDDs in many experimental systems, the use of physiologically based pharmacokinetic models has been successfully applied (Carrier *et al.*, 1995a,b). In almost all laboratory species which have been studied, elimination takes place through bile and faeces in the form of hydroxylated or conjugated metabolites that are rapidly eliminated from the body (Van den Berg *et al.*, 1994). Guinea-pigs seem to be an exception among rodents, as this species eliminates 2,3,7,8-TCDD more than its polar metabolites (Olson, 1986).

For 2,3,7,8-TCDD, whole body half-lives in the rat have been reported to range between 17 and 31 days, depending on the strain and experimental conditions used. Most studies with rodents have found similar elimination rates from the liver and adipose tissue for 2,3,7,8-TCDD (Piper *et al.*, 1973; Allen *et al.*, 1975; Fries & Marrow, 1975; Rose *et al.*, 1976; Abraham *et al.*, 1988, 1989; Pohjanvirta *et al.*, 1990a). In rats, lactation is a very effective route of elimination of 2,3,7,8-TCDD, leading to a 50% reduction of the half-life from the liver (Abraham *et al.*, 1988; Korte *et al.*, 1990). The half-life of 1,2,3,7,8-PeCDD elimination in the rat is approximately 30 days, which is

Figure 3. A generalized scheme of pathways for the biotransformation of PCDDs based on the information from mammalian studies *in vivo*



From Van den Berg *et al.* (1994)

similar to that of 2,3,7,8-TCDD (Wacker *et al.*, 1986). However, with more chlorine atoms present in a 2,3,7,8-substituted PCDD, elimination is much slower. For the Cl_6–Cl_8 congeners, half-lives between 75 days and seven years have been calculated for the rat (Birnbaum & Couture,1988; Van den Berg *et al.*, 1989a, 1994). In the mouse, half-lives for 2,3,7,8-TCDD are in the same range as those in the rat, but there are distinct strain differences (Gasiewicz *et al.*, 1983; Birnbaum, 1986). Thus, the half-life of 2,3,7,8-TCDD in the non-responsive DBA strain is approximately twice as long as that in the responsive C57BL strain (Gasiewicz *et al.*, 1983). In the Syrian hamster, the half-life of 2,3,7,8-TCDD is two- to three-fold lower than in the rat (Olson *et al.*, 1980). Although this faster elimination of 2,3,7,8-TCDD in the Syrian hamster might contribute to the relative insensitivity of this species towards acute toxicity, it can be assumed that the 100-fold difference in sensitivity between Syrian hamsters and other rodents is dominated more by genetic background than kinetics (Van den Berg *et al.*, 1994). The half-life of 2,3,7,8-TCDD elimination in guinea-pigs has been estimated as 30 days (i.e., the same as in rats) (Gasiewicz & Neal, 1979) and 94 days (Olson, 1986). No clear reason for the discrepancy was discovered.

The half-life of 2,3,7,8-TCDD is much longer in primate species than in rodent species. In seven adult female rhesus monkeys, an average half-life of about 391 days was determined after four years of dietary exposure to 25 ng/kg diet (0.67 ng/kg bw/day). Lactation during four months resulted in a 21% decrease of the maternal body burden. During the first year after birth, the half-life of 2,3,7,8-TCDD in breast-fed rhesus monkeys was approximately 181 days, which was significantly lower than that observed in the mothers (Bowman *et al.*, 1989). In marmosets, longer half-lives for the 2,3,7,8-substituted PCDDs than in rodents were also observed. In liver and adipose tissue, half-lives from eight weeks up to several years were measured (Neubert *et al.*, 1990a).

During pregnancy, elimination from the dam is approximately twice as fast as in the non-pregnant female (Weber & Birnbaum, 1985).

(*e*) *Kinetics and toxicity*

Kinetics to some extent influence the toxicity of individual PCDD and PCDF congeners. This was illustrated in experiments with B6C3F1 mice dosed with 2,3,7,8-TCDD or 2,3,7,8-TCDF, in which CYP1A1 activity was found to depend on the time period to acquire a steady-state situation (DeVito & Birnbaum, 1995). In addition, total body fat content in various species and strains of laboratory animals may contribute to some of the observed differences in species sensitivity for 2,3,7,8-TCDD (Geyer *et al.*, 1990). However, the available data seem to imply that kinetics are not a governing factor influencing the toxicity of these compounds, but genetic factors seem to predominate (Van den Berg *et al.*, 1994).

4.2 Toxic effects

4.2.1 *Humans*

Human exposure to 2,3,7,8-TCDD has been associated with many toxic effects other than cancer. The majority of these effects has been reported among occupationally exposed groups, such as chemical production workers, pesticide users and individuals who handled or were exposed to materials treated with 2,3,7,8-TCDD-contaminated pesticides, and among residents of areas contaminated with tainted waste oil and industrial effluent. These effects represent a complex network of responses ranging from changes in hepatic enzyme levels to observable alterations in the character and physiology of the sebaceous gland, as in chloracne. The Working Group noted that populations are exposed to a variety of chemicals and for some outcomes, it is difficult to separate the effects of the combined exposures. More comprehensive descriptions of studies cited in this section are included in other sections of this monograph (Taylor, 1979; Calvert *et al.*, 1992).

(*a*) *Chloracne and other effects on the skin*

Chloracne is a persistent acneiform condition characterized by comedones, keratin cysts and inflamed papules with hyperpigmentation and an anatomical distribution frequently involving the skin under the eyes and behind the ears. It occurs after acute or chronic exposure to a variety of chlorinated aromatic compounds by skin contact, ingestion or inhalation (Crow, 1978; Moses & Prioleau, 1985). This acne-like condition is reported to have occurred with or without other effects in at least a few workers after all reported accidents at TCP production facilities (Ashe & Suskind, 1950; Goldman, 1972; May, 1973; Zober *et al.*, 1990), among individuals involved in daily production of 2,3,7,8-TCDD-contaminated products (Bleiberg *et al.*, 1964; Poland *et al.*, 1971; Pazderova-Vejlupkova *et al.*, 1981; Moses *et al.*, 1984; Suskind & Hertzberg, 1984; Moses & Prioleau, 1985; Bond *et al.*, 1989b), among three laboratory workers exposed to pure 2,3,7,8-TCDD (Oliver, 1975) and among at least 193 (0.6%) Seveso residents, mostly children (at least 20% among children aged 0–14 years from Zone A) (Reggiani, 1978; Caramaschi *et al.*, 1981; Ideo *et al.*, 1985; Mocarelli *et al.*, 1986; Assennato *et al.*, 1989). Chloracne was not found among Missouri residents (Hoffman *et al.*, 1986; Webb *et al.*, 1989) examined 10 years after exposure or among Ranch Hand personnel (Roegner *et al.*, 1991). In United States Army Viet Nam veterans, chloracne-like skin lesions were rarely observed on examination (0.9% in Viet Nam veterans versus 0.8% in non-Viet Nam veterans; odds ratio, 1.4; 95% CI, 0.7–2.9) (Centers for Disease Control Vietnam Experience Study, 1988a).

Chloracne appears shortly after exposure to 2,3,7,8-TCDD-contaminated chemicals. In Seveso, the eruption of comedones, usually accompanied by cysts, was observed between two weeks and two months after the reactor release (Reggiani, 1980), and within six months of the explosion, 34 cases of chloracne were identified among children (Caramaschi *et al.*, 1981). In chemical workers involved in the TCP reactor release at the BASF plant in Ludwigshafen, Germany, most cases of chloracne developed within two days after first exposure (Zober *et al.*, 1990; Ott *et al.*, 1994). One case of chloracne

developed only two years after the accident, but the authors suggest that the etiology of this case is unclear.

Among Seveso residents, despite high serum 2,3,7,8-TCDD levels, the chloracne resolved in all but one person by 1983 (Assennato *et al.*, 1989; Mocarelli *et al.*, 1991). However, for TCP workers at the Nitro, WV, USA, plant, Moses *et al.* (1984) reported that the mean duration of chloracne was 26.1 ± 5.9 years.

Positive associations between serum and adipose tissue levels of 2,3,7,8-TCDD and other PCDDs and the risk of chloracne among chemical production workers have been reported (Ott *et al.*, 1987; Beck *et al.*, 1989b; Bond *et al.*, 1989b); the risk was also shown to be greater for subjects of young age at exposure, long exposure duration and a history of employment in production areas of high potential exposure.

Mocarelli *et al.* (1991) described chloracne in people from Seveso Zone A who had 2,3,7,8-TCDD levels in serum lipids ranging from 828 to 56 000 ng/kg (sampled within one year of the reactor release) (see **Table 21**). The study also included other individuals from Zone A, but without chloracne, who had serum 2,3,7,8-TCDD levels that ranged from 1770 to 10 400 ng/kg. With the exception of one person with chloracne who was 16 years old at the time of the accident, all of the cases were in children under the age of 11 years. This contrasts with higher serum levels measured in some adult chloracne subjects among German production workers (**Table 44**). Cases had estimated adipose levels of greater than 200 ng/kg 2,3,7,8-TCDD and over 2000 ng/kg lipid HxCDD at the time of diagnosis. However, a threshold level above which chloracne occurs has not been established.

Other dermatological alterations, including hypertrichosis and hyperpigmentation, have been reported among workers exposed to 2,3,7,8-TCDD in the United States (West Virginia and New Jersey) (Ashe & Suskind, 1950; Bleiberg *et al.*, 1964; Poland *et al.*, 1971), Germany (Bauer *et al.*, 1961; Goldman, 1972) and Czechoslovakia (Jirasek *et al.*, 1974). Increased prevalence of actinic or solar elastosis was observed only in West Virginia TCP workers (59.1% exposed versus 30.1% unexposed; $p < 0.01$). In the same exposed population, three cases of Peyronie's disease (a rare progressive scarring of the penile membrane) were identified (Suskind & Hertzberg, 1984).

(*b*) *Hepatic effects*

γ-Glutamyltransferase

The studies of Seveso children demonstrate an increase in γ-glutamyltransferase (GGT) levels that occured shortly after the explosion and then a gradual decline to near normal levels within five years (Caramaschi *et al.*, 1981; Mocarelli *et al.*, 1986).

Levels of GGT were also found to be elevated among some TCP production workers in the United Kingdom and the USA (West Virginia, Missouri and New Jersey) up to 30 years after last exposure to 2,3,7,8-TCDD-contaminated chemicals (May, 1982; Martin, 1984; Moses *et al.*, 1984; Calvert *et al.*, 1992). However, compared with controls, GGT was not elevated in another study of West Virginia workers (Suskind & Hertzberg, 1984).

Table 44. Chloracne and adipose tissue levels of 2,3,7,8-TCDD and HxCDD in German chemical workers

Population	2,3,7,8-TCDD level (ng/kg)[a]	HxCDD level (ng/kg)[a]	Year of chloracne diagnosis	Half-life extrapolated 2,3,7,8-TCDD[b] (ng/kg)	Half-life extrapolated HxCDD[b] (ng/kg)
Chemical workers[c]	174	247	1955	7 050	10 000
	99	166	1955	4 010	6 720
	147	5 101	1963	2 350	81 620
	61	172	[1955]	2 470	6 970
	50	517	1969	380	3 940
	16	58	1955	650	2 360
	1 280	1 019	1978	3 380	2 690
	49	3 442	[1974]	210	14 760
	50	9 613	[1972]	260	50 740
	2 252	3 087	1984	2 850	3 910
	158	1 191	[1977]	460	3 490
	6	283	1970	40	1 970

From Beck *et al.* (1989b)

Data on serum 2,3,7,8-TCDD levels in Seveso residents with or without chloracne are presented in Table 21.

[a] ng/kg lipid

[b] Half-life extrapolation calculated by authors (Beck *et al.*, 1989b) using the formula $C_0 = C_t \times 2^n$ where C_0 = original concentration of 2,3,7,8-TCDD or HxCDD, C_t = concentration at time t, n = number of half-life periods, and t = half-life of 5.8 years. Exposures occurred between 1949 and 1986.

[c] Measured in 1986

In a study of TCP production by Calvert *et al.* (1992), the increases in GGT were limited to workers with high serum 2,3,7,8-TCDD levels (> 100 ng/kg) and those with lifetime alcohol consumption of more than 30 alcohol–years (alcohol–year = 1 alcoholic beverage per day for one year). Contributions of other potentially confounding exposures were not explored.

Both the Viet Nam Experience Study and the United States Air Force Ranch Hand Study found statistically significant elevations in GGT levels among veterans (Centers for Disease Control Vietnam Experience Study, 1988a; Roegner *et al.*, 1991).

Aspartate aminotransferase and alanine aminotransferase

Reports on the Seveso children and on TCP production workers have found elevations in serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) that appear to be transient effects of acute exposure to 2,3,7,8-TCDD (May, 1973; Jirasek *et al.*, 1974; Caramaschi *et al.*, 1981; Mocarelli *et al.*, 1986). Epidemiological studies conducted 10–30 years after last exposure found no effects in exposed workers, Viet Nam veterans and Missouri residents compared with unexposed control groups (May, 1982; Bond *et al.*, 1983; Martin, 1984; Suskind & Hertzberg, 1984; Hoffman *et al.*,

1986; Centers for Disease Control Vietnam Experience Study, 1988a; Webb *et al.*, 1989; Roegner *et al.*, 1991; Calvert *et al.*, 1992; Ott *et al.*, 1994; Grubbs *et al.*, 1995) or in workers with or without chloracne (Moses *et al.*, 1984).

ALT was increased in five of 14 TCP workers from the United Kingdom who were inside a manufacturing building at the time of a TCP reactor explosion in 1968, but no elevation in AST or ALT was found when workers from the same facility were re-evaluated later (May, 1982).

In Seveso children, levels of ALT were elevated in those with chloracne (Caramaschi *et al.*, 1981).

D-Glucaric acid

D-Glucaric acid (DGA) excretion is an indirect but valid indicator of hepatic microsomal activity.

Ideo *et al.* (1985) reported that DGA excretion was significantly elevated in adults residing in Seveso, Italy, at the time of the reactor explosion compared with residents of unexposed communities (Seveso, 27.1 μmol/g creatinine versus unexposed, 19.8 μmol/g creatinine; $p < 0.05$). In 1976, DGA excretion was significantly higher in children from Zone A with chloracne (39 μmol/g creatinine) compared with those without chloracne (20.5 μmol/g creatinine). However, by 1981, levels were within the normal range.

A significantly higher DGA : creatinine ratio was also observed in exposed TCP workers tested within one year after exposure to 2,3,7,8-TCDD ceased and 10 years after a TCP reactor explosion (Martin, 1984).

Other studies did not find increased DGA excretion in exposed populations 10–37 years after last exposure to 2,3,7,8-TCDD-contaminated chemicals (Roegner *et al.*, 1991; Calvert *et al.*, 1992).

[In summary, none of the studies reporting elevations in ALT, AST or DGA identified clinical evidence of liver disease in the study populations. Therefore, it is possible that the increases in ALT and AST levels and DGA excretion are related to high-level, acute exposure to 2,3,7,8-TCDD-contaminated chemicals and that, barring additional exposure, the levels decrease with time.]

Porphyrin metabolism

Whether 2,3,7,8-TCDD is associated with porphyrin changes in humans, particularly porphyria cutanea tarda (PCT), is a subject of some debate. PCT is a form of acquired or inherited porphyria caused by a deficiency of the enzyme uroporphyrinogen decarboxylase and the resulting overproduction and excretion of uroporphyrin (Sweeney, 1986). The predominant characteristics of PCT include skin fragility, blistering upon sun exposure, dark pigmentation, excess hair growth, hepatomegaly, reddish-coloured urine, and urinary excretion of uro- and heptacarboxyporphyrins (Strik, 1979).

In 1964, increased uroporphyrins, urinary coproporphyrins and urobilinogen were found in 11 of 29 TCP production workers in New Jersey (Bleiberg *et al.*, 1964). In the NIOSH study (which included the same cohort as reported by Bleiberg *et al.*, 1964)

(Calvert *et al.*, 1994), no difference was found between workers and an unexposed control group in the prevalence of PCT (odds ratio, 0.9; 95% CI, 0.2–4.5) and there were no differences in the risk between workers and the control group for an out-of-range uroporphyrin concentration or an out-of-range coproporphyrin concentration. Because this study was conducted at least 15 years after last occupational exposure to 2,3,7,8-TCDD, it was not possible to determine whether porphyrinuria occurred during the earlier years after exposure. Changes in porphyrin levels were measured in only one other study of workers exposed to 2,3,7,8-TCDD, the study of West Virginia TCP workers, in which no evidence of porphyrinuria was observed (Moses *et al.*, 1984).

Sixty Seveso residents were tested for elevated porphyrins in 1977 and again in 1980. None developed PCT. However, 13 (22%) exhibited secondary coproporphyrinuria, five of whom showed a slight increase of urocarboxyporphyrins, heptacarboxyporphyrins and coproporphyrins classified as a 'transition constellation to chronic hepatic porphyria type A'. In 1980, porphyrin levels had returned to normal in 12 individuals (Doss *et al.*, 1984).

Lipid levels

A number of case reports and epidemiological studies have described increases in levels of serum lipid fractions, particularly total cholesterol and triglycerides, in TCP production workers, laboratory workers, Seveso and Missouri residents and Viet Nam veterans. Others report no differences between subject and reference levels. A summary of the reported levels is presented in **Tables 45 and 46**.

Total cholesterol

The majority of epidemiological studies of workers and community residents have reported no significant increases in total cholesterol levels among exposed populations compared with controls (Moses *et al.*, 1984; Suskind & Hertzberg, 1984; Hoffman *et al.*, 1986; Mocarelli *et al.*, 1986; Assennato *et al.*, 1989; Webb *et al.*, 1989; Ott *et al.*, 1994; Calvert *et al.*, 1996). However, in one study of British TCP production workers, one year after exposure to 2,3,7,8-TCDD had ceased, total cholesterol levels in exposed workers with chloracne (6.02 mmol/L) and without chloracne (6.14 mmol/L) were significantly elevated compared with those of unexposed controls (5.6 mmol/L) (Martin, 1984) (**Table 45**).

Similarly, a comparison of workers with persistent chloracne, no chloracne, or a history of chloracne revealed a significant association ($p < 0.05$) between the proportion of out-of-range low-density lipoprotein cholesterol values and persistent chloracne (Suskind & Hertzberg, 1984).

Among United States Army veterans, there was no difference in total cholesterol levels between groups who served in Viet Nam or in other countries (Centers for Disease Control Vietnam Experience Study, 1988a). In the United States Air Force Ranch Hand 1987 study, there was a statistically significant positive relationship between serum 2,3,7,8-TCDD levels above 33.3 ng/kg and total cholesterol levels (Roegner *et al.*, 1991). In the 1992 analysis, the difference was not observed (Grubbs *et al.*, 1995).

Table 45. Mean total cholesterol levels among Seveso and Missouri residents, TCP production workers, BASF accident cohort and Viet Nam veterans

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Mocarelli *et al.* (1986)	Seveso children				
	1977	16[a]	4.62 (95% CI, 3.26–5.98)	28[a]	4.45 (95% CI, 3.12–5.77)
	1982	182[a]	4.48 (95% CI, 2.97–5.99)	250[a]	4.41 (95% CI, 2.99–5.83)
Caramaschi *et al.* (1981)	Seveso children	138	15.2[b,c]	120	12.5[b,d]
Assennato *et al.* (1989)	Seveso residents				
	1976	193[c]	4.78 ± 0.99	–	–
	1982–1983	152[c]	4.06 ± 0.80	123[d]	4.14 ± 0.77
	1983–1984	142[c]	4.09 ± 0.88	196[d]	4.12 ± 0.86
	1985	141[c]	4.14 ± 0.91	167[d]	4.13 ± 0.78
May (1982)	TCP production workers in the United Kingdom	41[c]	5.97	31	6.6
Martin (1984)	TCP production workers in the United Kingdom	39[c]	6.02[e]	126	5.6
Poland *et al.* (1971)	TCP production workers in New Jersey	71	6.12 ± 1.14	–	–
Moses *et al.* (1984)	TCP production workers in West Virginia	105[c]	5.38 ± 0.88	101[d]	5.37 ± 0.85

Table 45 (contd)

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Suskind & Hertzberg (1984)	TCP production workers in West Virginia	200	5.46 ± 0.07	163	5.28 ± 0.08
	TCP production workers: chloracne versus never chloracne	105[c]	5.44 ± 0.08	28[d]	5.30 ± 0.18
Ott *et al.* (1994)	BASF accident cohort	135	6.14[f] ± 1.01	6 581	6.37[f] ± 1.17
Calvert *et al.* (1996)	TCP and 2,4,5-T production workers in Missouri and New Jersey	273	5.7[g,i]	259	5.6[g,i]
Hoffman *et al.* (1986)	Missouri residents in mobile home park	142	4.97[e] ± 0.96	148	5.2 ± 1.09
Webb *et al.* (1989)	Missouri residents				
	< 20 ng/kg[h]	16	5.88 ± 1.10	–	–
	20–60 ng/kg[h]	12	6.60 ± 0.93	–	–
	> 60 ng/kg[h]	12	6.76 ± 0.97	–	–
CDC Viet Nam Experience Study (1988a)	United States Army Viet Nam veterans	2 490	5.43[i,j]	1 972	5.36[i,j]
Roegner *et al.* (1991)	Ranch Hand personnel				
	Unknown ≤ 10 ng/kg[k]	338[l]	5.53	777	5.51
	Low 15–≤ 33.3 ng/kg[k]	191	5.55	–	–
	High > 33.3 ng/kg[k]	182	5.68[e]	–	–

Table 45 (contd)

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Grubbs *et al.* (1995)	Ranch Hand personnel				
	Background[n]	362	5.71[m]	1 025	5.69
	Low[n]	251	5.68[m]		
	High[n]	251	5.76[m]		

Data are means ± SD unless otherwise specified
[a] Number of samples
[b] % abnormal
[c] Chloracne
[d] No chloracne
[e] $p < 0.05$
[f] Adjusted for age, body mass index, smoking history
[g] Adjusted for age, smoking, current diabetes
[h] Adipose tissue levels of 2,3,7,8-TCDD in ng/kg of lipid
[i] Geometric mean
[j] % abnormal: Viet Nam veterans, 5.1; non-Viet Nam veterans, 4.7; odds ratio, 1.1; 95% CI, 0.8–1.5
[k] Serum 2,3,7,8-TCDD levels in ng/kg of lipid
[l] Contrasted to unexposed comparisons
[m] Adjusted mean
[n] Serum 2,3,7,8-TCDD levels in ng/kg of lipid; background: current level ≤10 ng/kg of lipid; low: current level > 10 ng/kg of lipid, 10 ng/kg < initial level ≤ 143 ng/kg of lipid; high: current level > 10 ng/kg of lipid, 10 ng/kg < initial level > 143 ng/kg of lipid

Table 46. Mean triglyceride levels among Seveso children and Missouri residents, TCP production workers, BASF accident cohort and Viet Nam veterans

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Mocarelli *et al.* (1986)	Seveso children				
	1977	38[a]	0.97 (95% CI, 0.60–1.50)	36[a]	0.95 (95% CI, 0.63–1.51)
	1982	207[a]	0.91 (95% CI, 0.52–1.60)	257[a]	0.86 (95% CI, 0.86–1.56)
Assennato *et al.* (1989)	Seveso residents				
	1976	193[b]	0.99 ± 0.43	–	–
	1982–1983	152[b]	0.87 ± 0.40	123[c]	0.85 ± 0.37
	1983–1984	142[b]	0.94 ± 0.59	196[c]	0.88 ± 0.46
	1985	141[b]	0.84 ± 0.44	167[c]	0.87 ± 0.55
May (1982)	TCP production workers, United Kingdom	41[b]	2.03	31[c]	1.83
Martin (1984)	TCP production workers, United Kingdom	39[b]	1.97[d] (95% CI, 0.4–4.0)	126[c]	1.41 (95% CI, 0.3–3.2)
Moses *et al.* (1984)	TCP production workers, West Virginia	93[b]	1.69[e] ± 1.26	93[c]	1.46 ± 0.73
Suskind & Hertzberg (1984)	TCP production workers, West Virginia	200	1.65 ± 0.08	163	1.76 ± 0.08
Ott *et al.* (1994)	BASF accident cohort	135	1.91[f] ± 1.19	4 471	1.97[f] ± 1.65

Table 46 (contd)

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Calvert *et al.* (1996)	TCP and 2,4,5-T production workers, Missouri and New Jersey	273	1.20[g,j]	259	1.15[g,j]
Hoffman *et al.* (1986)	Missouri residents in mobile home park	141	1.07 ± 0.73	146	1.19 ± 1.07
Webb *et al.* (1989)	Missouri residents				
	< 20 ng/kg[h]	16	2.17 ± 2.08	–	–
	20–60 ng/kg[h]	12	1.81 ± 1.19	–	–
	> 60 ng/kg[h]	12	2.69 ± 1.06	–	–
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2 490	1.06[i,j]	1 972	1.05[i,j]
Roegner *et al.* (1991)	Ranch Hand personnel				
	Unknown ≤ 10 ng/kg[k]	338	1.02[d,l]	777	1.16
	Low 15–≤ 33.3 ng/kg[k]	191	1.37[d,l]		
	High > 33.3 ng/kg[k]	182	1.35[d,l]		

Table 46 (contd)

Reference	Population	Exposed		Unexposed	
		No.	Mean level (mmol/L)	No.	Mean level (mmol/L)
Grubbs *et al.* (1995)	Ranch Hand personnel				
	Background	362	1.43[l]	1 025	1.47
	Low[m]	191	1.49[l]		
	High[m]	182	1.35[l]		

Data are means ± SD unless otherwise indicated
[a] Number of samples
[b] Chloracne
[c] No chloracne
[d] $p < 0.01$
[e] $p = 0.056$
[f] Adjusted for age, body mass index, smoking history
[g] Adjusted for body mass index, smoking, gender, race, current diabetes, use of β-blocker
[h] Adipose tissue levels of 2,3,7,8-TCDD in ng/kg of lipid
[i] % abnormal: Viet Nam veterans, 4.7; non-Viet Nam veterans, 5.3; odds ratio, 0.9; 95% CI, 0.7–1.2
[j] Geometric mean
[k] Serum 2,3,7,8-TCDD levels in ng/kg of lipid
[l] Contrasted to unexposed comparisons
[m] Serum 2,3,7,8-TCDD levels in ng/kg of lipid; background: current level ≤ 10 ng/kg of lipid; low: current level > 10 ng/kg of lipid, 10 ng/kg < initial level ≤ 143 ng/kg of lipid; high: current level > 10 ng/kg of lipid, 10 ng/kg < initial level > 143 ng/kg of lipid

Triglycerides

Elevated triglyceride levels were reported among British TCP workers with chloracne (Martin, 1984) and, in the United States Air Force Ranch Hand study, among subjects with serum 2,3,7,8-TCDD levels above 15 ng/kg lipid in 1987 (Roegner *et al.*, 1991) and only if above 33 ng/kg lipid in 1992 (Grubbs *et al.*, 1995). Among TCP production workers, there was a small rise in triglyceride levels with increasing serum 2,3,7,8-TCDD level, but this was found to be dependent on host factors (including sex and body mass index) in a multivariate regression analysis (Calvert *et al.*, 1996).

Triglyceride levels were not elevated in the BASF Ludwigshafen accident cohort (Ott *et al.*, 1994), in Missouri residents (Hoffman *et al.*, 1986; Webb *et al.*, 1989), in Seveso residents (Mocarelli *et al.*, 1986; Assennato *et al.*, 1989) or in United States Army Viet Nam veterans (Centers for Disease Control Vietnam Experience Study, 1988a) (**Table 46**).

(*c*) *Other gastrointestinal effects*

A variety of gastrointestinal disorders other than liver conditions have been reported following heavy, acute or chronic exposure of chemical workers (Ashe & Suskind, 1950; Baader & Bauer, 1951; Bauer *et al.*, 1961; Jirasek *et al.*, 1974). The most consistently reported symptoms were transient episodes of right upper quadrant pain, loss of appetite and nausea. None of the reports suggest an etiology for these symptoms and the symptoms were not reported in later follow-up studies of any cohort (Pazderova-Vejlupkova *et al.*, 1981; Moses *et al.*, 1984; Suskind & Hertzberg, 1984).

Three investigations of TCP production workers reported an increased prevalence of a history of upper gastrointestinal tract ulcer across all age strata of West Virginia workers (exposed, 20.7% versus unexposed, 5.5%) (Suskind & Hertzberg, 1984) and all digestive system diseases [not specified] among workers employed in a plant in Midland, MI (prevalence: exposed, 1.5% versus unexposed, 0.5%) (Bond *et al.*, 1983). The factors contributing to these conditions have not been examined fully. Neither the Ranch Hand study (Roegner *et al.*, 1991; Grubbs *et al.*, 1995) nor the NIOSH study (Calvert *et al.*, 1992) found an increased risk of upper gastrointestinal tract ulcers with increasing serum 2,3,7,8-TCDD level.

(*d*) *Thyroid function*

Effects in adults

Little or no information has been reported on the effects of 2,3,7,8-TCDD specifically on thyroid function in production workers or Seveso residents, two groups with documented high serum 2,3,7,8-TCDD levels.

No difference was found between West Virginia TCP production workers and controls for thyroxine (T4) and thyroxine-binding globulin (TBG), although quantitative results were not presented (Suskind & Hertzberg, 1984). Thyroid-stimulating hormone (TSH), T4 and TBG levels were within the normal range in workers exposed in the BASF Ludwigshafen (Germany) accident (Ott *et al.*, 1994). The 1987 Ranch Hand study indicated a nonsignificant reduction in the percentage of triiodothyronine (T3) uptake

(Roegner *et al.*, 1991). A slight increase in the mean level of TSH with increasing serum 2,3,7,8-TCDD level was noted in both 1987 and 1991, but did not reach statistical significance (Roegner *et al.*, 1991; Grubb *et al.*, 1995). Among Army Viet Nam veterans, mean TSH levels, but not mean free thyroxine index (FTI) (biologically active) levels, were statistically significantly higher than among non-Viet Nam veterans, after adjustment for the six entry characteristics of age and year of enlistment, race, enlistment status, general technical test score and primary military occupation (**Table 47**) (Centers for Disease Control Vietnam Experience Study, 1988a).

Table 47. Levels of triidothyronine percentage (T3%) uptake and free thyroxine index in Viet Nam veterans

Reference	Population	Exposed		Adjusted RR (95% CI)	Unexposed	
		No.	Mean level		No.	Mean level
T3% uptake						
Roegner *et al.* (1991)	Ranch Hand personnel				772	30.7
	Unknown ≤ 10[a]	338	30.7	1.1 (0.6–2.1)		
	Low 15–≤ 33.3[a]	194	30.4	0.9 (0.4–2.2)		
	High > 33.3[a]	181[b]	30.0	0.5 (0.1–1.5)		
	All Ranch Hand versus all comparisons	937	30.6	1.14 (0.7–1.8)	1 198	30.6
Free thyroxine index						
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2 490	2.2[c]	1.2[d] (0.9–1.5)	1 972	2.2[c]

[a] Serum 2,3,7,8-TCDD in ng/kg lipid
[b] $p < 0.05$ comparison of veterans at background level with ≥ 33.3 ng/kg 2,3,7,8-TCDD
[c] Geometric mean
[d] Adjusted odds ratio

Effects in infants

Two studies in the Netherlands examined thyroid function in infants and related this to PCDD, PCDF and/or PCB levels in breast milk, cord blood or third trimester maternal serum samples.

Pluim *et al.* (1992, 1993c) examined thyroid function among 38 full-term breast-fed infants in relation to the total I-TEQ per kg of breast milk fat of seven PCDDs (see **Table 30**) and 10 PCDFs. Total T4, TBG and TSH levels were measured sequentially in cord blood and in the blood of infants at one week of age and at 11 weeks of age (**Table 48**). Total T3 was measured in plasma at 11 weeks. Infants were classified as 'high' or 'low' with respect to the median of the range of total I-TEQ. At one week and 11 weeks postnatally, mean total T4 and total T4 : TBG ratios were significantly higher

Table 48. Levels of thyroxine-binding globulin (TBG), thyroxine (T4), free thyroxine (FT4), T4/TBG ratio and thyroid-stimulating hormone (TSH) in nursing infants and workers of the BASF accident cohort

Measurement	Reference	Population	Exposed[a]		Unexposed[b]	
			No.	Mean ± SD	No.	Mean ± SD
Nursing infants						
Total T4 (nmol/L)	Pluim *et al.* (1992, 1993c)	Neonates; Amsterdam, The Netherlands				
		At birth/cord blood	15	134.3 ± 4.8[d]	18	122.5 ± 4.1[d]
		1 week postnatal	19	178.7[c] ± 5.5	19	154.5 ± 6.3
		11 weeks postnatal	16	122.2[c] ± 3.0	18	111.1 ± 4.0
	Koopman-Esseboom *et al.* (1994b)	Neonates; Rotterdam, The Netherlands				
		2nd week postnatal	39	159.9[c] ± 31.6	39	177.5 ± 39.2
Free T4 (nmol/L)	Koopman-Esseboom *et al.* (1994b)	Neonates; Rotterdam, The Netherlands				
		2nd week postnatal	39	23.0[c] ± 3.3	39	24.6 ± 3.5
TBG (nmol/L)	Pluim *et al.* (1992, 1993c)	Neonates; Amsterdam, The Netherlands				
		At birth/cord blood	15	589.5 ± 30.5[d]	18	520.1 ± 27.2[d]
		1 week postnatal	19	546.2 ± 19.1	19	532.6 ± 16.3
		11 weeks postnatal	16	500.7 ± 13.0	18	519.0 ± 29.4
T4/TBG	Pluim *et al.* (1992, 1993c)	Neonates; Amsterdam, The Netherlands				
		At birth/cord blood	15	0.232 ± 0.008[d]	18	0.240 ± 0.007[d]
		1 week postnatal	19	0.332[c] ± 0.011	19	0.291 ± 0.009
		11 weeks postnatal	16	0.247[c] ± 0.009	18	0.220 ± 0.008
TSH (IU/ml)	Pluim *et al.* (1992, 1993c)	Neonates; Amsterdam, the Netherlands				
		At birth/cord blood	11	11.9 ± 1.9[d]	14	10.4 ± 1.3[d]
		1 week postnatal	11	2.56 ± 0.41	15	2.93 ± 0.41
		11 weeks postnatal	12	2.50 ± 0.26	18	1.81 ± 0.19
	Koopman-Esseboom *et al.* (1994b)	Neonates; Rotterdam, the Netherlands				
		At birth/cord blood	[e]	11.6[c] ± 8.0		8.5 ± 6.0
		2nd week postnatal	39	2.6[c] ± 1.5	39	1.9 ± 0.8
		3 months	39	2.3[c] ± 1.0	39	1.6 ± 0.6

Table 48 (contd)

Measurement	Reference	Population	Exposed[a]		Unexposed[b]	
			No.	Mean ± SD	No.	Mean ± SD
Ludwigshafen accident cohort						
TBG (mg/L)	Ott *et al.* (1994)	BASF chemical workers	131	12.7 ± 3.2	141	12.7 ± 2.9
T4 (µg/dL)	Ott *et al.* (1994)	BASF chemical workers	131	7.8 ± 1.9	141	8.3 ± 1.5
T4/TBG	Ott *et al.*(1994)	BASF chemical workers	131	6.3 ± 1.3	141	6.7 ± 1.6
TSH (IU/mL)	Ott *et al.* (1993, 1994)	BASF chemical workers	130	1.19 ± 0.90	–[f]	–

[a] High exposure group: 29.2–62.7 ng toxic equivalents/kg (I-TEQ/kg milk fat) (Pluim *et al.*, 1992, 1993c) or > 30.75–76.43 ng I-TEQ/kg fat (Koopman-Esseboom *et al.*, 1994b)

[b] Low exposure group: 8.7–28.0 ng I-TEQ/kg (Pluim *et al.*, 1992, 1993c) or 12.44–30.75 ng I-TEQ/kg fat (Koopman-Esseboom *et al.*, 1994b)

[c] $p < 0.05$ compared to the unexposed group

[d] Standard error of the mean

[e] Total for both high and low = 75

[f] No referent values

among infants in the 'high' group. At 11 weeks, TSH was also significantly higher in the 'high' group. Total T3 was unchanged.

Koopman-Esseboom *et al.* (1994b) examined thyroid function in 78 mother–infant pairs in relation to the I-TEQ levels for PCDDs, planar PCBs, non-planar PCBs and total PCBs–PCDDs in breast milk collected in the first and second weeks after delivery. Total T4, total T3, free T4 and TSH levels were measured in the mother during the last month of pregnancy and 9–14 days after delivery, in cord blood and in the blood of infants at 9–14 days and three months after birth (**Table 48**). All the I-TEQs (PCDDs, co-planar PCBs, non-planar PCBs and total PCDDs/PCBs) were significantly correlated with infant plasma levels of TSH at the second week and third month, and inversely correlated with total T3 pre-delivery, and with total T3 and total T4 post-delivery for the mothers. The non-planar PCB TEQ was not significantly correlated with the mothers' total T4 after delivery and the infants' third month TSH. The measurements in infants with higher PCDD TEQ (based on median TEQ) during their second week of life showed a significant increase in TSH and significant decreases for total T4 and free T4.

These two studies of nursing infants suggest that ingestion of breast milk with elevated levels of PCDDs may alter thyroid function. Both studies covered a short observation period which limits the examination of persistent or long-term changes in thyroid status and the analyses did not control for other factors which might affect thyroid status.

(*e*) *Diabetes*

Cross-sectional studies of workers from Nitro, West Virginia, found no difference in glucose levels between the exposed and control populations, although no quantitative values were presented in either report (Moses *et al.*, 1984; Suskind & Hertzberg, 1984). Similarly, the adjusted odds ratio for out-of-range fasting glucose levels comparing Viet Nam veterans with non-Viet Nam veterans was not statistically significant (odds ratio, 1.0; 95% CI, 0.4–2.2) (Centers for Disease Control Vietnam Experience Study, 1988a). Mean fasting glucose levels in the workers exposed in the BASF Ludwigshafen (Germany) accident were elevated compared with the control population and were associated with current levels of 2,3,7,8-TCDD ($p = 0.062$) but not with the levels back-extrapolated to the time of exposure (Ott *et al.*, 1994).

In the Ranch Hand study, diabetic status was assessed by measuring fasting serum glucose and 2-h postprandial glucose and by using a case definition of diabetes. Diabetes was defined as having a verified history of diabetes or an oral glucose tolerance test of ≥ 11.1 mmol/L (200 mg/dL) (Roegner *et al.*, 1991). The analyses of all three parameters suggested a consistent association between serum 2,3,7,8-TCDD levels above 33.3 ng/kg and an increased risk of diabetes. Adjusted relative risks in Ranch Hand veterans with serum 2,3,7,8-TCDD above 33.3 ng/kg compared with an unexposed group were statistically significantly elevated for fasting serum glucose levels and diabetes (glucose RR, 3.0; $p < 0.001$; diabetes RR, 2.5; $p < 0.001$) (**Table 49**) and for the 2-h postprandial glucose test (RR, 2.4; $p = 0.035$). In addition, Ranch Hand personnel meeting the case definition for diabetes were also more likely to have earlier onset of diabetes than the unexposed group (Wolfe *et al.*, 1992).

Table 49. Adjusted relative risk (RR) for fasting serum glucose levels, cases of diabetes and mean 2-h postprandial glucose levels by category of lipid-adjusted serum 2,3,7,8-TCDD level in Ranch Hand veterans

Serum 2,3,7,8-TCDD (ng/kg)	Fasting serum glucose (RR)	Diabetes (RR)[a]	2-H postprandial glucose level (RR)[b]
Unknown: ≤ 10 ng/kg lipid	0.66	0.82	0.88
Low: 15–≤ 33.3 ng/kg lipid	1.18	1.01	0.60
High: > 33.3 ng/kg lipid	2.95[d]	2.51[d]	2.35[c]

From Roegner *et al.* (1991)
[a]Defined as having a verified history of diabetes or 2-h postprandial glucose level of ≥ 11.1 mmol/L (200 mg/dL)
[b]Comparison of diabetics and normals
[c]$p = 0.035$
[d]$p < 0.001$

(*f*) *Immunological effects* (**Tables 50–57**)

Nine epidemiological studies and one case report have assessed immunological function in populations exposed to 2,3,7,8-TCDD (Reggiani, 1978; Hoffman *et al.*, 1986; Centers for Disease Control Vietnam Experience Study, 1988a; Evans *et al.*, 1988; Jennings *et al.*, 1988; Webb *et al.*, 1989; Roegner *et al.*, 1991; Ott *et al.*, 1994; Tonn *et al.*, 1996). With the exception of the Tonn *et al.* (1996) study, none has found a clear relationship between exposure and impaired immunological status. Among the Seveso children resident in the area of highest 2,3,7,8-TCDD contamination, immunoglobulins, complement levels, lymphocyte subpopulations and lymphocyte activity analyses were within the normal range, with no differences from those of unexposed controls (Reggiani, 1978).

In Missouri residents with potential exposure to 2,3,7,8-TCDD-contaminated soil (see Section 1.3.2(*c*)), depression in cell-mediated immunity (delayed hypersensitivity) was reported by Hoffman *et al.* (1986). However, a follow-up study did not confirm the presence of anergy (Evans *et al.*, 1988). In a later study of a different cohort of Missouri residents, Webb *et al.* (1989) found no clinical evidence of immunosuppression in 40 individuals whose adipose 2,3,7,8-TCDD levels ranged from below 20 ng/kg to over 430 ng/kg [top of range not given].

The effect of past occupational exposure on parameters of the immune system was examined in 18 British workers who were evaluated 17 years after accidental industrial exposure to chemicals contaminated with 2,3,7,8-TCDD (Jennings *et al.*, 1988). There were no significant differences in the levels of immunoglobulins, T and B lymphocytes, responsiveness to phytohaemagglutinin A or the CD4 and CD8 counts. Three measurements were different in workers compared with controls: antinuclear antibodies (8 workers versus 0 controls, $p < 0.01$), immune complexes (11 workers versus 3 controls, $p < 0.05$) and natural killer cells (workers, 0.40×10^6/L versus controls, 0.19×10^6/L; $p < 0.002$).

Table 50. CD4/CD8 ratios in Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed			Unexposed		
		No.	Mean ratio (SD)		No.	Mean ratio (SD)	
Roegner *et al.* (1991)	Ranch Hand personnel						
	Unknown: ≤ 10 ng/kg[a]	126	1.72		301	1.89	
	Low: 15–≤ 3.3 ng/kg[a]	72	1.91				
	High: > 33.3 ng/kg[a]	72	1.99				
Grubbs *et al.* (1995)	Ranch Hand personnel						
	Background[b]	139	1.50		399	1.48	
	Low[b]	94	1.58				
	High[b]	106	1.57				
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2 490	1.8[c]	Odds ratio < reference range, 0.9 Odds ratio > reference range, 1.1	1 972	1.8[c]	
Hoffman *et al.* (1986)	Missouri residents in mobile home park	135	1.9 (0.8)	% abnormal, 8.2	142	1.9 (0.6)	% abnormal, 6.3
Webb *et al.* (1989)	Missouri residents				–	–	
	< 20 ng/kg[d]	16	2.0 (0.7)				
	20–60 ng/kg[d]	12	2.1 (1.0)				
	> 60 ng/kg[d]	12	1.4 (0.7)				
Ott *et al.* (1994)	BASF accident cohort	132	1.6 (0.9)		42[e]	1.5 (0.6)	

[a] Serum 2,3,7,8-TCDD level in ng/kg of lipid

[b] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin> 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg

[c] Geometric mean

[d] Adipose tissue 2,3,7,8-TCDD level in ng/kg of lipid

[e] From Zober *et al.* (1992)

Table 51. Total lymphocytes in 2,4,5-T production workers, Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed				Unexposed		
		No.	Mean level[a]	SD		No.	Mean level[a]	SD
Roegner *et al.* (1991)	Ranch Hand personnel							
	Unknown: ≤ 10 ng/kg[b]	127	1954	–		301	1972	–
	Low: 15 ≤ 33.3 ng/kg[b]	73	2011	–				
	High: > 33.3 ng/kg[b]	74	2032	–				
Grubbs *et al.* (1995)	Ranch Hand personnel							
	Background[c]	141	2067[d]			400	2022	
	Low[c]	95	1989					
	High[c]	108	2034					
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	1973	–	1.0[e]/1.2[f]	1972	1936	–
Hoffman *et al.* (1986)	Missouri residents in mobile home park	135	2465	724		142	2311	634
Webb *et al.* (1989)	Missouri residents				% lymphocytes			
	< 20 ng/kg[g]	16	2200	830	32	–	–	
	20–60 ng/kg[g]	12	2300	600	32			
	> 60 ng/kg[g]	12	2200	720	28			
Jennings *et al.* (1988)	2,4,5-T production workers exposed 17 years earlier	18	1980	840		15	2020	470

Table 51 (contd)

Reference	Population	Exposed				Unexposed			
		No.	Mean level[a]	SD		No.	Mean level[a]	SD	
Ott *et al.* (1994)	BASF accident cohort	133	1978	805	% lymphocytes 33.4 (± 9.4)	42	2268	838	% lymphocytes 36 (± 12.4)

[a] Units, lymphocyte per mm^3
[b] Serum 2,3,7,8-TCDD level in ng/kg of lipid
[c] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[d] Adjusted mean
[e] Odds ratio < reference range
[f] Odds ratio > reference range
[g] Adipose tissue 2,3,7,8-TCDD level in ng/kg of lipid

Table 52. B1 Lymphocytes in 2,4,5-T production workers, Missouri residents, Viet Nam veterans, BASF accident cohort and extruder personnel

Reference	Population	Exposed				Unexposed			
		No.	Mean level[a]	SD		No.	Mean level[a]	SD	
Roegner *et al.* (1991)	Ranch Hand personnel								
	Unknown: ≤ 10[b]	127	176	–		301	172	–	
	Low: 1.5 ≤ 33.3[b]	71	183	–					
	High: > 33.3[b]	73	191	–					
Grubbs *et al.* (1995)	Ranch Hand personnel								
	Background[c]	140	245[d]	–		400	214	–	
	Low[c]	95	224	–					
	High[c]	106	220	–					
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	240[e]	–	1.1[f] 1.2[g]	1972	230[e]	–	
Webb *et al.* (1989)	Missouri residents				% B1 cells				
	< 20 ng/kg[h]	16	190	865	9.1	–	–		–
	20–60 ng/kg[h]	12	189	983	8.3				
	> 60 ng/kg[h]	12	171	573	7.8				
Jennings *et al.* (1988)	2,4,5-T production workers exposed 17 years earlier	18	210	110		15	160	80	

Table 52 (contd)

Reference	Population	Exposed			Unexposed		
		No.	Mean level[a]	SD	No.	Mean level[a]	SD
Ott *et al.* (1994)	BASF accident cohort	133	10.4[i]	6.0	42	12.3[i,j]	5.1

[a] Units, cell/mm^3
[b] Serum 2,3,7,8-TCDD level in ng/kg of lipid
[c] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[d] Adjusted mean
[e] Geometric mean
[f] Odds ratio < reference range
[g] Odds ratio > reference range
[h] Adipose tissue 2,3,7,8-TCDD level in ng/kg of lipid
[i] % B1 cells
[j] From Zober *et al.* (1992)

Table 53. CD4 Lymphocytes in production workers, Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed				Unexposed			
		No.	Mean level[a]	SD		No.	Mean level[a]	SD	
Roegner *et al.* (1991)	Ranch Hand personnel								
	Unknown: ≤ 10 ng/kg[b]	127	867	–		301	907	–	
	Low: 1.5 ≤ 33.3 ng/kg[b]	72	945	–					
	High: > 33.3 ng/kg[b]	72	929	–					
Grubbs *et al.* (1995)	Ranch Hand personnel								
	Background[c]	141	961[d]	–		403	923	–	
	Low[c]	95	917	–					
	High[c]	108	962	–					
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	1020[e]	–	1.0[f] 1.4[g]	1972	990[e]	–	
Hoffman *et al.* (1986)	Missouri residents in mobile home park	135	1021	353	% abnormal, 0.7	142	1033	346	% abnormal, 0.0
Webb *et al.* (1989)	Missouri residents				% CD4 cells				
	< 20 ng/kg[h]	16	1084	485	48	–	–		
	20–60 ng/kg[h]	12	1198	391	51				
	> 60 ng/kg[h]	12	963	403	42				
Jennings *et al.* (1988)	2,4,5-T production workers exposed 17 years earlier	18	950	340		15	1040	290	
Tonn *et al.* (1996)	2,4,5-TCP production and maintenance workers	11	–		% CD4 cells, 47.6 (± 8.1)	10	–		% CD4 cells, 48.5 (± 10.6)

Table 53 (contd)

Reference	Population	Exposed			Unexposed			
		No.	Mean level[a]	SD	No.	Mean level[a]	SD	
Ott *et al.* (1994)	BASF accident cohort	133	–	% CD4 cells, 42.5 (± 10.4)	42[i]	–		% CD4 cells, 45.1 (± 8.9)

[a] Units, cells/mm^3
[b] Serum 2,3,7,8-TCDD in ng/kg of lipid
[c] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[d] Adjusted mean
[e] Geometric mean
[f] Odds ratio < reference range
[g] Odds ratio > reference range
[h] Adipose tissue 2,3,7,8-TCDD level in ng/kg of lipid
[i] From Zober *et al.* (1992)

Table 54. CD8 Lymphocytes in production workers, Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed				Unexposed			
		No.	Mean level[a]	SD		No.	Mean level[a]	SD	
Roegner *et al.* (1991)	Ranch Hand personnel								
	Unknown: ≤ 10 ng/kg[b]	126	485	–		301	473	–	
	Low: 1.5 ≤ 33.3 ng/kg[b]	71	465	–					
	High: > 33.3 ng/kg[b]	73	475	–					
Grubbs *et al.* (1995)	Ranch Hand personnel								
	Background[c]	140	645[d]	–		400	634	–	
	Low[c]	95	606	–					
	High[c]	106	618	–					
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	560[e]	–	1.0[f] 0.9[g]	1972	550	–	
Hoffman *et al.* (1986)	Missouri residents in mobile home park	135	592	223	% abnormal, 1.5	142	578	198	% abnormal, 0.0
Webb *et al.* (1989)	Missouri residents				% CD8 cells				
	< 20 ng/kg[h]	16	562	215	26	–	–		
	20–60 ng/kg[h]	12	645	225	28				
	> 60 ng/kg[h]	12	807	381	35				
Jennings *et al.* (1988)	2,4,5-T production workers exposed 17 years earlier	18	630	280	–	15	590	230	

Table 54 (contd)

Reference	Population	Exposed			Unexposed		
		No.	Mean level[a] SD		No.	Mean level[a] SD	
Ott *et al.* (1994)	BASF accident cohort	132	–	% CD8 cells, 31.9 (± 10.4)	42[i]	–	% CD8 cells, 32.0 (± 7.1)

[a] Units, cells/mm^3
[b] Serum 2,3,7,8-TCDD level in ng/kg of lipid
[c] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[d] Adjusted mean
[e] Geometric mean
[f] Odds ratio < reference range
[g] Odds ratio > reference range
[h] Adipose tissue 2,3,7,8-TCDD level in ng/kg of lipid
[i] From Zober *et al.* (1992)

Table 55. IgG levels in Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed				Unexposed		
		No.	Mean level[a]	SD		No.	Mean level[a]	SD
Roegner *et al.* (1991)	Ranch Hand personnel							
	Unknown: ≤ 10[b]	335	1087	–		757	1120	–
	Low: 1.5 ≤ 33.3[b]	190	1122	–				
	High: > 33.3[b]	175	1122	–				
Grubbs *et al.* (1995)	Ranch Hand personnel							
	Background[c]	364	1126[d]	–		1035	1113.9	–
	Low[c]	243	1111	–				
	High[c]	251	1115	–				
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	1078[e]	–	1.0[f] 1.0[g]	1972	1077[e]	–
Webb *et al.* (1989)	Missouri residents							
	< 20 ng/kg[h]	16	1064[i]	273		–	–	
	20–60 ng/kg[h]	12	1146	193				
	> 60 ng/kg[h]	12	1151	223				
Ott *et al.* (1994)	BASF accident cohort	132	1200[d]	262		194	1183	310

[a] Units, mg/dL
[b] Serum 2,3,7,8-TCDD in ng/kg of lipid
[c] Comparison: current dioxin ≤ 10 ng/kg of lipid; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[d] Significant positive relationship between IgG and current 2,3,7,8-TCDD level and back-extrapolated 2,3,7,8-TCDD ($p < 0.01$)
[e] Geometric mean
[f] Odds ratio < reference range
[g] Odds ratio > reference range
[h] Adipose tissue 2,3,7,8-TCDD in ng/kg of lipid
[i] $p < 0.05$ trend

Table 56. IgM levels in Missouri residents, Viet Nam veterans and BASF accident cohort

Reference	Population	Exposed				Unexposed			
		No.	Mean level[a]	SD	Ratio	No.	Mean level[a]	SD	Ratio
Roegner *et al.* (1991)	Ranch Hand personnel								
	Unknown: ≤ 10 ng/kg[b]	335	107	–	–	757	103	–	–
	Low: 1.5 ≤ 33.3 ng/kg[b]	190	96	–					
	High: > 33.3 ng/kg[b]	175	106	–					
CDC Vietnam Experience Study (1988a)	United States Army Viet Nam veterans	2490	121[c]	–	1.0[d] 1.0[e]	1972	121[c]	–	–
Webb *et al.* (1989)	Missouri residents								
	< 20 ng/kg[f]	16	128	90	–	–	–		
	20–60 ng/kg[f]	12	157	57					
	> 60 ng/kg[f]	12	114	44					
Ott *et al.* (1994)	BASF accident cohort	132	140	65	–	192	135	70	

[a] Units, mg/dL
[b] Serum 2,3,7,8-TCDD in ng/kg of lipid
[c] Geometric mean
[d] Odds ratio < reference range
[e] Odds ratio > reference range
[f] Adipose tissue 2,3,7,8-TCDD in ng/kg of lipid

Table 57. Levels of natural killer cells in Missouri residents, Viet Nam veterans and extruder personnel

Reference	Population	Exposed			Unexposed		
		No.	Mean level	SD	No.	Mean level	SD
Roegner *et al.* (1991)	Ranch Hand personnel						
	Unknown ≤ 10 ng/kg[a]	126	455[b,c]		291	414[b]	
	Low 15–≤ 33.3 ng/kg[a]	70	378				
	High > 33.3 ng/kg[a]	72	386				
Grubbs *et al.* (1995)	Ranch Hand personnel		*CD16+CD56*				
	Background[d]	139	242[b,e]	–	399	248[b,e]	
	Low[d]	94	219	–			
	High[d]	106	237	–			
Tonn *et al.* (1996)	2,4,5-TCP production and		*% CD56*			*% CD56*	
	maintenance workers	11	5.4	1.9	10	5.5	1.6
Jennings *et al.* (1988)	2,4,5-T production workers exposed 17 years earlier	18	400[b]	210[f]	15	190[b]	150

[a] Serum 2,3,7,8-TCDD in ng/kg of lipid
[b] Units, cells/mm^3
[c] Net response
[d] Comparison: current dioxin ≤ 10 ng/kg of lipid.; background: current dioxin > 10 ng/kg; low: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin ≤ 143 ng/kg; high: current dioxin > 10 ng/kg, 10 ng/kg < initial dioxin > 143 ng/kg
[e] Adjusted mean
[f] $p < 0.05$

Levels of IgA, IgG, IgM and complement C4 and C3 were higher in exposed workers than in the unexposed control population in the BASF accident study (Ott *et al.*, 1994).

Flow cytometric analysis of lymphocytes from workers with moderately increased serum 2,3,7,8-TCDD (25–140 ng/kg fat) and other PCDDs/PCDFs (maximum, 522 ng I-TEQ/kg fat) did not indicate any decrease of specific cellular components of the immune system (Neubert *et al.*, 1993a, 1995). Moderate increases in the 2,3,7,8-TCDD body burden did not induce any medically significant change in the capacity for proliferation of lymphocytes, measured as [^{3}H]thymidine incorporation (Neubert *et al.*, 1995).

Tonn *et al.* (1996) examined lymphocyte function among 11 workers employed between 1966 and 1976 in TCP production. Lipid-adjusted serum 2,3,7,8-TCDD levels, measured in 1989–92, ranged from 43 to 874 ng/kg. Although the numbers of lymphocyte subsets were normal, there was a small but significant difference in the response to alloantigen challenge of T-cells and their proliferative response to interleukin (IL)-2. In addition, compared with unexposed controls, the lymphocytes of the exposed workers displayed a suppressive activity that inhibited an on-going allo-response of HLA-unrelated lymphocytes.

Two studies extensively evaluated parameters of the immune system in Viet Nam veterans. No significant differences were detected between United States Army ground troops and the comparison population in lymphocyte subset populations, T-cell populations or serum immunoglobulins (**Tables 50–57**) (Centers for Disease Control Vietnam Experience Study, 1988a). In the United States Air Force Ranch Hand Study (Roegner *et al.*, 1991), significant positive associations were found between IgA and serum 2,3,7,8-TCDD levels. The authors suggest that the rise in IgA is consistent with a subclinical inflammatory response of unspecified origin.

One report (Weisglas-Kuperus *et al.*, 1995) examined direct and surrogate measures of immune status in 207 babies in Rotterdam. The surrogate measures were derived from questionnaires given to the mothers, that covered incidence of rhinitis, bronchitis, tonsillitis and otitis in children up to 18 months of age. Almost all of the children (205) were vaccinated against measles, rubella and mumps; the children's antibody levels were subsequently measured to assess humoral immunity. No relationship was found between pre- and postnatal PCB/PCDD exposure and respiratory tract symptoms (i.e., number of periods with rhinitis, bronchitis, tonsillitis and otitis) or humoral antibody production. However, high prenatal exposure, estimated by cord blood levels, was associated with alterations in T-cell subsets. All values were within clinically normal ranges, and the small changes observed do not necessarily mirror alterations in the cell composition of lymphoid and non-lymphoid organs, nor do they necessarily reflect functional defects. The long-term effects of such subtle shifts in the distribution of outcome measures remain unknown.

(*g*) *Neurological effects*

Adults: Several studies have evaluated the relationship between neurobehavioural and neurological function among chemical production workers and community residents exposed to 2,3,7,8-TCDD (Sweeney *et al.*, 1989). Two studies have reported significant neuropathy in Seveso residents (Filippini *et al.*, 1981) and in TCP production workers

(Moses *et al.*, 1984), but these findings have not been reproduced in subsequent studies of the same populations. Studies of TCP production workers and Viet Nam veterans have found no neurological disorder associated with PCDD exposure (Suskind & Hertzberg, 1984; Assennato *et al.*, 1989; Hoffman *et al.*, 1986; Centers for Disease Control Vietnam Experience Study, 1988a,b; Assennato *et al.*, 1989; Webb *et al.*, 1989; Sweeney *et al.*, 1993).

Infants: A series of studies of infants born in either Rotterdam or Groningen in the Netherlands evaluated their neurological and behavioural development in a series of tests conducted at two weeks and three, seven and 18 months, in relation to breast milk levels of PCBs, PCDDs and PCDFs (see **Table 31**). Exposed infants were breast-fed; the controls were formula-fed. Neurological status of 418 neonates (10–21 days) measured using the Prechtl neonatal neurological examination (Huisman *et al.*, 1995a) was related to total I-TEQ (odds ratio, 3.2; 95% CI, 1.4–7.5) and specifically to higher chlorinated PCDDs, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF. Multiple tests were conducted in the 207 Groningen children at three, seven and 18 months to evaluate psychomotor development (Bayley Scales of Infant Development) (Koopman-Esseboom *et al.*, 1996) and visual recognition memory (Fagan Test of Infant Intelligence) at three and seven months (Koopman-Esseboom *et al.*, 1995a,b). Results of the Fagan Tests and the psychomotor and mental developmental indices were no different in breast-fed or formula-fed infants. Similarly, the neurological status of children at 18 months was not significantly affected by PCB/PCDD exposure through breast milk (Huisman *et al.*, 1995b).

(*h*) *Circulatory system*

Several studies have described mortality from diseases affecting the circulatory system among populations exposed to 2,3,7,8-TCDD (Bond *et al.*, 1987; Bertazzi *et al.*, 1989; Zober *et al.*, 1990; Coggon *et al.*, 1991; Fingerhut *et al.*, 1991b; Bertazzi *et al.*, 1992; Bueno de Mesquita *et al.*, 1993; Collins *et al.*, 1993; Flesch-Janys *et al.*, 1995) (**Table 58**).

Mortality from all diseases of the circulatory system was similar to that of general populations in studies of workers from the Netherlands (Plant A) (Bueno de Mesquita *et al.*, 1993), the United States (Nitro, West Virginia) (Collins *et al.*, 1993) and the United Kingdom (Coggon *et al.*, 1991). In two studies of workers with chloracne, mortality was not significantly different from that of the national comparison groups (Bond *et al.*, 1987; Zober *et al.*, 1990). However, in a study of German chemical workers who manufactured TCP and 2,4,5-T in addition to chemicals contaminated with higher chlorinated PCDDs and PCDFs (Flesch-Janys *et al.*, 1995), mortality from all circulatory diseases was positively related to estimated 2,3,7,8-TCDD levels and significantly related to estimated total I-TEQ concentrations above 39 ng/kg (lipid-adjusted).

Mortality from more specific endpoints, including ischaemic heart disease and cerebrovascular disease, has been reported in some studies of TCP production workers (Bond *et al.*, 1987; Bueno de Mesquita *et al.*, 1993; Fingerhut *et al.*, 1991b; Flesch-Janys *et al.*, 1995). With the exception of an increase in risk for ischaemic heart disease with

Table 58. Mortality from diseases of the circulatory system in populations exposed to 2,3,7,8-TCDD

Reference	Population	Outcome	No. of deaths	SMR[a]	95% CI	Cohort size	Years of follow-up
Fingerhut *et al.* (1991b)	TCP and 2,4,5-T production workers, United States	Diseases of the heart (ICD 390–398, 402–404, 410–414, 420–429)	393	1.0	0.9–1.1	5172	1942–87
		Diseases of the circulatory system (ICD 401, 403, 405, 415–417, 430–438, 440–459)	67	0.8	0.6–1.0		
Zober *et al.* (1990)	2,4,5-T production workers with chloracne, Germany	Diseases of the circulatory system (ICD 390–458)		1.2	0.8–1.7[b]	127	1953–87
Coggon *et al.* (1991)	2,4,5-T synthesis or formulation, United Kingdom	Diseases of the circulatory system	74	1.2	0.9–1.5	2239	1975–87
Bond *et al.* (1987)	United States chemical production workers with chloracne (TCP + 2,4,5-T) (Michigan)	Diseases of the circulatory system	19	1.0	0.6–1.6	322	1940–82
		Atherosclerotic heart disease	13	1.0	0.5–1.6		
		Vascular lesions of CNS	4	2.1	0.6–5.4		
	United States chemical production: workers without chloracne (TCP + 2,4,5-T) (Michigan)	Diseases of the circulatory system	130	1.0	0.8–1.1	2026	1940–82
		Atherosclerotic heart disease	106	1.1	0.9–1.3		
		Vascular lesions of CNS	10	0.6	0.3–1.2		
Bueno de Mesquita *et al.* (1993)	TCP and 2,4,5-T production workers, The Netherlands	Diseases of the circulatory system (ICD 390–458)	28	1.0	0.7–1.4	549	1955–85
		Ischaemic heart disease (ICD 410–414)	20	1.0	0.6–1.6		
		Cerebrovascular disease (ICD 430–438)	5	1.2	0.4–2.7		

Table 58 (contd)

Reference	Population	Outcome	No. of deaths	SMR[a]	95% CI	Cohort size	Years of follow-up
Asp *et al.* (1994)	Herbicide sprayers, 2,4,5-T and 2,4-D, Finland	Ischaemic heart disease Other heart disease Cerebrovascular disease Other vascular disease	148 20 26 9	0.9 1.0 0.7 0.7	0.8–1.1 0.6–1.6 0.5–1.0 0.3–1.4	1909	1972–89
Michalek *et al.* (1990)	United States Air Force Ranch Hand personnel	Diseases of the circulatory system	25	1.0	0.8–1.7	1261	1961–87
CDC Vietnam Experience Study (1988c)	United States Army Viet Nam veterans	Diseases of the circulatory system (ICD 390–459)	12	0.5[c]	0.3–1.0	9324	1965–83
Fett *et al.* (1987b)	Australian Viet Nam veterans; served > 12 months	Diseases of the circulatory system	20[c]	1.7	0.9–3.0	19 205 Viet Nam veterans 25 677 Non-Viet Nam veterans	1966–85
Bertazzi *et al.* (1989)	Residents of Seveso, Italy, aged 20–74, Zone A (high TCDD region)	Diseases of the circulatory system (ICD 390–459) Ischaemic heart disease Cerebrovascular disease	11 6 2 5	M: 1.8[c] F: 1.9[c] M: 1.3[c] M: 3.3[c]	1.0–3.2 0.8–4.2 0.5–3.3 1.4–8.0	556[d]	1976–86
Bertazzi *et al.* (1992)	Residents of Seveso, Italy, aged 1–19 years, Zone A (high TCDD region)	Diseases of the circulatory system (ICD 390–459)	0 2	M: NR F: 1.6[d]	– 0.3–8.1	306[d]	1976–86

Table 58 (contd)

Reference	Population	Outcome	No. of deaths	SMR[a]	95% CI	Cohort size	Years of follow-up
Flesch-Janys *et al.* (1995)	2,4,5-T, TCP, Bromophos and lindane production workers, Hamburg, Germany	**Cardiovascular disease** (ICD 390–459)	414			1189	1952–92
		Estimated 2,3,7,8-TCDD (ng/kg of blood fat)					
		0–2.8		1.2[c]	0.8–1.8		
		2.81–14.4		0.9	0.5–1.4		
		14.5–49.2		1.4	0.9–2.0		
		49.3–156.7		1.6	1.1–2.4		
		156.8–344.6		1.5	1.0–2.4		
		344.7–3890.2		2.0	1.2–3.3		
		Ischaemic heart disease					
		Estimated 2,3,7,8-TCDD (ng/kg of blood fat)					
		0–2.8		1.4	0.8–2.4		
		2.81–14.4		0.8	0.4–1.6		
		14.5–49.2		1.2	0.7–2.2		
		49.3–156.7		0.9	0.5–1.8		
		156.8–344.6		1.6	0.9–3.0		
		344.7–3890.2		2.5	1.3–4.7		

Table 58 (contd)

Reference	Population	Outcome	No. of deaths	SMR[a]	95% CI	Cohort size	Years of follow-up
		Cardiovascular disease (ICD 390–459)	414				
		Estimated total TEQ levels (ng/kg of blood fat)					
		1.0–12.2		0.9	0.6–1.5		
		12.3–39.5		0.9	0.6–1.5		
		39.6–98.9		1.5	1.0–2.2		
		99.0–278.5		1.6	1.1–2.2		
		278.6–545.0		1.6	1.0–2.6		
		545.1–4361.9		2.1	1.2–3.5		
		Ischaemic heart disease					
		Estimated total TEQ levels (ng/kg of blood fat)					
		1.0–12.2		1.0	0.5–2.0		
		12.3–39.5		1.0	0.5–1.8		
		39.6–98.9		1.0	0.5–1.8		
		99.0–278.5		1.1	0.6–2.0		
		278.6–545.0		1.7	0.9–3.3		
		545.1–4361.9		2.7	1.5–5.0		
Collins *et al.* (1993)	TCP and 2,4,5-T production workers, USA	Diseases of the circulatory system (ICD 390–458)	188	0.9	0.8–1.0	754	1949–87

[a] SMR, Standardized mortality ratio
[b] 90% confidence interval
[c] Relative risk
[d] Zone A males and females combined

levels of 2,3,7,8-TCDD exposure in one study (Flesch-Janys *et al.*, 1995), ischaemic heart disease mortality was consistent with background population rates.

The SMRs for circulatory system diseases reported in the various mortality studies are close to 1.00, suggesting that the 'healthy worker effect' is not seen in these studies. Because employed workers are usually healthier than the general population, the SMR for cardiovascular disease in employed populations tends to be lower than 1.00 (Fox & Collier, 1976; McMichael, 1976). The absence of a healthy worker effect, in the light of the positive results of experiments with animals, suggests that more detailed analyses should be conducted for cardiovascular outcomes in the exposed populations.

Studies of Viet Nam veterans have reported nonsignificant increases in the relative mortality ratio for circulatory diseases (Fett *et al.*, 1987b; Michalek *et al.*, 1990; Wolfe *et al.*, 1994).

Bertazzi *et al.* (1989, 1992) reported increased circulatory disease mortality among residents of Seveso Zone A (the most highly contaminated region) in both men (RR, 1.8; 95% CI, 1.0–3.2) and women (RR, 1.9; 95% CI, 0.8–4.2). The study was limited by the small number of subjects and the crude measure of 2,3,7,8-TCDD exposure.

Several other effects of 2,3,7,8-TCDD on the cardiovascular system have been reported (Bond *et al.*, 1983; Moses *et al.*, 1984; Suskind & Hertzberg, 1984; Centers for Disease Control Vietnam Experience Study, 1988a; Roegner *et al.*, 1991; Grubbs *et al.*, 1995). Statistically significant associations with 2,3,7,8-TCDD exposure were found only in the Ranch Hand study for diastolic blood pressure, arrhythmias detected on the electrocardiogram and peripheral pulse abnormalities (Roegner *et al.*, 1991). Significant increases in blood pressure were found in subjects with serum 2,3,7,8-TCDD levels from 15 to 33.3 ng/kg, but not in those with higher levels.

(*i*) *Pulmonary effects*

There is conflicting evidence from controlled epidemiological studies regarding an association between chronic respiratory system effects and human exposure to substances contaminated with 2,3,7,8-TCDD. One study of workers involved in production of TCP and 2,4,5-T suggested that 2,3,7,8-TCDD exposure increases the risk for abnormal ventilatory function (Suskind & Hertzberg, 1984). This study found statistically significant decreases in pulmonary function as measured by spirometric evaluation. However, the exposed workers were, on average, 10 years older than controls and therefore had greater potential exposure to 2,4,5-T formulated in powder form, as used in earlier periods of production, which may have presented a greater risk of impaired lung function than the liquid form used later.

No association between ventilatory function and serum 2,3,7,8-TCDD was found in the NIOSH study (Calvert *et al.*, 1991). The Ranch Hand study found significant declines in the mean FEV_1 and the mean FVC for subjects with serum 2,3,7,8-TCDD levels above 33.3 ng/kg (adjusted mean FEV_1, 91.3%; mean FVC, 87.4%) compared with an unexposed comparison group (adjusted mean FEV_1, 93.5%; mean FVC, 91.7%) (Roegner *et al.*, 1991). Smoking appeared to have a greater influence on lung function than 2,3,7,8-TCDD exposure. In a follow-up examination conducted in 1992, no consistent relation-

ship was found between serum 2,3,7,8-TCDD concentration and respiratory parameters (Grubbs *et al.*, 1995).

(*j*) *Renal effects*

There is little evidence in the human data to suggest that exposure to 2,3,7,8-TCDD is related to renal or bladder dysfunction. No major renal or bladder dysfunction was noted among Ranch Hand veterans (Roegner *et al.*, 1991) or among TCP production workers from West Virginia (Suskind & Hertzberg, 1984) or New Jersey (Poland *et al.*, 1971).

4.2.2 *Experimental systems*

(*a*) *Species comparisons of toxic effects*

(i) *Lethality and other major effects*

2,3,7,8-TCDD-induced mortality does not occur immediately after exposure, but only after several days or several weeks, and it is therefore not reasonable to perform animal experiments studying specific organ functions during the lag phase between exposure to potentially lethal doses and death.

A very large number of studies on the acute, subchronic and chronic toxicity of PCDDs and PCDFs has revealed that the toxic outcome of treatment with a certain congener or with a mixture of congeners strongly depends on the species, strain and toxicological endpoint investigated. Furthermore, there are remarkable differences in the potency of individual PCDD or PCDF congeners. The situation is further complicated by the fact that the relative potency, and probably the dose–response relationship, of a given congener varies with the experimental system and the parameters of toxicity measured. Finally, the large differences in doses or dose ranges used contribute to the sometimes considerable uncertainty when an 'effect' of PCDDs/PCDFs on a certain biological parameter is discussed.

However, there is no doubt that potent PCDDs/PCDFs have a number of characteristic toxic effects in common, which make them an almost unique example for a distinct class of toxic substances. Among the toxic responses consistently observed in all mammalian species tested so far are a progressive loss of body weight, a reduced intake of food, atrophy of the thymus, gastrointestinal haemorrhage and delayed lethality (Safe, 1986; Vanden Heuvel & Lucier, 1993). Other characteristic signs of toxicity are frequently found in the liver, skin and organs of the endocrine system (see **Tables 59** and **60** for 2,3,7,8-TCDD data). The most sensitive endpoints for 2,3,7,8-TCDD effects are listed in **Table 61**.

An early report on the acute toxicity of 2,3,7,8-TCDD, 2,7-DCDD, an unspecified HxCDD and OCDD in animals was published by Schwetz *et al.* (1973). The authors reported the much higher acute toxicity of 2,3,7,8-TCDD compared with HxCDD or OCDD in guinea-pigs and rabbits. Furthermore, the acnegenic, teratogenic and hepatotoxic properties of 2,3,7,8-TCDD and HxCDD were described.

Table 59. Acute lethality of 2,3,7,8-TCDD in various animal species and strains

Species/strain (sex)	Administration	LD_{50} (μg/kg)	Time of death (days after exposure)	Follow-up (days)	Body weight loss[a] (%)	References
Guinea-pig/Hartley (male)	Oral	0.6–2.0	5–34	NR 30	50	Schwetz *et al.* (1973); McConnell *et al.* (1978a); DeCaprio *et al.* (1986)
Mink/NR (male)	Oral	4.2	7–17	28	31	Hochstein *et al.* (1988)
Chicken/NR	Oral	< 25	12–21	NR	NR	Greig *et al.* (1973)
Rhesus monkey (female)	Oral	*c.* 70	14–34	42–47	13–38	McConnell *et al.*. (1978b)
Rat/Long-Evans (male)	Intraperitoneal	*c.* 10	15–23	48–49	39	Tuomisto & Pohjanvirta (1987)
Rat/Sherman, Spartan (male) (female)	Oral	 22 13–43	9–27	NR	NR	Schwetz *et al.* (1973)
Rat/Sprague-Dawley (male)	Oral	43	28–34	30 or until death	NR	Stahl *et al.* (1992a)
Rat/Sprague-Dawley (male) (female) (weanling male)	Intraperitoneal	 60 25 25	NR	20	NR	Beatty *et al.* (1978)
Rat/Fischer Harlan (male)	Oral	340	28[b]	30	43	Walden & Schiller (1985)
Rat/Han-Wistar (male)	Intraperitoneal	> 3000	23–34	39–48	40–53	Pohjanvirta & Tuomisto (1987); Pohjanvirta *et al.* (1987)

Table 59 (contd)

Species/strain/sex	Administration	LD_{50} (μg/kg)	Time of death (days post-exposure)	Follow-up (days)	Body weight loss[a] (%)	References
Mouse C57BL/6 (male) DBA2/2J (male) B6D2F1 (male)	Oral	181 2570 296	24[b] 21[b] 25[b]	30	25 33 34	Chapman & Schiller (1985)
Mouse C57BL/6 DBA2 B6D2F1	Intraperitoneal	132 620 300	NR	NR	NR	Neal *et al.* (1982)
Rabbit/New Zealand White (male and female)	Oral Dermal	115 275	6–39 12–22	NR 22	NR NR	Schwetz *et al.* (1973)
Rabbit/New Zealand White (male and female)	Intraperitoneal	ca. 50	7–10	10–20	14.5	Brewster *et al.* (1988)
Syrian hamster (male and female)	Oral	1157–5051	2–43	50	NR	Henck *et al.* (1981)
Syrian hamster (male and female)	Intraperitoneal	> 3000	14–32	50	1[c]	Olson *et al.* (1980)

[a] Of dead animals
[b] Mean time to death
[c] Data from five animals
NR, not reported

Table 60. Acute toxic responses following exposure to 2,3,7,8-TCDD: species differences[a]

Response	Rhesus monkey	Guinea-pig	Mink	Rat	Mouse	Rabbit[b]	Chicken[b]	Syrian hamster
Hyperplasia or metaplasia								
Gastric mucosa	++	0	+	0	0			0
Intestinal mucosa	+		±					++
Urinary tract	++	++		0	0			
Bile duct and/or gall-bladder	++	0			++			0
Lung: focal alveolar				++				
Skin	++	0		0	0	++		0
Hypoplasia, atrophy, or necrosis								
Thymus	+	+		+	+		+	+
Bone marrow	+	+			±		+	
Testicle	+	+		+	+		+	+
Other lesions								
Liver lesions	+	±		+	++	+	+	±
Porphyria	0	0		+	++		+	0
Oedema	+	0		0	+		++	+
Haemorrhage	+	+	+	+	+			+

0, lesion not observed; +, lesion observed; ++, severe lesion observed; ±, lesion observed to a very limited extent; blank, no evidence reported in the literature.

[a] References: Kimmig & Schulz (1957a); Allen & Lalich (1962); Vos & Koeman (1970); Vos & Beems (1971); Greig *et al.* (1973); Gupta *et al.* (1973); Norback & Allen (1973); Schwetz *et al.* (1973); Vos *et al.* (1974); Allen *et al.* (1977); Kociba *et al.* (1976, 1978); McConnell *et al.* (1978a,b); Kociba *et al.* (1979); Moore *et al.* (1979); McConnell (1980); Olson *et al.* (1980); Henck *et al.* (1981); Turner & Collins (1983); Hochstein *et al.* (1988)

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

Table 61. No observed-effect and lowest-observed-effect levels (NOEL and LOEL) of 2,3,7,8-TCDD for mammalian species

Species	Experimental design	NOEL	LOEL	Effect	Reference
Rhesus monkey	0.5 µg/kg in food, 9 months		12 ng/kg/d	Death	Allen *et al.* (1977)
	2 µg/kg in food, 61 days		50 ng/kg/d	Death	McNulty (1977)
	0.05 µg/kg in food, 20 months		1.5 ng/kg/d	Hair loss	Schantz *et al.* (1979)
	5 and 25 ng/kg in food to mother, 4 years		0.126 ng/kg/d	Object recognition, juvenile	Bowman *et al.* (1989b)
	5 and 25 ng/kg in food to mother, 4 years		0.642 ng/kg/d	Prenatal death	Bowman *et al.* (1989a); Hong *et al.* (1989)
	9 × 22–111 ng/kg to mother, days 20–40 of pregnancy		9 ×111 ng/kg	Prenatal death	McNulty (1984)
	25 ng/kg in food to mother, 4 years		0.642 ng/kg/d	Change in lymphocytes	Hong *et al.* (1989)
	5 and 25 ng/kg in food to mother, 4 years		0.126 ng/kg/d	Endometriosis	Rier *et al.* (1993)
Marmoset	3 ng/kg, 1 × orally		3 ng/kg	Induction of CYP1A2	Krueger *et al.* (1990)
	0.3 ng/kg/wk × 24 wk		0.135 ng/kg/d chronic.[a]	Change in lymphocytes	Neubert *et al.* (1992)
	1.5 ng/kg/wk × 6 wk + 0 × 12 wk				
Sprague-Dawley rat	2 ng/kg, 1 × orally	0.6 ng/kg	2 ng/kg	Induction of CYP1A1	Kitchin & Woods (1979)
	1–100 ng/kg/d, orally, 2 years	1 ng/kg/d	10 ng/kg/d	Porphyria	Kociba *et al.* (1978)
	14–1024 ng/kg/d, orally, 3 months	< 14 ng/kg/d	14 ng/kg/d	Less vitamin A	van Birgelen *et al.* (1995a)
	14–1024 ng/kg/d, orally, 3 months	0.3 ng/kg/d[a]	14 ng/kg/d	Induction of CYP1A1	van Birgelen *et al.* (1995a)
	14–1024 ng/kg/d, orally, 3 months	0.5 ng/kg/d[a]	14 ng/kg/d	Induction of CYP1A2	van Birgelen *et al.* (1995a)
	1–100 ng/kg to mother, chronic	1 ng/kg/d mth	10 ng/kg/d	Prenatal death	Murray *et al.* (1979)
	30 ng/kg/d to mother, days 6–15 of pregnancy	30 ng/kg/d mth		Prenatal death	Sparchu *et al.* (1971)

Table 61 (contd)

Species	Experimental design	NOEL	LOEL	Effect	Reference
Sprague-Dawley rat	100–10 000 ng/kg/d, 30 d		100 ng/kg/d	Lower serum glucose	Zinkl *et al.* (1973)
	Calculated with data from Tritscher *et al.* (1992) and Sewall *et al.* (1993); 3.5-10, 7–35, 7–125 ng/kg/d, 30 wk	0.01 ng/kg/d 0.1 ng/kg/d 0.1 ng/kg/d 0.1 ng/kg/d 0.1 ng/kg/d	0.1 ng/kg/d 1 ng/kg/d 1 ng/kg/d 1 ng/kg/d 1 ng/kg/d	CYP1A induction CYP1A2 induction Ah receptor induction EGF receptor induction Oestrogen receptor induction	Kohn *et al.* (1993)
Holtzmann rat	64 ng/kg/d to mother, day 15 of pregnancy		64 ng/kg/d	Decrease in male reproductive capacity	Mably *et al.* (1992a,b)
C57BL/6 mouse	1 ng/kg/wk, 4 wk intraperitoneally		1 ng/kg/wk	Immunosuppression, low CTL generation	Clark *et al.* (1983)
B6C3F1 mouse	10 ng/kg, 7 days after fertilization		10 ng/kg, d 7	Increase in viral infection	Lebrec & Burleson (1994)
Guinea-pig	8–200 ng/kg/wk, 8 wk		8 ng/kg/wk 200 ng/kg/wk	Immunosuppression Lower response to tetanus toxin	Vos *et al.* (1973)

wk, week(s); d, day(s); EGF, epidermal growth factor; CTL, cytotoxic T-lymphocyte

[a] Calculated

Guinea-pig

Guinea-pigs are among the most sensitive species towards 2,3,7,8-TCDD and other PCDDs and PCDFs, with regard to lethality (Plüss *et al.*, 1988a), with 90% dying from a single 3 µg/kg dose (Harris *et al.*, 1973). The mean time interval until death was 18 days, and a large weight loss over a period of days preceded death. Thymic atrophy was observed after treatment with weekly doses of 0.008 µg/kg 2,3,7,8-TCDD over four to five weeks (Gupta *et al.*, 1973). Other lesions were haemorrhages, hyperplasia of the urinary bladder mucosa and atrophy of the adrenal zona glomerulosa. In a subchronic study, DeCaprio *et al.* (1986) treated guinea-pigs with various doses of 2,3,7,8-TCDD in the diet for up to 90 days. At a total dose of 0.44 µg/kg, animals exhibited a decreased rate of body weight gain and increased relative liver weights. Male animals also displayed a reduction in relative thymus weights and elevated serum levels of triglycerides, while females showed hepatocellular cytoplasmic inclusion bodies and lowered serum ALT activities. A no-observed-effect level of approximately 0.65 ng/kg per day for prolonged exposure was calculated for these lesions.

After a single lethal dose of ≥ 10 µg/kg 2,3,7,8-TCDD, there was a marked reduction in the size of the thymus, loss of body fat and reduction of muscle mass in guinea-pigs. Histopathological examination revealed epithelial hyperplasia in the renal pelvis, ureter and urinary bladder, hypocellularity of the bone marrow and seminiferous tubules and loss of lymphocytes in thymic cortex, spleen and Peyer's patches, whereas no marked pathological alterations of the liver were evident (Moore *et al.*, 1979). After treatment of guinea-pigs with 20 µg/kg 2,3,7,8-TCDD, hepatocellular hypertrophy, steatosis, focal necrosis and hyalin-like cytoplasmic bodies as well as hepatic focal necrosis were observed in a study by Turner and Collins (1983).

Rat

The single oral LD_{50} value of four PCDDs in male Sprague-Dawley rats was determined to be 43 µg/kg for 2,3,7,8-TCDD, 206 µg/kg for 1,2,3,7,8-PeCDD, 887 µg/kg for 1,2,3,4,7,8-HxCDD and 6325 µg/kg for 1,2,3,4,6,7,8-HpCDD (Stahl *et al.*, 1992a). A mixture containing all four congeners in a predicted equitoxic amount showed strictly additive toxic effects.

The discovery of a more than 300-fold difference in acute LD_{50} values for 2,3,7,8-TCDD between the Long-Evans and Han/Wistar rat strains led to a number of studies aimed at elucidating the biochemical basis of this difference. Han/Wistar rats are extraordinarily resistant towards 2,3,7,8-TCDD (Pohjanvirta & Tuomisto, 1987). Doses of 125–1400 µg/kg resulted in marked decreases in food consumption, and in a decrease of 20–25% in body weight. However, the lethality to the animals was low (two of 40 died). A similar strain difference exists for 1,2,3,7,8-PeCDD, whereas it was much less pronounced for 1,2,3,4,7,8-HxCDD (Pohjanvirta *et al.*, 1993). Genetic crossings between Long-Evans and Han/Wistar rats suggest that 2,3,7,8-TCDD resistance is the dominant trait in the rat. Two (or possibly three) genes seem to regulate resistance (Pohjanvirta, 1990). However, even within a given rat strain, such as Long-Evans or Han/Wistar, substrains with markedly differing sensitivity towards 2,3,7,8-TCDD may exist.

Therefore, a critical analysis of 2,3,7,8-TCDD sensitivity in the used animals is required (Pohjanvirta & Tuomisto, 1990a). Most toxic effects are similar in both strains, the Long-Evans strain, however, being more sensitive. In the livers of Long-Evans rats, a (lethal) dose of 50 μg/kg bw 2,3,7,8-TCDD caused marked hepatocyte swelling, vacuoles probably containing fat in the cytoplasm, multinuclear cells (up to eight nuclei) and accumulation of inflammatory cells in hepatic sinusoids (Pohjanvirta *et al.*, 1989a). At the same dose, Han/Wistar rats showed slight cytoplasmic swelling and irregular haematoxylin–eosin staining. Thymic atrophy was seen in both strains, the Long-Evans strain being about 10-fold more sensitive than the Han/Wistar strain. Furthermore, pronounced reduction in serum T4 and increases in TSH, corticosterone and free fatty acids were observed in Long-Evans rats.

In CD rats, moderate thymic atrophy was observed after treatment with 1 μg/kg per day over four to five weeks (Gupta *et al.*, 1973). Other effects were haemorrhages, degenerative changes in kidney and thyroid, increase in megakaryocytes in spleen, lymphoid depletion of the spleen and lymph nodes and severe liver lesions. Female CD rats were slightly more sensitive than males (Harris *et al.*, 1973). A more sensitive indicator of 2,3,7,8-TCDD exposure than body weight reduction was a decrease in thymus weight. Blood coagulation was not significantly affected in five-week-old rats that had received 1 μg/kg 2,3,7,8-TCDD (Bouwman *et al.*, 1992).

In a 13-week oral toxicity study in adult Sprague-Dawley rats (Kociba *et al.*, 1976; Goodman & Sauer, 1992), 1 μg/kg 2,3,7,8-TCDD per day caused some mortality, inactivity, decreased body weights and food consumption, icterus, pathomorphological changes in the liver, lymphoid depletion of the thymus and other lymphoid organs and minimal alterations of some haematopoietic components. Doses of 0.1 μg/kg 2,3,7,8-TCDD per day also caused decreased body weights and food consumption and lymphoid depletion. Effects seen only in males given this dose level included a depression in packed blood cell volume, red blood cells and haemoglobin. Lower doses (0.01 or 0.001 μg/kg 2,3,7,8-TCDD per day) did not affect any of the measured parameters, except for a slight increase in the mean liver-to-body weight ratio in rats given 0.01 μg/kg 2,3,7,8-TCDD per day. A single dose of 1 or 10 μg/kg 2,3,7,8-TCDD led to a pronounced reduction in blood glucose. In female Sprague-Dawley rats, chronic daily intake of 47 ng/kg 2,3,7,8-TCDD resulted in a decrease in plasma concentration of thyroid hormone (total T4) and body weight. More sensitive parameters influenced at 14 ng/kg per day were a decrease in relative thymus weight, loss of hepatic retinoids and induction of CYP1A1 and CYP1A2 activities in the liver (van Birgelen *et al.*, 1995a). Hepatic CYP1A1 and CYP1A2 were induced at comparable doses of 2,3,7,8-TCDD in both mice and rats (Smialowicz *et al.*, 1994).

Hamster

In the Syrian hamster, 2,3,7,8-TCDD treatment led to loss of body weight and thymic atrophy, while no histopathological changes were seen in the liver, spleen, kidney adrenals or heart (Olson *et al.*, 1980). Oral treatment resulted in moderate to severe ileitis and peritonitis in many animals, associated with marked hyperplasia with mild to severe haemorrhage and necrosis of the mucosal epithelium.

Inter-species rodent comparisons

In a comparative study in rats, mice and guinea-pigs (Zinkl *et al.*, 1973), 2,3,7,8-TCDD was found to cause acute/subacute increases in serum levels of alanine aminotransferase (ALT, previously SGPT) and aspartate aminotransferase (AST, previously SGOT), cholesterol and bilirubin in rats after a single dose of 1 or 10 µg/kg bw. Histopathological examination also suggested that the liver is highly sensitive to 2,3,7,8-TCDD in rats but not in guinea-pigs and mice. Furthermore, significant reductions in numbers of platelets in the peripheral blood of rats and guinea-pigs, and lymphopenia in mice and guinea-pigs were evident.

In male C57BL/6 mice (Gasiewicz *et al.*, 1980) and guinea-pigs, various PCDD congeners were administered as a single oral dose (McConnell *et al.*, 1978a). LD_{50}s of 2 µg/kg in guinea-pigs and 283.7 µg/kg in mice were determined for 2,3,7,8-TCDD. The acute toxicity of 2,3,7,8-substituted PCDD was in the following rank order: 1,2,3,4,6,7,8 < 1,2,3,7,8,9 = 1,2,3,6,7,8 = 1,2,3,4,7,8 < 1,2,3,7,8 < 2,3,7,8. In both species, 2,3,7,8-TCDD strongly reduced the thymus weight due to a reduction in cortical lymphocytes. Significant macroscopic or histopathological hepatic effects including porphyria were observed only in the mouse. Hyperplasia of the transitional epithelium in the urinary tract was found in guinea-pigs.

The relative toxicity of four different congeners was found by Rozman *et al.* (1993) to be similar in guinea-pigs and Sprague-Dawley rats following acute, subchronic or chronic dosing. Furthermore, the dose–response relationships for 2,3,7,8-TCDD concerning the endpoints mortality, porphyria and carcinogenicity were very similar. Therefore, the authors suggested that the product of average tissue concentration and time can describe the toxicity for a given congener.

Rhesus monkey

In rhesus monkeys (*Macaca mulatta*) fed toxic fat containing 2,3,7,8-TCDD, gastric hyperplasia and ulceration, hydropericardium, ascites, reduced spermatogenesis, focal liver necrosis, decreased haematopoiesis, skin lesions and eventual mortality were observed (Allen *et al.*, 1977). In an earlier study (Norback & Allen, 1973), atrophy of the lymph tissue, liver enlargement and a reduction in the number of spermatocytes in the testes were also reported.

Following administration of a single dose of 70 µg/kg 2,3,7,8-TCDD by gastric instillation to rhesus monkeys, McConnell *et al.* (1978b) observed weight loss, blepharitis, loss of fingernails and eyelashes, facial alopecia with acneform eruptions, mild anaemia, neutrophilia, lymphopenia and a decrease in serum cholesterol with an increase in serum triglyceride concentrations. The liver, adrenal gland and kidney showed increased relative organ weight, whereas the thymus was dramatically reduced in size due to a loss of cortical lymphocytes. Histopathological examination revealed hyperplastic and metaplastic changes in sebaceous glands, especially in the eyelid (meibomian glands) and ear canal, together with epithelial hyperplasia in the renal pelvis, stomach, gall-bladder and bile duct, whereas the histology of the liver parenchyme was relatively normal.

Cachexia

One of the most striking effects of 2,3,7,8-TCDD in all mammalian species tested so far is body weight loss combined with a reduced intake of food. From a pair-feeding experiment, hypophagia was suggested to be the major factor leading to wasting in guinea-pigs, Fischer 344 rats and C57BL/6 mice (Kelling *et al.*, 1985). However, lethality was only partially due to body weight loss, since the lethality, in particular in rats and mice, was significantly lower among pair-fed controls than among 2,3,7,8-TCDD-treated animals. Similarly, total parenteral nutrition, preventing the body weight loss associated with 2,3,7,8-TCDD toxicity in rats after a single dose of 100 µg/kg bw, did not prevent death (Gasiewicz *et al.*, 1980).

Seefeld *et al.* (1984a) reported that 2,3,7,8-TCDD-treated rats maintained their body weight at a lower, nearly constant percentage of that of control rats fed *ad libitum*. The authors concluded from this and a number of additional experiments that the target level for body weight is reduced under the influence of 2,3,7,8-TCDD. Hypophagia relative to untreated animals was suggested to be the key symptom of this down-regulation of body weight, whereas malabsorption or less efficient feed utilization did not play a decisive role (Seefeld *et al.*, 1984b). The role of hypophagia in weight loss, and the changes in carbohydrate and lipid metabolism observed after 2,3,7,8-TCDD treatment, were questioned by Chapman and Schiller (1985), who found no influence of 2,3,7,8-TCDD on the cumulative amount of feed consumption by C57BL/6 mice until the animals became moribund. In addition, the alterations in serum glucose and lipid concentrations in fasted mice were very different from those induced by 2,3,7,8-TCDD exposure. In DBA/2J mice bearing a low-affinity Ah receptor, dose–response experiments showed comparable changes in glucose and lipid parameters when the animals were exposed to 10-fold higher doses of 2,3,7,8-TCDD than C57BL/6 mice.

In a series of experiments, Pohjanvirta and Tuomisto (1990b) administered sodium chloride, 2-deoxy-D-glucose, sodium mercaptoacetate, insulin, naloxone, glucose or fructose to 2,3,7,8-TCDD-treated or control rats. 2,3,7,8-TCDD caused an attenuation of feeding responses to metabolic deficits, resulting, for example, in severe hypoglycaemia (Pohjanvirta *et al.*, 1991a), but induced hypersensitivity to peripheral satiety signals. The central nervous system was suggested to play a crucial role in these effects. Transfusion of blood from rats with 2,3,7,8-TCDD-induced appetite suppression into untreated rats did not affect their feed intake, whereas transfusion of blood from normally sated rats did (Rozman *et al.*, 1991). This indicated that 2,3,7,8-TCDD treatment does not increase a satiety-signalling factor in blood but may rather suppress the formation of hunger-related signals. Evidence for the involvement of a serotonergic mechanism was derived from the finding that 2,3,7,8-TCDD increased the levels of tryptophan and its metabolites serotonin and 5-hydroxyindoleacetic acid in blood and brain.

2,3,7,8-TCDD treatment also produced a longer-lasting suppression of feed intake after oral treatment with glucose than in untreated rats (Pohjanvirta *et al.*, 1991b) and induced aversion to eating energy-providing food, irrespective of its type (Pohjanvirta & Tuomisto, 1990c). From another series of experiments in rats, including behavioural, biochemical and antiemetic approaches, it was concluded that nausea cannot explain the

lethal wasting syndrome (Pohjanvirta *et al.*, 1994a). Food intake and body weight loss after 2,3,7,8-TCDD treatment of Long-Evans rats did not change after either vagotomy or portocaval anastomosis resulting in a circumvention of the liver and a direct blood flow from the intestine to extrahepatic organs (Tuomisto *et al.*, 1995).

Cachexia observed after 2,3,7,8-TCDD treatment can also be induced with certain cytokines such as the endotoxin-responsive tumour necrosis factor α (TNFα) (Döhr *et al.*, 1994), which led to the suggestion that TNFα might mediate the acute toxicity of 2,3,7,8-TCDD. In fact, mortality following a single 2,3,7,8-TCDD dose of 300 μg/kg in male C57BL/6J mice was significantly reduced by treatment with the anti-inflammatory corticoid dexamethasone or with anti-TNFα antibodies (Taylor *et al.*, 1992). In endotoxin-non-responsive C3H/HeJ mice, Clark and Taylor (1994) found no trend in body weight loss after a single 2,3,7,8-TCDD dose of 350 μg/kg, while endotoxin-responsive C57BL/6 mice demonstrated a statistically significant decline in body weight. However, pathological effects, such as peritoneal infiltration and hepatotoxicity, were also observable in C3H/HeJ mice.

Mechanistic studies of toxicity

Early attempts to investigate the mechanism of action of 2,3,7,8-TCDD in cell culture were made by Knutson and Poland (1980). In 23 cultured cell types including rat hepatoma cells and primary hepatocytes, mouse fibroblasts and human epithelial cell lines, no morphological abnormalities were observed with 10^{-7}–10^{-11} M 2,3,7,8-TCDD. Analysis of trypan blue exclusion or cell proliferation in a number of cell types also failed to show any toxicity. Also, aryl hydrocarbon hydroxylase activity was inducible only in a subgroup of cell types including most hepatoma cell lines, some mouse embryo cell lines and primary rat and chicken hepatocytes.

The fundamental role of the Ah receptor for the toxicity of 2,3,7,8-TCDD (see Section 4.3) was demonstrated by Poland and Glover (1979). DBA/2J mice, which have a low-affinity receptor, were approximately 10-fold less sensitive to thymic involution after 2,3,7,8-TCDD treatment than C57BL/6J mice, a result that precisely matches the 10-fold difference in affinity of 2,3,7,8-TCDD for the Ah receptor (Ema *et al.*, 1994). Furthermore, the capacity of other halogenated aromatic hydrocarbons to produce thymic atrophy corresponded to their capacity to bind to the receptor. The same dependence on receptor affinity applied to the production of cleft palate and to the reduction of the adipose-type (type 4) glucose transporter (GLUT) in adipose tissue and of the brain-type transporter GLUT1 in brain (Liu & Matsumura, 1995).

Comparison of low-affinity Ah receptor (Ah^d/Ah^d) with congenic wild-type high-affinity (Ah^b/Ah^b) Ah receptor mice revealed LD_{50} values for 2,3,7,8-TCDD of 3351 and 159 μg/kg, respectively. A similar difference in dose–response was found for the decrease in body weight, increase in liver weight and decreases in the weight of thymus, spleen, testes and epididymal fat pad (Birnbaum *et al.*, 1990). Okey *et al.* (1995) pointed out the strong genetic link between the Ah receptor and the sensitivity of different mouse strains towards a wide variety of toxic outcomes *in vivo* such as thymic atrophy, induction of cleft palate and porphyria (Jones & Sweeney, 1980; Poland & Knudson, 1982).

It has been claimed, however, that the discrepancy between the Han/Wistar and Long-Evans rats cannot be explained on the basis of Ah receptor concentration, and that differences in the acute toxicity of 2,3,7,8-TCDD between Long-Evans and Sprague-Dawley rats are not related to Ah receptor-mediated induction of ethoxyrufin-*O*-deethylase activity (Fan & Rozman, 1994). In a reply, Okey *et al.* (1995) pointed out that the very diversity of tissue-selective and species-selective responses elicited by 2,3,7,8-TCDD almost requires that the receptor is part of a multicomponent system. Therefore, it is unlikely that the differences in dose–response are related solely to differences in Ah receptor concentrations or affinities in various species or tissues. In particular, conclusions on the relative susceptibility of different species cannot be drawn on the basis of receptor data only.

Fernandez-Salguero *et al.* (1995) demonstrated that Ah receptor-deficient mice are relatively unaffected by doses of 2,3,7,8-TCDD (2000 μg/kg) 10-fold higher than those found to induce severe toxic and pathological effects in litter mates expressing a functional Ah receptor.

(ii) *Skin*

The occurrence of cutaneous lesions after treatment with 2,3,7,8-TCDD and related compounds has been described primarily in humans and non-human primates; most rodent species do not represent suitable experimental models for the study of such lesions. The development of a strain of hairless mice susceptible to the skin toxicity of 2,3,7,8-TCDD was an important first step to provide such a model.

In haired and hairless newborn and adult mice, skin application of 2,3,7,8-TCDD caused an involution of sebaceous glands (Puhvel & Sakamoto, 1988). Epidermal hyperplasia and hyperkeratinization, however, were induced in the hairless mice only. The density of inflammatory cell infiltrates in the skin was not reduced by topical treatment with anti-inflammatory agents. The distinct pattern of chloracne observable in hairless mice (Puhvel *et al.*, 1982) did not include the hyperkeratinization of the sebaceous follicles that is typical of human chloracne. Histopathological changes observed with all acnegenic compounds were epidermal hyperkeratosis and hyperplasia, loss of sebaceous glands, keratinization of intradermal pilar cysts and diffuse lymphohistiocytic infiltration of the dermis. Atrophy or complete absence of the hair follicles was evident in severe lesions (Hébert *et al.*, 1990b). In these cases, the epidermis was atrophic with keratinization.

Skin application of 2,3,7,8-TCDD to hairless mice produced an increase in relative liver weight after treatment with 5 ng per week, and a decrease in relative thymus weight after treatment with 20 ng per week over 20 weeks (Hébert *et al.*, 1990a).

Connor *et al.* (1994) showed that the acute systemic toxicity of 2,3,7,8-TCDD is even higher in mice bearing the recessive mutation hairless (hr). Vitamin A deficiency enhanced the dermal but not the systemic toxicity of 2,3,7,8-TCDD after dermal application to hairless mice (Puhvel *et al.*, 1991). 2,3,7,8-TCDD treatment did not affect cutaneous vitamin A levels. Using an ear bioassay for acnegenic activity, Schwetz *et al.* (1973) showed that the rabbit is highly sensitive to the acnegenic effect of 2,3,7,8-TCDD; a single treatment with a solution containing 4 ng/mL 2,3,7,8-TCDD led to a

positive result. In rhesus monkeys, a single dose of 1000 ng/kg bw 2,3,7,8-TCDD induced chloracne (McNulty, 1975).

The ability of 2,3,7,8-TCDD and 1,2,4,7,8-PeCDD to induce a flat, cobblestone-like morphology was studied in a non-keratinizing derivative (XBF) of the keratinizing XB mouse epithelial cell line cocultured with irradiated 3T3 feeder cells. The minimal concentrations required to produce these changes from the normal spindle-shape cells, over a 14-day exposure period, were 0.0032 μg/L (ppb) for 2,3,7,8-TCDD and 0.359 μg/L for 1,2,4,7,8-PeCDD (Gierthy & Crane, 1985).

The growth inhibitory effect of transforming growth factor (TGF) β1 in the human squamous carcinoma cell lines, SCC-15G and SCC-25, was not affected by 10^{-8} M 2,3,7,8-TCDD (Hébert *et al.*, 1990b). Furthermore, 2,3,7,8-TCDD had no effect on binding of ^{125}I-labelled TGFβ1 to SCC-15G cells or secretion of TGFβ1 by them, while TGFβ1 suppressed the induction of 7-ethoxyresorufin *O*-deethylase (EROD) activity. The authors concluded that 2,3,7,8-TCDD and TGFβ1 exert their opposite effects on proliferation of the SCC lines by independent mechanisms.

In two human squamous carcinoma cell lines, 0.1 or 1 nM 2,3,7,8-TCDD induced proliferation (Hébert *et al.*, 1990c). This effect was detectable only in cells exposed at subconfluent density, indicating that 2,3,7,8-TCDD prevented normal density-dependent growth arrest. In addition, 2,3,7,8-TCDD inhibited differentiation, measured as keratin staining, and envelope formation in the presence of calcium ionophore. When XB cells derived from a mouse teratoma were cultured at high density, cocultured with irradiated 3T3 feeder-cells, 5×10^{-11} M 2,3,7,8-TCDD produced maximal keratinization (Knutson & Poland, 1980). The potency of other Ah receptor agonists to induce keratinization correlated with their binding affinities to the receptor.

In human cultured epidermal cells, 2,3,7,8-TCDD decreased the number of small (basal) cells and DNA synthesis, while increasing the number of cells containing spontaneous envelopes (which consist of insoluble cross-linked proteins beneath the plasma membrane; an indicator of terminal differentiation of epidermal keratinocytes), as well as the number of envelope-competent cells (Osborne & Greenlee, 1985). On the basis of these findings, it was proposed that 2,3,7,8-TCDD enhances terminal differentiation in epidermal basal cells (Hudson *et al.*, 1986), leading, for example, to hyperkeratinization. T3 and T4 did not produce hyperkeratinization in human epidermal cells or in the SCC-12F human keratinocyte cell line, indicating that 2,3,7,8-TCDD and thyroid hormone effects on the skin are mediated by different mechanisms (Osborne *et al.*, 1987).

In non-transformed human keratinocytes, 2,3,7,8-TCDD treatment caused an increase in the state of differentiation, as judged by an increase in cross-linked envelope formation, and an increase in stratification and keratin staining (Gaido & Maness, 1994).

(iii) *Nervous system*

The possible links between the anorexigenic potency of 2,3,7,8-TCDD and biogenic amines at central nervous sites controlling body weight and food intake were investigated in a number of reports. After measuring noradrenaline, dopamine, dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan

and histamine in the brain and pituitary gland of 2,3,7,8-TCDD-treated male Long-Evans rats, Tuomisto *et al.* (1990) concluded that 2,3,7,8-TCDD caused minor changes in brain neurotransmitter systems, which were not likely to cause 2,3,7,8-TCDD-induced hypophagia. Eight days after intraperitoneal injection with 1000 μg/kg 2,3,7,8-TCDD, a significant increase in tryptophan concentration of about 20% was found in the lateral hypothalamic area and in lateral and medial accumbens nuclei (Unkila *et al.*, 1993a). Furthermore, a slight tendency to diminished dopamine, serotonin and/or 5-hydroxy-indoleacetic acid levels in various brain sites was found during the first day after exposure. Histamine concentrations were not changed in a number of discrete brain nuclei but in the median eminence, 25 h after a single intraperitoneal dose of 1000 μg/kg 2,3,7,8-TCDD (Tuomisto *et al.*, 1991). Unkila *et al.* (1994a) found a selective increase in brain serotonin turnover in 2,3,7,8-TCDD-susceptible Long-Evans but not in 2,3,7,8-TCDD-resistant Han/Wistar rats. The authors related this to increased plasma levels of tryptophan, possibly resulting from inhibited tryptophan catabolism in the liver. Direct application of 2,3,7,8-TCDD into the lateral cerebral ventricle of rats, leading to much higher brain concentrations than intravenous administration of the same dose, did not cause appetite suppression or loss of body weight, whereas animals treated intravenously displayed the cachectic syndrome (Stahl & Rozman, 1990). In contrast, Pohjanvirta *et al.* (1989b) reported that intracerebroventricular injection of 2,3,7,8-TCDD into male Han/Wistar or Long-Evans rats depressed food intake more severely than subcutaneous administration.

Hanneman *et al.* (1996) recently reported that 2,3,7,8-TCDD induces rapid calcium uptake in rat hippocampal neuronal cells, accompanied by decreased mitochondrial membrane potential and increased neuronal membrane protein kinase C activity.

In young male Han/Wistar rats, a single intraperitoneal dose of 1000 μg/kg 2,3,7,8-TCDD did not lead to changes in behaviour or motility (Sirkka *et al.*, 1992). In particular, spontaneous motor activity, anxiety scores, passive avoidance learning, motor coordination and nociception were not altered markedly. A slowing of sensory and motor conduction velocities was observed in male Wistar rats 10 months after a single intravenous injection of 2.2 μg/kg 2,3,7,8-TCDD. Histopathological examination of peripheral nerves revealed a progressive, and proximally accentuated neuropathy (Grehl *et al.*, 1993).

(iv) *Liver*

In male rhesus monkeys, an oral dose of 5 μg/kg bw 2,3,7,8-TCDD caused an initial mild increase in indocyanine green blood clearance followed by a slight decrease (Seefeld *et al.*, 1979). Serum glutamic pyruvate transaminase and sorbitol dehydrogenase levels were increased. Light microscopy of the livers revealed fatty infiltration with minimal hepatocellular necrosis. In rats, hepatotoxic reactions were characterized by swelling of hepatocytes, fatty metamorphosis and ultimately necrosis after treatment with 10 μg/kg 2,3,7,8-TCDD per day over 10–13 days (Gupta *et al.*, 1973). At this time, there was also an increase in serum transaminase activities. Thereafter, the hepatic lesions progressed and more parenchymal tissue was destroyed. Besides these degenerative lesions, large multinucleated giant hepatocytes were also seen.

A high dose of 2,3,7,8-TCDD (25 μg/kg bw) led to impairment of the clearance and biliary excretion of phenol-3,6-dibromophthalein and sulfobromophthalein, reduced bile flow, swelling and occasional necrosis of hepatocytes and infiltration of mononuclear inflammatory cells in the liver of male Holtzman rats (Yang *et al.*, 1977). Twenty-five days after treatment with 10 μg/kg bw, however, an enhancement of biliary excretion of the bromophthaleins was observed.

In 2,3,7,8-TCDD-sensitive C57BL/6J mice, 3 μg/kg bw 2,3,7,8-TCDD caused mild to moderate hepatic lipid accumulation in the absence of both inflammation and necrosis (Shen *et al.*, 1991), while severe fatty change and mild inflammation and necrosis occurred after treatment with 150 μg/kg. In contrast, DBA mice exposed to 30 μg/kg 2,3,7,8-TCDD developed hepatocellular necrosis and inflammation without any fatty change. The authors concluded that the Ah locus plays a role in determining the sensitivity of mice to the steatotic effects of 2,3,7,8-TCDD in the liver. In female CD1 mice, 2,3,7,8-TCDD produced a centrilobular pattern of hepatocellular degeneration and necrosis with perivascular infiltration of inflammatory cells (MacKenzie *et al.*, 1992). This effect was potentiated by tamoxifen, which is possibly associated with decreased hepatic excretion of 2,3,7,8-TCDD.

A single low-lethality oral dose of 75 μg/kg 2,3,7,8-TCDD induced hepatic porphyria in both male and female C57BL/10 mice (Smith *et al.*, 1981), which was associated with decreased activity of hepatic uroporphyrinogen decarboxylase. DBA/2 mice, bearing a low-affinity Ah receptor, were much less sensitive to these effects of 2,3,7,8-TCDD. In male C57BL/6 mice, chronic administration of 25 μg/kg 2,3,7,8-TCDD per week resulted in hepatic porphyrin accumulation and inhibition of porphyrinogen decarboxylase activity (Cantoni *et al.*, 1984). Partial antagonism of 2,3,7,8-TCDD-induced hepatic porphyrin accumulation in male C57BL/6 mice with 6-methyl-1,3,8-trichlorodibenzofuran was not related to suppression of induction of CYP1A activities or to a less pronounced suppression of uroporphyrinogen decarboxylase (Yao & Safe, 1989). In female Sprague-Dawley rats, administration of 1 μg/kg 2,3,7,8-TCDD per week over 16 weeks also resulted in hepatic porphyria (Goldstein *et al.*, 1982). After the administration period, recovery from the porphyrogenic effects of 2,3,7,8-TCDD was very slow and did not correlate with the biological half-life of 2,3,7,8-TCDD. In female Sprague-Dawley rats treated with 2,3,7,8-TCDD and various PCBs, a significant correlation was observed between CYP1A2 activities and hepatic porphyrin levels. In addition, the non-dioxin-like 2,2′,4,4′,5,5′-hexachlorobiphenyl caused a strong synergistic effect on 2,3,7,8-TCDD-induced hepatic porphyric (800 times) (van Birgelen *et al.*, 1996a,b). An interaction between 2,3,7,8-TCDD and iron was noted by Jones *et al.* (1981). In iron-deficient mice, the liver toxicity of 2,3,7,8-TCDD (25 μg/kg per week over 12 weeks) was much less pronounced than in iron-supplemented animals, while extrahepatic effects of 2,3,7,8-TCDD were not affected.

A somewhat controversial issue is the effect of 2,3,7,8-TCDD on the proliferation of hepatocytes *in vivo* and *in vitro*. In male and female Harlan-Sprague-Dawley rats, Dickins *et al.* (1981) observed a significantly higher increase in liver DNA synthesis after a one-third hepatectomy when the animals were treated with 2,3,7,8-TCDD

(5 μg/kg bw) five days before surgery. Interestingly, the effect was also observable after laparotomy only (Christian & Peterson, 1983). In female CD (Sprague-Dawley) rats and in male B6C3F1 mice, 2,3,7,8-TCDD caused an acute 1.5–1.7-fold increase in liver DNA synthesis (Büsser & Lutz, 1987), while no increase in total hepatic BrdU labelling index was observed in male or female Sprague-Dawley rats one and two weeks after 2,3,7,8-TCDD treatment designed to achieve quasi-steady-state liver concentrations of 0.03, 30 or 150 ng/g liver (Fox *et al.*, 1993). However, a slight increase was seen in the periportal hepatocyte proliferation pattern. Inhibition of hepatocellular proliferation, stimulated by a two-thirds hepatectomy, was also observed in female Sprague-Dawley rats after 14 days of a dosing regimen designed to achieve and maintain a steady-state concentration of 30 ng 2,3,7,8-TCDD/g liver (Bauman *et al.*, 1995). Furthermore, 2,3,7,8-TCDD induced a periportal pattern of cell proliferation as compared to the pan-lobular pattern in the control partial hepatectomy group.

In female Sprague-Dawley rats either ovariectomized or sham-operated, and then treated first with *N*-nitrosodiethylamine (NDEA) and, from one week later, with 100 ng/kg 2,3,7,8-TCDD per day (for details, see Section 3.6.3(*b*)), 2,3,7,8-TCDD-induced cell proliferation was observed in the intact rats (Lucier *et al.*, 1991). The average BrdU labelling index values were 6.0 and 7.3 in intact rats treated with 2,3,7,8-TCDD alone and with NDEA followed by 2,3,7,8-TCDD. Control values were 0.32 in intact rats and 1.09 in ovariectomized rats. The average BrdU labelling index values were 0.97 in rats treated with 2,3,7,8-TCDD alone and 1.15 in rats treated with NDEA followed by 2,3,7,8-TCDD. Intact rats treated with NDEA alone had a slightly higher labelling index (0.8) than untreated controls. Large interindividual variations were observed in the effects of 2,3,7,8-TCDD on cell proliferation. Similarly, large interindividual variations were seen for the development of GGT- and GSTP-positive foci. Livers from animals undergoing more rapid cell proliferation had the greatest number of GSTP-positive foci. The correlation coefficient for the two parameters was 0.85 ($p = 0.007$). A similar positive correlation was seen for GGT-positive foci and cell proliferation ($r = 0.67$; $p = 0.05$). The volume percentage of PGST-positive foci of the livers having the four highest proliferation rates was 1.9, whereas the corresponding value for the four lowest was 0.7.

A further series of female Sprague-Dawley rats were given similar treatment using a range of 2,3,7,8-TCDD doses (for details, see Section 3.6.3(*b*)). For all rats treated only with NDEA, $BrdU^+$ S-phase nuclei were randomly distributed throughout the hepatic lobules. In contrast, there was a periportal distribution of $BrdU^+$ S-phase nuclei in several non-NDEA-treated, 2,3,7,8-TCDD-treated rats. Overall, there was a statistically significant increasing trend in labelling index as a function of dose of 2,3,7,8-TCDD, with an interaction between 2,3,7,8-TCDD and NDEA. This trend suggests dose-dependence, but the results in comparison to the controls were not statistically significant. The trend in increasing labelling index was stronger in initiated rats than in non-initiated rats. There was a significant decrease in labelling index in the low-dose group of initiated rats (Maronpot *et al.*, 1993).

Groups of female Wistar rats were pretreated with NDEA, then given either a single ('acute') or repeated ('chronic') treatment with 2,3,7,8-TCDD (for details, see Section 3.6.3(*b*)). Proliferation as measured by BrdU labelling index was not significantly increased (NDEA, 6.7%; NDEA + 'acute' 2,3,7,8-TCDD, 8.8%; NDEA + 'chronic' 2,3,7,8-TCDD, 9.5%). However, apoptosis was markedly decreased (NDEA, 6.2%; NDEA + 'acute' 2,3,7,8-TCDD, 3.7%; NDEA + 'chronic' 2,3,7,8-TCDD, 0.8%) (Stinchcombe *et al.*, 1995). [The Working Group noted that measurement of apoptosis by the fluorescent method used in this study has not been validated.]

In rodent hepatocytes in primary culture, Schrenk *et al.* (1992, 1994b) found that 2,3,7,8-TCDD concentrations between 10^{-12} M and 10^{-9} M did not affect DNA synthesis. However, the response of DNA synthesis to the epidermal growth factor (EGF) was enhanced at low and attenuated at high 2,3,7,8-TCDD concentrations, also depending on cell density. Hepatocytes from Ah^d/Ah^d (low-affinity receptor) C57BL/6J mice were about 10-fold less sensitive than those from Ah^b/Ah^b (high-affinity receptor) mice, consistent with the involvement of the Ah receptor in these effects. In rat hepatocytes, the enhancement of the EGF response by 2,3,7,8-TCDD was further increased when ethinyloestradiol was added to the cultures. A synergistic effect of 2,3,7,8-TCDD on DNA synthesis in cultured rat hepatocytes stimulated with EGF or insulin was also reported by Wölfle *et al.* (1993). In contrast, Hushka and Greenlee (1995) did not detect a 2,3,7,8-TCDD-mediated increase in proliferation of either untreated or EGF-treated cultured hepatocytes from male or female Sprague-Dawley rats. 2,3,7,8-TCDD rather caused an inhibition of DNA synthesis, with an EC_{50} of 10^{-11} M.

In 5L cells derived from the rat hepatoma cell line H4IIEC3, 2,3,7,8-TCDD reduced proliferation by about 50%, with half-maximal inhibition at $1–3 \times 10^{-10}$ M (Göttlicher & Wiebel, 1991; Wiebel *et al.*, 1991), while the parental line was insensitive to the growth-inhibitory effect of 2,3,7,8-TCDD. Flow cytometric analysis revealed that 2,3,7,8-TCDD blocked the entry of 5L cells into S-phase, without affecting their progression through S and G_2/M to the G_1 phase. This effect is associated with the presence of the Ah receptor (Göttlicher *et al.*, 1990). In WB-F344 rat liver epithelial cells, however, 10^{-9} M 2,3,7,8-TCDD increased DNA synthesis two- to three-fold (Münzel *et al.*, 1996).

(v) *Endocrine system*

Studies on the interactions of 2,3,7,8-TCDD with a variety of hormone systems have demonstrated that a number of links exist, in particular to the sex steroids, corticosteroids and thyroid hormones.

The effects on gonads and on levels and function of gonadal steroids are described in Section 4.4.

A target organ for 2,3,7,8-TCDD is the pituitary, where it disrupted the normal feed-back mechanisms between plasma testosterone, 5α-dihydrotestosterone or 17β-oestradiol and luteinizing hormone (LH) secretion (Bookstaff *et al.*, 1990a). In male Sprague-Dawley rats, an oral dose of 20 μg/kg 2,3,7,8-TCDD (ED_{50}) inhibited the compensatory increases in pituitary gonadotropin-releasing hormone (GnRH) receptor number, and the LH secretory responsiveness of the pituitary to GnRH and plasma LH concentrations

which should have occurred in response to 2,3,7,8-TCDD-induced decreases in plasma testosterone concentration (Bookstaff *et al.*, 1990b).

A single oral dose of 2,3,7,8-TCDD (50 μg/kg) resulted in a significant and sustained increase in plasma adrenocorticotropin in male Sprague-Dawley rats (Bestervelt *et al.*, 1993a). Plasma corticosterone levels were slightly but significantly increased at days 1–5, but were below those of untreated controls at days 10 and 14, indicating that the pituitary–adrenal axis was disturbed at these later time points. This conclusion was supported by in-vitro findings using primary cultures of rat anterior pituitary cells and adrenal cells (Bestervelt *et al.*, 1993b).

Neal *et al.* (1979) concluded from determinations of the corticosteroid-inducible tyrosine aminotransferase that 2,3,7,8-TCDD (200 μg/kg bw) does not mimic glucocorticoid activity. No direct interference with T3 was found, although application of T3 caused a delay of up to 50% in 2,3,7,8-TCDD mortality. In a receptor-binding experiment using rat liver cytosol, 2,3,7,8-TCDD did not displace dexamethasone from the glucocorticoid receptor.

The hypothalamic/endorphin concentration initially increased after 2,3,7,8-TCDD treatment (50 μg/kg) of male Sprague-Dawley rats and then was depressed (Bestervelt *et al.*, 1991), while brain mu opioid receptor number was increased by 60%.

2,3,7,8-TCDD also affects serum melatonin levels in rats, changing the concentration of this hormone in the pineal gland (Pohjanvirta *et al.*, 1989c; Linden *et al.*, 1991; Pohjanvirta *et al.*, 1996). This response appears to be related to increased extrahepatic metabolism of melatonin (Pohjanvirta *et al.*, 1996).

Total T4, T3, TSH serum levels and uridine diphosphate-glucuronosyl transferase (UDP-GT) activity were measured in 21-day-old Sprague-Dawley rats whose mothers had been treated with 2,3,7,8-TCDD doses of 0.025 or 0.10 μg/kg bw per day on days 10–16 of gestation (Seo *et al.*. 1995). With regard to total T4, no difference between the undivided groups was observed; however, a small (20.4%), but significant reduction in total T4 was found in female but not male rats derived from the high-dose group. Neither total T3 nor TSH was affected by treatment. Significant increases were observed in both low- and high-dose-derived weanling rats, with regard to hepatic UDP-GT activity (310% in the high-dose group); there was no evidence of a sex difference.

Administration of 2,3,7,8-TCDD by gastric instillation to 12-week-old female Sprague-Dawley rats once every two weeks for 30 weeks induced a dose-dependent decrease in serum T4 level that was significant at daily dose equivalents of 0.035 μg/kg bw or more. Total T3 levels were not significantly affected, but TSH was increased 2–3-fold at a daily dose equivalent of 0.125 μg/kg bw. In this same dose group, the hepatic mRNA levels for a UDP-GT (UGT1A1) and a cytochrome P450 (CYP1A1) were increased about 2.5- and 250-fold respectively (Sewall *et al.*, 1995). Decreases in total T4 plasma level following treatment of rats with 2,3,7,8-TCDD were related to the induction of UGT1A1, which catalyses the conjugation of T4 and thereby facilitates its excretion (van Birgelen *et al.*, 1995b). The resulting elevation of TSH level has been suggested to lead to the follicular cell hyperplasia and hypertrophy observed in 2,3,7,8-TCDD-treated rats (Sewall *et al.*, 1995).

A pronounced reduction in serum T4 level was observed (Potter *et al.*, 1983) along with decreases in blood levels of insulin and glucose in male Sprague-Dawley rats treated with 45 µg/kg 2,3,7,8-TCDD. Furthermore, the body temperature of the animals dropped to 34.5 °C. In pair-fed controls, no hypothyroxinaemia, hypothermia or hypoglycaemia was observed. 2,3,7,8-TCDD did not cause significant alterations in serum glucagon or somatostatin levels. A dose-dependent decrease in serum T4 (free and total), but not in TSH or T3, was also observed by Górski and Rozman (1987); T4 levels returned to normal 32 days after 2,3,7,8-TCDD dosage. Other effects of 2,3,7,8-TCDD were a decrease in serum levels of insulin and glucose after high dosage. The hypoinsulinaemic rats were hypersensitive towards insulin, so that otherwise non-toxic insulin doses were lethal. The authors concluded that hypoinsulinaemia is part of an adaptive response of the organism to reduce toxicity after 2,3,7,8-TCDD exposure.

Thyroidectomy with ^{131}I has been shown to protect male Sprague-Dawley rats (10 per group) from the acute toxicity of 2,3,7,8-TCDD at a dose of 100 µg/kg bw, whereas substitution of T4 re-established sensitivity towards 2,3,7,8-TCDD. Percentage mortality figures after 45 days in non-thyroidectomized rats, thyroidectomized + T4 rats and thyroidectomized rats were 78%, 70% and 0%, respectively (Rozman *et al.*, 1984). The authors concluded that thyroid hormone(s) play(s) an important role in mediating the toxicity of 2,3,7,8-TCDD. Radiothyroidectomy protected rats against the 2,3,7,8-TCDD-induced immunotoxicity, as assessed by the spleen anti-sheep red blood cell (SRBC) plaque-forming assay (Pazdernik & Rozman, 1985). The authors suggested that the T4 decrease induced by 2,3,7,8-TCDD may also represent a protective response of the organism to reduce 2,3,7,8-TCDD toxicity including immunotoxicity.

(vi) *Other systems*

Treatment of rats with 2,3,7,8-TCDD (50–100 µg/kg bw) led to a significant increase in serum levels of gastrin (Mably *et al.*, 1990; Theobald *et al.*, 1991).

High, generally toxic doses of 2,3,7,8-TCDD alter cardiac function and morphology, as shown in several animal species (Buu-Hoi *et al.*, 1972; Gupta *et al.*, 1973; Allen *et al.*, 1977; Kociba *et al.*, 1979; Poland & Knutson, 1982; Rifkind *et al.*, 1984; Kelling *et al.*, 1987).

Rozman (1984) suggested that the brown adipose tissue was a target for 2,3,7,8-TCDD toxicity. In fact, 2,3,7,8-TCDD induced a progressive lipid depletion in the brown adipose tissue followed by alterations in glycogen, widening of intercellular spaces, mitochondrial swelling and enhanced lysosomal activity (Rozman *et al.*, 1986). In 3T3-L1 cells, 10 nM 2,3,7,8-TCDD suppressed differentiation into fat cell colonies induced by dexamethasone and isobutylmethylxanthine (Phillips *et al.*, 1995). In contrast, 2,3,7,8-TCDD had no effect on the maintenance of the adipose phenotype in differentiated cells.

(*b*) *Immunological responses*

Immunological responses induced by PCDDs and PCDFs in mammals have been observed for the last 25 years with doses varying over many orders of magnitude. Initial studies were performed with doses in the mg/kg and µg/kg range in mice and rats, but it

is now known that alterations of some immune responses can be observed after exposure to ng/kg doses of 2,3,7,8-TCDD in mice and non-human primates.

Immunological responses observed after treatment with doses which cause overt toxicity or even mortality are without relevance to the human situation in relation to environmental or occupational exposures.

Studies in mice and rats performed at doses which induce thymic atrophy should be interpreted with great caution.

(i) *Effects of 2,3,7,8-TCDD*

The effects of 2,3,7,8-TCDD and other polyhalogenated dibenzo-*para*-dioxins and polyhalogenated dibenzofurans on the mammalian immune system have been reviewed several times. 2,3,7,8-TCDD causes suppression of both cell-mediated and humoral immunity, but little is known about the underlying mechanisms (Vos & Luster, 1989; Holsapple *et al.*, 1991a,b; Vos *et al.*, 1991; Kerkvliet, 1994; Kerkvliet & Burleson, 1994; Holsapple, 1995).

Effects on thymus and role of Ah receptor

Poland and Glover (1979) studied the dose–response relationship for thymic atrophy produced by 2,3,7,8-TCDD in two strains of mice. C57BL/6 mice, which have a high-affinity Ah receptor, were approximately 10-fold more sensitive to thymic involution than DBA/2 mice, which have a lower-affinity receptor.

Germolec *et al.* (1996) have described CYP1A1 induction in lymphoid tissues from Fischer 344 rats exposed to a single oral dose of 100 µg/kg bw 2,3,7,8-TCDD. Immunohistochemical localization of CYP1A1 in immune tissues indicated that cells other than the lymphoid populations are responsible for the increased CYP1A1 expression.

The presence of the Ah receptor and the protein Arnt was demonstrated in T cells, and a combined exposure to 2,3,7,8-TCDD and the T-cell activator anti-CD3 caused the Ah receptor to translocate to the nucleus, but DNA binding activity of the murine T-cell Ah receptor was not detected (Lawrence *et al.*, 1996).

Ah receptor-deficient mice showed decreased accumulation of lymphocytes in the spleen and lymph nodes, but not in the thymus (Fernandez-Salguero *et al.*, 1995). However, corresponding results were not observed in Ahr^{-}/Ahr^{-} mice generated by another group (Schmidt *et al.*, 1996).

Kerkvliet and Brauner (1990) showed that, in C57BL/6 mice treated with 2 µg/kg bw 2,3,7,8-TCDD, the percentage of double positive $CD4^{+}CD8^{+}$ (DP) thymocytes was decreased, whereas the percentage of double negative $CD4^{-}$ $CD8^{-}$ (DN) thymocytes was increased.

In C57BL/6 mice treated with a single intraperitoneal injection of 50 µg/kg bw 2,3,7,8-TCDD, a decrease was seen in cell number in the thymus mainly of the DP and DN populations (Lundberg *et al.*, 1990a; Lundberg, 1991).

Silverstone *et al.* (1994a) showed that thymic atrophy after a single intraperitoneal dose of 30 µg/kg bw 2,3,7,8-TCDD in BALB/c mice resulted from a proportional loss of all classes of thymocytes. There was no significant relative reduction in terminal deoxy-

nucleotidyl transferase $(TdT)^+$ recombinase activating gene $(RAG\text{-}1)^+$ cells in the thymus, but a slow and persistent reduction of TdT and RAG-1 in bone marrow [TdT and RAG-1 are markers for lymphocyte stem cells].

A single intraperitoneal 2,3,7,8-TCDD dose of 30 µg/kg bw to sham-operated or neonatally thymectomized BALB/c mice reduced the bone marrow levels of mRNA for TdT and RAG-1. Thus, neonatal thymectomy had no effect on the 2,3,7,8-TCDD-elicited reduction of TdT or RAG-1 mRNAs. Corresponding effects of 2,3,7,8-TCDD were also observed in athymic nu/nu mice (Frazier *et al.*, 1994a).

Severe combined immunodeficient C.B-17 *scid/scid* (SCID) mice engrafted with human fetal thymus and liver tissue fragments (SCID-hu mice) were used to assess the sensitivity of the human thymus to 2,3,7,8-TCDD. The relative size of the cortex showed a dose-dependent decrease in grafted human thymus as well as in rat thymus, which was significant after exposure to 25 µg/kg 2,3,7,8-TCDD. A dose-dependent increase in keratinization of Hassal's corpuscles in the medullary areas of the thymus grafts was observed (de Heer *et al.*, 1995)

Muralidhara *et al.* (1994) observed decreased activity of the enzyme adenosine deaminase in thymic tissue of BALB/c mice (but not in DBA/2 mice) after treatment with a single intraperitoneal injection of 28.8, 57.5 or 115 µg/ kg bw 2,3,7,8-TCDD or more. The lowest dose tested (11.5 µg/kg) did not cause a significant reduction in the enzyme activity.

Thymocytes from 15-day-old C57BL/6 mice, treated with 50 µg/kg bw 2,3,7,8-TCDD four days before sacrifice, showed an earlier response and a higher maximal cell proliferation than thymocytes from control mice upon stimulation with concanavalin A *in vitro* (Lundberg *et al.*, 1990b).

The effects of 2,3,7,8-TCDD on the murine fetal thymus were studied in an organ culture system. A concentration of 5×10^{-10} M caused a 50% inhibition of lymphoid development. At 10^{-9} M, reduction of DP cells was most pronounced (Dencker *et al.*, 1985; d'Argy *et al.*, 1989; Lundberg *et al.*, 1990a).

Greenlee *et al.* (1985) studied the effects of 2,3,7,8-TCDD on primary cultures of thymic epithelial cells from C57BL/6 mice. Treatment of the cultures with 10 nM 2,3,7,8-TCDD resulted in altered maturation of cocultured thymocytes as judged by the suppression (40% of control) of thymic epithelium-dependent responsiveness of thymocytes to the mitogens concanavalin A and phytohaemagglutinin.

With human thymic epithelial cells, marked differences were seen in the sensitivity of the cells from different donors to 2,3,7,8-TCDD. Cytochrome P450 activities were inducible in these cells *in vitro* (EC_{50} value, approximately 1 nM), with maximal increases in 7-ethoxycoumarin-*O*-deethylase (ECOD) and EROD activities of 3–400-fold and 1–21-fold, respectively (Cook *et al.*, 1987).

Thymic atrophy in rats following exposure to 2,3,7,8-TCDD (at doses of 1 and 10 mg/kg bw) was first described in the Wistar strain by Buu-Hoi *et al.* (1972).

In Fischer 344 rats, Rice *et al.* (1995) observed a significant reduction in the relative thymus weight (thymus weight/body weight) after a single intraperitoneal injection of 0.3 µg/kg bw 2,3,7,8-TCDD.

de Heer *et al.* (1994a,b) reported a significant reduction in the number of immature $CD4^+CD8^+$ thymocytes after single oral doses of 1 μg/kg bw 2,3,7,8-TCDD or more in Wistar rats. Numbers of mature CD3 medullary thymocytes were not affected at doses of up to 25 μg/kg bw 2,3,7,8-TCDD. A detailed study of the time course of the effect after treatment with 25 μg/kg 2,3,7,8-TCDD revealed a recovery in proliferative activity in the thymic cortex (after six days) and an increase in cellularity after day 13.

Kurl *et al.* (1993) studied the time course of events which precede 2,3,7,8-TCDD-induced thymic apoptosis. They showed that, in thymocytes from immature rats incubated *in vitro*, nuclear accumulation of 2,3,7,8-TCDD reaches maximal levels within 60 min, paralleling 2,3,7,8-TCDD-induced increases in RNA synthesis and poly(A)polymerase activity.

Pronounced thymic atrophy was induced in PVG rats by treatment with a single dose of 50 μg/kg bw 2,3,7,8-TCDD. Thymus lobes were transplanted into control rats and evaluated 20 days later, when they did not differ from controls, indicating that the 2,3,7,8-TCDD-induced damage in rat thymus is rapidly reversible (van Loveren *et al.*, 1991).

de Waal *et al.* (1992, 1993) investigated rat thymus by immunohistochemistry and electron microscopy after treatment with single doses of 50 and 150 μg/kg bw 2,3,7,8-TCDD. They observed changes which mainly affected the cortical epithelial cells, but, because both dose levels used in this study were lethal to the animals, the relevance of the results is doubtful.

Cytotoxic T-cells

Doses of 4 ng/kg bw 2,3,7,8-TCDD altered the ability of adult male C57BL/6 mice to generate cytotoxic T-lymphocytes (CTL) in response to alloantigen challenge. The cellular basis of the 2,3,7,8-TCDD-induced suppression was shown to be an enhanced suppressor T-cell activity of CTL responses, whereas CTL precursers and IL-2 production appeared to be intact in 2,3,7,8-TCDD-exposed mice. CTL activity generated *in vitro* following allogenic stimulation was not impaired when spleen cells from 2,3,7,8-TCDD-treated DBA/2 mice were used (Clark *et al.*, 1981, 1983; Nagarkatti *et al.*, 1984). However, these results at very low dose levels could not be corroborated by other investigators.

Hanson and Smialowicz (1994) specifically designed a study to re-evaluate the effect of 2,3,7,8-TCDD on CTL response as described by Clark *et al.* (1981). Neither the in-vivo- nor the in-vitro-generated CTL response was altered following a single intraperitoneal injection of 2,3,7,8-TCDD at doses ranging from 0.24 to 7.2 μg/kg bw. Also, no effect was observed on the in-vivo-generated CTL response following four weekly exposures to 2,3,7,8-TCDD at doses of 0.01 to 3.0 μg/kg bw. Similarly, 2,3,7,8-TCDD did not alter the in-vitro-generated CTL response at these dose levels. [The Working Group noted that the sex of the C57BL/6 mice was different (female instead of male), but it is unlikely that this accounts for the discrepancy in the results.]

Kerkvliet *et al.* (1990a) observed a significant suppression of CTL response in C57BL/6 mice (Ah^b/Ah^b) at doses of 5–20 µg/kg bw 2,3,7,8-TCDD. Ah^d/Ah^d mice were less susceptible.

Single oral doses of 2.5–40 µg/kg 2,3,7,8-TCDD suppressed the activity of CTL in C57BL/6 mice with a calculated ID_{50} (50% immunosuppressive dose) of 7.2 µg/kg. Glucocorticoid levels (corticosterone) were not altered at doses below 40 µg/kg, indicating that 2,3,7,8-TCDD-induced CTL suppression is not dependent on elevated glucocorticoid levels (de Krey & Kerkvliet, 1995).

Rice *et al.* (1995) treated Fischer 344 rats with single oral doses of 2,3,7,8-TCDD up to 30 µg/kg bw and examined CTL activities 24 days following treatment. Syngenic in-vivo tumour-specific CTL were generated that model cell-mediated immune reactions against neoplastically transformed self antigens. Under these conditions, CTL activity showed no significant dose-dependent alteration due to 2,3,7,8-TCDD exposure, but relative thymus weight was significantly decreased at the lowest dose studied (0.3 µg/kg bw).

Effects on lymphocytes in vivo

Oughton *et al.* (1995) studied the effects of 2,3,7,8-TCDD on lymphocytes by flow cytometry. Female C57BL/6 mice were treated weekly with 200 ng/kg bw 2,3,7,8-TCDD for 14–15 months. Besides an age-matched vehicle control, a group of four-month-old mice was evaluated to assess alterations associated with ageing. In the thymus of the 2,3,7,8-TCDD-treated mice, the proportion of $CD4^-CD8^-$ cells was increased, as was the proportion of *gamma-delta*$^+$ thymocytes. The most definite change in 2,3,7,8-TCDD-treated mice was a decrease in the frequency of memory T helper cells, defined as $CD4^+Pgp\text{-}1^{hi}CD45RB^{lo}$, with a concomitant increase in the proportion of naive T helper cells identified as $CD4^+Pgp\text{-}1^{lo}CD45RB^{hi}$. [Pgp-1 and CD45RB are the murine analogues to the human markers CD29 and CD45RA. The results of this paper are consistent with the findings in marmosets described below.]

Following a single subcutaneous injection of 10 ng/kg bw 2,3,7,8-TCDD into four mature female marmosets, a pronounced decrease was observed in the number of cells from a defined lymphocyte subpopulation carrying both the CD4 and the CD29 epitopes ('helper-inducer' or memory cells). Also, the percentage of pan-B cells (B1, $CD20^+$) was clearly lower than that in the controls (Neubert *et al.*, 1990b).

In a 42-week study with lower doses, an opposite effect was initially observed, whereas the results of the first study were confirmed after an increase of the weekly dose. The study consisted of three parts: (*a*) weekly subcutaneous doses of 0.3 ng/kg bw 2,3,7,8-TCDD were given to seven marmosets for 24 weeks; (*b*) the weekly dose was then raised to 1.5 ng/kg bw 2,3,7,8-TCDD for another six weeks; (*c*) finally, this was followed by a dose-free period of 12 weeks. An increase in the percentage as well as the absolute number of $CD4^+CD29^+$ cells was observed after the 24-week treatment period with weekly injections of 0.3 ng/kg bw 2,3,7,8-TCDD. In the second part of the study, this lymphocyte subpopulation decreased after weekly injections of 1.5 ng/kg bw 2,3,7,8-TCDD. The effect was completely reversed during the 12-week recovery period. In addition, several changes were observed in other lymphocyte subpopulations: there was a

change in $CD4^+CD45R^+$ cells contrary to that of the helper-inducer cells. Furthermore, a transient increase in $CD8^+CD56^+$ cells ('cytotoxic T-cells') was found, and a decrease in pan-B cells ($CD20^+$), which were again reversible after discontinuation of the dosing (Neubert *et al.*, 1992a,b).

After subcutaneous injection of a single dose of 300 ng/kg bw 2,3,7,8-TCDD into three marmosets, the number of total lymphocytes decreased and at doses of 1 μg/kg or more the number of total leukocytes in peripheral blood of two marmosets was significantly reduced (Neubert *et al.*, 1993b).

Effects on lymphocytes in vitro

Neubert *et al.* (1991) studied the effects of 2,3,7,8-TCDD on pokeweed mitogen-stimulated proliferation and differentiation of peripheral lymphocytes *in vitro* with cells from a marmoset and from two healthy human donors. They observed a pronounced decrease in the percentage of B-cells ($CD20^+$) and $CD4^+$ cells and a concomitant increase in the percentage of $CD8^+$ cells at dose levels as low as 10^{-12}–10^{-14} M 2,3,7,8-TCDD.

Using a similar experimental approach, Lang *et al.* (1994) exposed human lymphocytes from peripheral blood *in vitro* to concentrations of 10^{-7}–10^{-14} M 2,3,7,8-TCDD. Cells were stimulated by pokeweed mitogen or anti-CD3 monoclonal antibody. Analysis of surface markers (e.g., $CD4^+$, $CD29^+$, $CD19^+$) by flow cytometry showed no significant alterations under the given conditions.

Effects on antibody-producing cells in vivo

Intraperitoneal injections of single 2,3,7,8-TCDD doses ranging from 1.2 to 30 μg/kg bw suppressed primary antibody production to SRBC plaque-forming cell (PFC) response in C57BL/6J and in less sensitive DBA/2 mice (Vecchi *et al.*, 1980, 1983).

A single oral 2,3,7,8-TCDD dose ranging from 0.2 to 5 μg/kg bw given two days before SRBC sensitization produced a suppression of anti-SRBC PFC response on day 5 in C57BL/6 mice; the ID_{50} was 0.74 μg/kg (Kerkvliet & Brauner, 1990).

Narasimhan *et al.* (1994) determined the splenic antibody PFC response to SRBC nine days after administration of 2,3,7,8-TCDD to B6C3F1 mice. Suppression of PFC response expressed per spleen or per 10^6 cells was observed at 100 ng/kg bw 2,3,7,8-TCDD and higher doses (results with 50 ng/kg bw 2,3,7,8-TCDD were not significantly different from controls).

Kerkvliet *et al.* (1990b) compared the effects of 2,3,7,8-TCDD in two congenic strains of C57BL/6 mice that differed at the Ah locus. Using the haemolytic antibody isotope release assay, both Ah^b/Ah^b and Ah^d/Ah^d mice exhibited dose-dependent suppression of the anti-trinitrophenyl (TNP) response following 2,3,7,8-TCDD exposure (T-cell-independent). The ID_{50} values were 7.0 μg/kg (Ah^b/Ah^b mice) and 30.8 μg/kg (Ah^d/Ah^d mice). Suppression of the antibody response to the T-cell-dependent antigen SRBC occurred at lower doses (0.6 μg/kg bw 2,3,7,8-TCDD in Ah^b/Ah^b mice) and the reaction in Ah^d/Ah^d mice showed an apparent biphasic dose–response relationship.

When spleen cells from mice treated two days previously with 5 μg/kg bw 2,3,7,8-TCDD were transferred to an irradiated host, no suppression of the PFC response to SRBC was observed, while a similar dose in a non-irradiated animal produced profound

suppression of the PFC response. Cultures of spleen cells from SRBC-primed 2,3,7,8-TCDD-treated (5 μg/kg) C57BL/6 mice produced fewer anti-TNP PFC when immunized with TNP-SRBC, as compared to cells from primed vehicle-treated controls (Tomar & Kerkvliet, 1991).

Dooley and Holsapple (1988) showed that the B lymphocyte is the primary target for suppression of the T-dependent antibody response in B6C3F1 mice by demonstrating that the suppression of several humoral responses (the polyclonal response to lipopolysaccharide (LPS), the T-cell-independent response to dinitrophenyl (DNP)–Ficoll and the T-cell-dependent response to SRBC) was characterized by dose–response–effect curves that were approximately parallel. They used separation/reconstitution assay techniques to show that the suppression of antibody responses to 2,3,7,8-TCDD is the predominant result of a specific effect on the functional capacities of the B-cell.

Dooley *et al.* (1990) treated B6C3F1 mice orally on five consecutive days with daily doses of 1 μg/kg bw 2,3,7,8-TCDD. They observed no effect of 2,3,7,8-TCDD exposure on [^{3}H]thymidine uptake in splenocytes of these mice 24 h after in-vitro stimulation with the T-cell mitogen concanavalin A. Titration of T-cells from 2,3,7,8-TCDD-treated mice into naïve splenocyte cultures did not suppress the humoral response to either a T-cell-dependent (SRBC) or -independent (DNP–Ficoll) antigen. Data suggest that an alteration of T-cell function following 2,3,7,8-TCDD exposure does not play a role in the suppression of the antibody response elicited by antigen stimulation of splenocytes from B6C3F1 mice.

Morris *et al.* (1992) showed that the sensitivity of DBA/2 mice to suppression of the antibody response increased significantly when 2,3,7,8-TCDD was administered daily over two weeks rather than as an acute exposure. This change in sensitivity was not paralleled by a shift in the sensitivity to other effects of 2,3,7,8-TCDD, including thymic atrophy and liver enzyme induction.

Smialowicz *et al.* (1994) studied the effects of 2,3,7,8-TCDD on the antibody PFC response to SRBC comparatively in B6C3F1 mice and in Fischer 344 rats. Their data for mice were in agreement with the results from other laboratories (ED_{50}, 0.7 μg/kg bw 2,3,7,8-TCDD). In contrast, 2,3,7,8-TCDD failed to suppress, and in fact enhanced, the PFC response to SRBC in rats at doses of 3 and 30 μg/kg bw. Flow cytometric analysis of thymocytes and splenic lymphocytes from 2,3,7,8-TCDD-dosed (3, 10 and 30 μg/kg bw) and SRBC-immunized mice and rats revealed that $CD4^-CD8^+$ splenocytes were reduced and IgM^+ splenocytes were increased in a dose-related manner in rats only.

Corresponding experiments were performed with a T-cell-independent antigen TNP-LPS in B6C3F1 mice and in Fischer 344 rats. The dose of 2,3,7,8-TCDD required to suppress the immune response in rats was higher (30 μg/kg) than in mice (10 and 30 μg/kg). Thus, species differences were demonstrable also under these conditions; however, they were not as pronounced as with the T-cell-dependent antigen SRBC (Smialowicz *et al.*, 1996).

Harper *et al.* (1993) studied the effects of a single intraperitoneal injection of 2,3,7,8-TCDD on the suppression of the splenic PFC response to the T-cell-independent antigen

TNP-LPS in C57BL/6 and DBA/2 mice. The ED_{50} values (PFC/10^6 viable cells) were 1.5 μg/kg bw (C57BL/6 mice) and 9.7 μg/kg bw (DBA/2 mice).

Effects on antibody-producing cells in vitro

Tucker *et al.* (1986) observed a direct effect of 2,3,7,8-TCDD on cultured lymphcytes resulting in a selective inhibition of the differentiation of B-cells into antibody-secreting cells. Using lymphocytes from congenic mice differing only at the Ah locus, it was determined that the Ah^b/Ah^b-derived cells were inhibited by 2,3,7,8-TCDD *in vitro*, whereas the Ah^d/Ah^d-derived cells were not.

Holsapple *et al.* (1986a) reported a reduction in the number of antibody-producing murine spleen cells which developed in response to LPS, DNP–Ficoll and SRBC with 2,3,7,8-TCCD at concentrations ranging from 5 to 20 nM. Direct addition of 2,3,7,8-TCDD had no effect on mitogen-induced proliferation. There was no suppression when 2,3,7,8-TCDD was added to the medium 3 h after LPS (200 μg/mL). 2,3,7,8-TCDD suppressed the antibody response of cells from DBA/2 mice at concentrations similar to those required to suppress the cells from B6C3F1 mice, suggesting that the observed effect is independent of the Ah locus.

In agreement with these results, Davis and Safe (1991) reported that 2,3,7,8-TCDD and other congeners produced a similar concentration-dependent suppression of the in-vitro anti-SRBC response using cells from either C57BL/6 mice or DBA/2 mice.

Morris *et al.* (1991) showed that, with spleen cells from B6C3F1 mice, the suppression of the in-vitro T-cell-dependent humoral immune response by 2,3,7,8-TCDD is dependent on the lot of serum used. Only three of 23 commercial lots supported a full dose-responsive suppression.

Morris *et al.* (1994) compared the influence of calf serum and mouse serum on the results of in-vitro splenocyte antibody response experiments with cells derived from B6C3F1 or DBA/2 mice. With calf serum, a similar degree of suppression of antibody responses by 2,3,7,8-TCDD in splenocytes from both strains was found. In contrast, responses in the presence of mouse serum showed an Ah receptor-dependence that was characterized by a dose-related suppression of antibody responses by B6C3F1 splenocytes only.

Morris and Holsapple (1991) observed an increase in proliferation of dense resting B-cells in the presence of 30 and 60 nM 2,3,7,8-TCDD in the medium.

When 2,3,7,8-TCDD was added at concentrations between 0.3 and 30 nM to activated low-density B-cells isolated from whole spleen cell suspensions from B6C3F1 mice, suppression of cell proliferation and antibody response was observed. In contrast, neither the proliferation nor the antibody response of high-density B-cells (small resting cells) stimulated with LPS was affected by 2,3,7,8-TCDD (Morris *et al.*, 1993).

Wood *et al.* (1992) studied the effects of 2,3,7,8-TCDD on cultured murine splenocytes ((C57BL/6 × C3H)F1 mice) and compared the effects with those seen with human tonsillar lymphocytes. 2,3,7,8-TCDD at concentrations between 0.3 and 30 nM caused a significant reduction in the proliferation of both human and murine cells;

however, the substance had no effect on pokeweed mitogen-induced proliferation or antibody production.

In low-density human B-cells (isolated from human tonsils), 2,3,7,8-TCDD suppressed background proliferation and IgM secretion at concentrations between 0.3 and 30 nM. 2,3,7,8-TCDD-induced suppression was less pronounced when cells were stimulated with LPS or T-cell replacing factor. 2,3,7,8-TCDD did not alter background or stimulated proliferation of high-density human B-cells (Wood *et al.*, 1993).

Wood and Holsapple (1993) examined the effects of 2,3,7,8-TCDD upon human tonsillar lymphocytes stimulated with toxic shock syndrome toxin-1. 2,3,7,8-TCDD at concentrations < 30 nM had no effect upon T-cell or B-cell proliferation, but B-cell differentiation, as manifested by IgM secretion, was significantly suppressed at all concentrations tested (0.3–30 nM). The sensitivity to 2,3,7,8-TCDD varied considerably, with IC_{50} values ranging from < 0.3 nM to 25 nM with cells from four different donors.

Karras and Holsapple (1994a,b) observed inhibitory effects of 2,3,7,8-TCDD on proliferative responses of murine low-density B-cells to activation by anti-IgM.

In another study, Karras *et al.* (1996) reported that 2,3,7,8-TCDD suppressed murine B-cell IgM secretion induced by either soluble or insolubilized anti-IgM plus lymphokines, but did not affect IgM secretion stimulated by activated T_H-cells and lymphokines. Their data indicate that 2,3,7,8-TCDD elevates resting intracellular calcium levels in murine B-cells and may selectively inhibit calcium-dependent signalling pathways linked to surface immunoglobulin.

Karras *et al.* (1995) investigated whether the immunosuppression mediated by direct exposure of murine B-cells to 2,3,7,8-TCDD *in vitro* is due to an IL-4-like biological activity. However, 2,3,7,8-TCDD failed to demonstrate any of the activities of IL-4 observed in parallel cultures.

Masten and Shiverick (1995) found that 25 nM 2,3,7,8-TCDD decreased steady-state levels of CD19 mRNA by 67% in a human B-lymphocyte cell line (IM-9). They identified a DNA-binding complex in nuclear extracts that appeared to be the Ah receptor. The Ah receptor complex recognized a DNA-binding site for B-cell lineage-specific activator protein in the promoter region of the human CD19 gene that was similar to the Ah receptor DNA-binding site.

Effects on macrophages, neutrophils and natural killer (NK) cells

Mantovani *et al.* (1980) found no alteration in macrophage-mediated or NK cell-mediated cytotoxicity in C57BL/6J mice after treatment with single doses of up to 30 µg/kg bw 2,3,7,8-TCDD.

Kerkvliet and Oughton (1993) showed that, in C57BL/6 mice, the inflammatory response following intraperitoneal injection of SRBC was aggravated when mice were treated with 5 µg/kg bw 2,3,7,8-TCDD compared with vehicle-treated controls. The increased number of peritoneal cells reflected significant increases in both neutrophils and macrophages. 2,3,7,8-TCDD treatment did not significantly alter expression of the macrophage activation markers F4/80 or I-A on Mac-1^+ cells. The antigen-presenting function of the peritoneal exudate cells was unaltered by 2,3,7,8-TCDD.

Ackermann *et al.* (1989) observed a reduced cytolytic and cytostatic activity of polymorphonuclear neutrophils (PMN) from B6C3F1 mice (but not those from DBA/2 mice) after a single oral exposure to 5 or 10 µg/kg bw 2,3,7,8-TCDD. Supernatants recovered from PMN cell cultures of B6C3F1 mice (but not those of DBA/2 mice) showed reduced killing capacity for actinomycin D-treated L929 tumour cells.

Funseth and Ilbäck (1992) treated male A/J mice with a loading dose of 5 µg/kg bw 2,3,7,8-TCDD and three weekly maintenance doses of 1.42 µg/kg, given intraperitoneally. NK cell activity increased significantly in blood and spleen (3.4-fold and 2.2-fold, respectively). The effects were still present on day 120 after the treatment.

Effects on popliteal lymph nodes after local stimulation

In C57BL/6 mice, a single intraperitoneal injection of 50 µg/kg bw 2,3,7,8-TCDD suppressed the normal immune response in popliteal and inguinal lymph nodes to ovalbumin injected into the hind foot pads four days after 2,3,7,8-TCDD treatment. Increase of the lymph node cell number was inhibited and a reduced frequency of antigen-specific B-cells was observed. The antigen-specific T-cell proliferation and IL-2 production in response to ovalbumin were significantly suppressed by 2,3,7,8-TCDD (Lundberg *et al.*, 1991, 1992).

An anti-CD3 monoclonal antibody was injected into both rear footpads of female C57BL/6 mice and the draining popliteal and inguinal lymph nodes were removed 24 h later. 2,3,7,8-TCDD enhanced anti-CD3-induced [^{3}H]thymidine incorporation and increased the percentage of both $CD4^+$ and $CD8^+$ cells cycling in S and G_2M phases. The authors concluded that 2,3,7,8-TCDD appeared to be targeting T-cells undergoing activation rather than resting cells (Neumann *et al.*, 1993).

Schmidt *et al.* (1992) treated NMRI mice with single subcutaneous injections of up to 3 µg/kg bw 2,3,7,8-TCDD. One week later they injected streptozotocin or other stimulants into one hind foot pad of the animals. Lymph node enlargement (weight or cell number) in 2,3,7,8-TCDD-treated mice was not significantly different from that of controls.

Korte *et al.* (1991a) and Stroh *et al.* (1992) used the popliteal lymph node assay to investigate effects of 2,3,7,8-TCDD on immune reactions in Wistar rats. Animals were treated with single subcutaneous injections of 2,3,7,8-TCDD at doses of up to 600 ng/kg or 3 µg/kg bw, respectively. Seven days later, either erythrocytes or streptozotocin were injected into one hind foot pad and the weight and cell number of the lymph node were determined after a further seven days. 2,3,7,8-TCDD had no significant effect on the results of this test under the given conditions.

Fan *et al.* (1995) injected 2,3,7,8-TCDD directly into one foot pad of Sprague-Dawley rats (approximately 10 µg/kg bw) and observed a slightly increased weight index (1.59 $\pm$ 0.2 versus 1.07 $\pm$ 0.2 in controls; mean $\pm$ SEM; $n = 4$ and 6).

Theobald *et al.* (1983) first reported that the paw oedema formation after subplantar injection of carrageenan was enhanced in Sprague-Dawley rats treated with 2,3,7,8-TCDD. The ED_{50} of 2,3,7,8-TCDD for this effect was 6 µg/kg bw.

Katz *et al.* (1984) injected various irritants into the subplantar surface of one hind paw of Sprague-Dawley rats or C57BL/6 mice pretreated with 10 µg/kg bw 2,3,7,8-TCDD. 2,3,7,8-TCDD increased the oedemagenic potency of carrageenan, dextran, bradykinin and histamine, but not that of prostaglandin E2 or serotonin.

Effects on complement

Treatment of B6C3F1 mice with daily doses of 0.01–2.0 µg/kg bw 2,3,7,8-TCDD for two weeks suppressed serum total haemolytic complement activity (CH50). Serum levels of complement component C3 were decreased at daily doses of 0.5 µg/kg or more. Immediately after treatment with single doses of 10–40 µg/kg bw 2,3,7,8-TCDD, a rapid, but transient, dose-dependent increase in liver intracellular C3 levels was observed. No inhibitory effect of 2,3,7,8-TCDD on C3 production was detected when cells of a hepatoma cell line (Hepa 1c1c7) were exposed to 2,3,7,8-TCDD (White *et al.*, 1986; Lin & White, 1993a,b,c).

Host resistance assays

Host resistance assays are often used for assessing effects of xenobiotics on the mammalian immune system, because more than one aspect of the specific and non-specific defence mechanisms are included. Several authors have described the potential of PCDDs to suppress resistance to bacterial, viral, parasitic and neoplastic challenges in mice.

Acute exposure of adult mice to 2,3,7,8-TCDD results in hypersensitivity to endotoxin (Vos *et al.*, 1978; Rosenthal *et al.*, 1989). Clark *et al.* (1991b) investigated the effects of 2,3,7,8-TCDD on the endotoxin-induced release of TNFα. They observed a significant increase in TNFα in the serum of endotoxin-exposed mice (Ah^b/Ah^b) after exposure to a single oral dose of 10 µg/kg bw 2,3,7,8-TCDD.

Studies of effects of 2,3,7,8-TCDD on host resistance in mice are summarized in **Table 62**. To date, the finding of Burleson *et al.* (1996), that a single dose of 2,3,7,8-TCDD at 0.1, 0.05 or 0.01 µg/kg bw resulted in an increase in mortality from Hong Kong influenza virus when B6C3F1 mice were challenged seven days after 2,3,7,8-TCDD administration, represents the most sensitive adverse effect yet reported for 2,3,7,8-TCDD.

Only a few host resistance studies have been performed with PCDDs in rats.

Virus-augmented NK activity assessed 48 h after infection in the lung was significantly suppressed in Fischer 344 rats treated with 3, 10 or 30 µg/kg bw 2,3,7,8-TCDD. Significantly higher virus titres were observed on days 2, 3 and 4 after infection in the lungs of rats treated with 10 µg/kg bw (Yang *et al.*, 1994).

Using the parasite *Trichinella spiralis* in five-week-old Wistar rats that were exposed perinatally to 2,3,7,8-TCDD (one subcutaneous injection of 0.3 or 3 µg/kg bw on day 19 of gestation), no differences were found in the antibody titres or the number of *T. spiralis* larvae in muscle (Korte *et al.*, 1991b).

In Fischer 344 rats treated with single doses of 30 µg/kg bw 2,3,7,8-TCDD, no evidence for an immunosuppressive effect was seen in the *T. spiralis* host resistance assay, but proliferative responses of lymphocytes cultured with parasite antigen were

Table 62. Effects of 2,3,7,8-TCDD on host resistance in mice

Strain	Effect	Dose	No./time of exposures	Reference
C57BL/6	No effect on resistance to *Herpes suis*	20 µg/kg p.o.	once a week/4 weeks	Thigpen *et al.* (1975)
C57BL/6	Reduced resistance to *Salmonella bern*	1 µg/kg p.o.	once a week/4 weeks	Thigpen *et al.* (1975)
C57BL/6	Reduced resistance to Herpes simplex type II	0.04 µg/kg i.p.	once a week/4 weeks	Clark *et al.* (1983)
Swiss	Reduced resistance to endotoxin	1.5 µg/kg p.o.	once a week/4 weeks	Vos *et al.* (1978)
Swiss	Reduced resistance to endotoxin	1 µg/kg diet	pre/postnatal	Thomas & Hinsdill (1979)
Swiss	No effect on resistance to *Listeria monocytogenes*	10 µg/kg diet	pre/postnatal	Thomas & Hinsdill (1979)
Swiss	Reduced resistance to *Salmonella* and *Listeria*	50 µg/kg diet	5 weeks	Hinsdill *et al.* (1980)
B6C3F1	Reduced resistance to *Listeria monocytogenes*	5 µg/kg p.o.	4 times (pre/postnatal)[a]	Luster *et al.* (1980)
B6C3F1	Reduced resistance to *Plasmodium yoelli*	5 µg/kg p.o.	once	Tucker *et al.* (1986)
B6C3F1	Reduced resistance to *Streptococcus pneumoniae*	1 µg/kg p.o.	once a day/2 weeks	White *et al.* (1986)
B6C3F1	No effect on resistance to *Listeria monocytogenes*	10 µg/kg i.p.	once	House *et al.* (1990)
B6C3F1	Reduced resistance to influenza virus	0.1 µg/kg i.p.	once	House *et al.* (1990)
B6C3F1	Reduced resistance to *Trichinella spiralis*	10 µg/kg i.p.	once	Luebke *et al.* (1994)
B6C3F1	Reduced resistance to influenza virus	0.01 µg/kg p.o.	once	Burleson *et al.* (1996)

p.o., oral; i.p., intraperitoneal

[a]Day 14 of gestation and days 1, 7 and 14 after birth

enhanced (Luebke *et al.*, 1995). This is in contrast to results obtained with the *T. spiralis* test in mice from the same laboratory (**Table 62**). It was shown in this study that infection delayed elimination from the host: infected mice had higher 2,3,7,8-TCDD levels than non-infected mice (Luebke *et al.*, 1994).

A/J mice infected with Coxsackievirus B3 (CB3) had increased tissue concentrations of 2,3,7,8-TCDD in the brain, pancreas, heart, spleen and liver compared with uninfected controls (Funseth & Ilbäck, 1994).

Direct exposure of isolated human erythrocytes to 10 nM 2,3,7,8-TCDD caused a two-fold increase in the infectivity of *Plasmodium falciparum* after 48 h, when the parasites were in a synchronized state of growth. Additional treatment with sodium orthovanadate, an inhibitor of plasma membrane Ca-ATPase and phosphotyrosine phosphatase, completely blocked the 2,3,7,8-TCDD-induced increase in parasitaemia (Kim *et al.*, 1994).

Pre- and perinatal exposure

Vos and Moore (1974) treated female Fischer 344 rats and C57BL/6 mice with doses of up to 5 µg/kg bw 2,3,7,8-TCDD pre- and postnatally (rats: gestation days 11 and 18, postnatally on days 4, 11 and 18; mice: gestation days 14 and 17, postnatally on days 1, 8 and 15). The high-dose regimen was lethal to 31/34 rat offspring. The authors observed a depletion of lymphocytes in the thymic cortex of the offspring which, histologically evaluated, was not due to lymphocyte destruction. Cellular immunity was impaired. Allograft rejection times were prolonged in rats and mice. Graft-versus-host activity of spleen cells was reduced, as well as the response of rat thymus and spleen cells to phytohaemagglutinin. In four-month-old mice treated with six weekly doses of 25 µg/kg bw 2,3,7,8-TCDD, these effects were not observed.

Fine *et al.* (1988, 1990a,b) studied the effects of 2,3,7,8-TCDD on lymphocyte stem cell function in BALB/c mice following direct or perinatal exposure (maternal treatment with 10 or 15 µg/kg bw 2,3,7,8-TCDD on day 14 of gestation). In the fetus and the neonate, significant reductions were found in both the biosynthesis and mRNA levels of the lymphocyte stem cell-specific DNA polymerase TdT. Thymic biosynthesis was relatively unaffected, suggesting that alterations of early events of T-cell lymphopoiesis (prothymocytes) can contribute to 2,3,7,8-TCDD-induced thymic atrophy. A slight reduction in the expression of the thymocyte surface marker Lyt-2$^+$L3T4$^+$ was demonstrated by flow cytometry.

Holladay *et al.* (1991) and Blaylock *et al.* (1992) treated pregnant C57BL/6 mice on nine consecutive days (gestation days 6–14) with daily doses of 1.5 or 3.0 µg/kg bw 2,3,7,8-TCDD by gastric instillation (total doses of 13.5 and 27 µg/kg bw 2,3,7,8-TCDD). 2,3,7,8-TCDD treatment (both groups) resulted in significant decreases in fetal thymic weight and the percentage of CD4$^+$CD8$^+$ fetal thymocytes (DP), as well as significantly increased CD4$^-$CD8$^-$ (DN) and CD4$^-$CD8$^+$ thymocytes on gestation day 18 as analysed by flow cytometry. 2,3,7,8-TCDD induced a significant shift in T-cell receptor (TCR) expression of thymocytes with a decrease in alpha-beta-TCR and a concomitant increase in gamma-delta-TCR expression. On postnatal day 6, no significant differences were detectable by flow cytometry; however, a significantly depressed CTL activity was

demonstrable until eight weeks postnatally (offspring had been cross-fostered to avoid exposure via milk). There were no significant differences at postnatal weeks 7–8 between controls and 2,3,7,8-TCDD-exposed mice in lymphocyte proliferation to mitogens or antibody PFC response.

Faith and Moore (1977) showed that when Fischer 344 rats were exposed to 2,3,7,8-TCDD prenatally (day 18 of gestation) and postnatally (days 0, 7, 14 of lactation), body weight and thymus/body weight ratios were suppressed up to 145 days. The effects were less pronounced when the rats were exposed only postnatally (days 0, 7, 14 of lactation).

Badesha *et al.* (1995) fed a 2,3,7,8-TCDD-containing diet to lactating Leeds rats starting on postnatal day 1. Total doses of 0.2, 1.0 or 5.0 μg/kg were administered over a period of 18 days. At the age of 130 days, body weights remained significantly reduced in all three groups of female offspring and the two highest-dose groups of males. Immunocompetence of the offspring was affected: in-vitro T-cell-dependent and T-cell-independent responses and mitogen-induced in-vitro production of IL-1 and IL-2 were suppressed at postnatal day 130.

(ii) *Effects of other PCDDs*

Only seven of 75 possible PCDD congeners and five out of 135 PCDF congeners have been studied for their effects on the mammalian immune system (Holsapple, 1995).

Mason *et al.* (1986) demonstrated that in immature male Wistar rats 2,3,7,8-TCDD was the most active congener out of a series of six compounds with respect to their potency to induce thymic atrophy after intraperitoneal injection. The ED_{50} values (dose that caused a 50% decrease in thymus/body weight ratio compared with control rats) were: 0.09 μmol/kg for 2,3,7,8-TCDD, 0.17 μmol/kg for 1,2,3,7,8-PeCDD, 1.07 μmol/kg for 1,2,3,4,7,8-HxCDD, 11.2 μmol/kg for 1,2,4,7,8-PeCDD, 98.1 μmol/kg for 2,3,7-triCDD and 100 μmol/kg for 1,3,7,8-TCDD.

The effects of 1,2,3,4,6,7,8-HpCDD on antibody responses to T-helper cell-dependent (SRBC) and T-helper cell-independent antigens (TNP-LPS and DNP-Ficoll) were studied in C57BL/6 mice. The results indicated that sensitivity to HpCDD-induced suppression directly correlated with the sensitivity of the response to T-cell regulation. T-cell deficient nu/nu mice were significantly more resistant to the immunosuppressive effects of 1,2,3,4,6,7,8-HpCDD as compared with their nu/+ littermates. Following treatment with 100 μg/kg bw 1,2,3,4,6,7,8-HpCDD, the response of nu/nu mice was unaffected, whereas the response of nu/+ mice was significantly suppressed, indicating that the primary immunological defect induced by the substance is at the level of regulatory T-cells. However, after treatment with a dose of 500 μg/kg bw 1,2,3,4,6,7,8-HpCDD, the response of nu/nu mice was also suppressed (Kerkvliet & Brauner, 1987).

Holsapple *et al.* (1986b) compared the effects of 2,3,7,8-TCCD, 2,7-DCDD and OCDD on the antibody response to SRBC and to DNP-Ficoll in B6C3F1 mice. 2,3,7,8-TCDD and 2,7-DCDD suppressed antibody responses to both antigens (compared with 2,3,7,8-TCDD, 10-fold higher doses of 2,7-DCDD induced less pronounced effects). OCDD was without effect.

Treatment of B6C3F1 mice with daily doses of 0.01 μg/kg bw 2,3,7,8-TCDD for two weeks or more suppressed serum total haemolytic complement activity (CH50). 1,2,3,6,7,8-HxCDD was less active than 2,3,7,8-TCDD. Suppression of complement activity was seen after multiple doses of 0.1 to 10 μg/kg bw with decreased C3 levels at 10 μg/kg. Recovery studies showed that complement activity in animals treated with 2,3,7,8-TCDD (1 μg/kg) or 1,2,3,6,7,8-HxCDD (10 μg/kg) was suppressed until 50 days after treatment. Interestingly, CH50 levels were elevated after low doses (0.1 and 1.0 μg/kg) of 1,2,3,6,7,8-HxCDD (White *et al.*, 1986).

4.3 Interactions with Ah receptors and their early molecular consequences and other biochemical responses

The toxic effects elicited by 2,3,7,8-TCDD and other PCDDs are accompanied by modulation of numerous biochemical responses in target tissues and organs. Although there is extensive support for the role of the intracellular dioxin or aryl hydrocarbon (Ah) receptor in mediating PCDD-induced biochemical and toxic effects, the direct link between a series of biochemical alterations and any specific toxicity is unclear. This section highlights the effects of 2,3,7,8-TCDD and other PCDDs on various biochemical parameters, interactions of these compounds with the Ah receptor and the molecular consequences of these reactions.

As outlined below, intracellular signal transduction by PCDDs and PCDFs, most notably 2,3,7,8-TCDD and 2,3,7,8-TCDF, is mediated by the Ah receptor. This protein is a ubiquitous regulatory factor that binds 2,3,7,8-TCDD and its planar aromatic congeners in a saturable manner and with high affinity (in the case of 2,3,7,8-TCDD and 2,3,7,8-TCDF with dissociation constants (K_d, a measure of binding affinity) in the subnanomolar range; reviewed by Poland & Knutson, 1982; Safe, 1986; Bradfield *et al.*, 1988; Gillner *et al.*, 1993). At the molecular level, high-affinity Ah receptor ligands such as 2,3,7,8-TCDD and 2,3,7,8-TCDF are very potent inducers of transcription of a distinct network of target genes encoding xenobiotic-metabolizing enzymes such as cytochrome P4501A1 (CYP1A1), P4501A2 (CYP1A2) and glutathione *S*-transferase Ya (for recent reviews, see Fujii-Kuriyama *et al.*, 1992; Swanson & Bradfield, 1993; Whitlock, 1994; Hankinson, 1994; Poellinger, 1995). Although there is a paucity of data regarding primary Ah receptor target genes that do not encode drug-metabolizing enzymes, 2,3,7,8-TCDD has been reported to modulate expression of the growth modulatory genes for IL-1β and plasminogen activator inhibitor-2 (PAI-2) (Sutter *et al.*, 1991). (See the section below on modulation of growth factors, growth factor receptors, lymphokines and related factors.)

Binding affinities of individual PCDDs to the Ah receptor are strongest for those congeners having a 2,3,7,8-chlorine substitution pattern, with 2,3,7,8-TCDD being the most potent. Within this group of congeners, increasing chlorination on the 1, 4, 6 and 9 positions leads to a significant decrease in the binding affinity of up to several orders of magnitude (Poland & Knutson, 1982; Safe, 1990).

The binding affinity (K_d) of PCDDs for the rat hepatic Ah receptor ranges between 10^{-10} and 10^{-5} M or higher. Across and within species, the Ah-receptor binding affinities

for, e.g., 2,3,7,8-TCDD can vary by one or two orders of magnitude. For example, in both man and mouse, two forms of Ah receptor have been identified which show a 5–10-fold difference in binding affinity for 2,3,7,8-TCDD. In the human forms, one has a K_d for 2,3,7,8-TCDD of 0.4 nM, while the other form has a K_d of about 2 nM. In addition, variability in Ah-receptor binding is influenced by the cell type, tissue, sex, age, experimental conditions and assay used (Okey *et al.*, 1989; Bradfield *et al.*, 1988; Safe, 1990; Poland & Knutson, 1982).

4.3.1 *The Ah receptor*

The Ah receptor is a member of the basic helix–loop–helix (bHLH) family of gene regulatory proteins (Burbach *et al.*, 1992; Ema *et al.*, 1992). The bHLH motif represents a well studied dimerization and DNA-binding domain common to a large group of regulatory factors that are generally involved in cell growth and differentiation and include the proto-oncogene c-*myc* and the muscle developmental factor MyoD (reviewed by Jan & Jan, 1993; Weintraub, 1993; Dorschkind, 1994). Thus, the Ah receptor is distinct from the superfamily of ligand-activated nuclear receptors that encompasses steroid hormone, thyroid hormone, vitamin D and retinoic acid receptors and contains the structurally well characterized 'zinc finger' DNA binding motif (see Gronemeyer & Laudet, 1995, for a comprehensive review).

The receptor functions as a ligand-dependent transcription factor that, upon exposure to ligand, recognizes DNA of target genes as a heterodimeric complex with the structurally related factor Arnt (Reyes *et al.*, 1992; Dolwick *et al.*, 1993; Matsushita *et al.*, 1993; Whitelaw *et al.*, 1993a). Both the Ah receptor and Arnt (Hoffman *et al.*, 1991) belong to a distinct subgroup, bHLH/PAS (Per–Arnt–Sim homology region) (Littlewood & Evan, 1995), of the bHLH family transcription factors, and are characterized by an N-terminal arrangement of the bHLH DNA-binding motif contiguous with a second structural motif, PAS. The PAS domain of about 250 amino acids contains two imperfect repeats and is also conserved in the product of the *Drosophila* gene *period* (Per) (Takahashi, 1992 and references therein), that is involved in circadian rhythm regulation, the *Drosophila* neurodevelopmental factor *single-minded* (Sim) (Nambu *et al.*, 1991), the *Drosophila* factor *trachealess*, important for insect tubulogenesis (Isaac & Andrew, 1996; Wilk *et al.*, 1996), as well as the hypoxia-inducible factor (HIF-1α) and E-PAS of mammals (Wang *et al.*, 1995; Gradin *et al.*, 1996; Tian *et al.*, 1997) and a human *single-minded* homologue identified as a putative Down syndrome-critical factor (Dahmane *et al.*, 1995). In addition, a mammalian factor, Arnt 2, closely related to Arnt (Drutel *et al.*, 1996; Hirose *et al.*, 1996) has recently been cloned. This factor supports Ah receptor functions in hepatoma cells deficient in Arnt (Hirose *et al.*, 1996).

Ligand-dependent activation of Ah receptor function is a multi-step process. In the absence of ligand, the latent, non-DNA-binding form of the receptor is recovered in cytosolic cellular extracts as a ~300 kDa heteromeric complex associated with the molecular chaperone hsp90 (heat shock protein 90) (Denis *et al.*, 1988; Perdew, 1988; Wilhelmsson *et al.*, 1990; Chen & Perdew, 1994). 2,3,7,8-TCDD and other known receptor ligands induce nuclear import of the Ah receptor (Jain *et al.*, 1994; Pollenz *et al.*, 1994, and

references therein) and release of the hsp90 chaperone (Wilhelmsson *et al.*, 1990) and regulate dimerization with Arnt (Whitelaw *et al.*, 1993a), enabling both proteins to bind to DNA (Reyes *et al.*, 1992; Dolwick *et al.*, 1993; Matsushita *et al.*, 1993; Whitelaw *et al.*, 1993a). The ligand-activated Ah receptor–Arnt complex induces transcription of target promoters via potent transactivation domains that are contained within the C-terminus of both the Ah receptor and Arnt (Jain *et al.*, 1994; Li *et al.*, 1994; Whitelaw *et al.*, 1994). The Ah receptor may also modulate biochemical and cellular responses via non-DNA dependent mechanisms (Matsumura, 1994; Birnbaum, 1995a; Weiss *et al.*, 1996).

In the absence of ligand, it appears that hsp90 blocks receptor–Arnt dimerization (Whitelaw *et al.*, 1993a), resulting in repression of receptor function. Ligand-induced release of hsp90 is facilitated *in vitro* by concomitant dimerization of the receptor with Arnt (McGuire *et al.*, 1994), indicating that these two processes are functionally interdigitated. Ligand (Dolwick *et al.*, 1993; Whitelaw *et al.*, 1993b) and hsp90 (Whitelaw *et al.*, 1993b) binding activities of the receptor are co-localized within the PAS domain. In contrast, the PAS domain of Arnt does not mediate association with hsp90 (Probst *et al.*, 1993; McGuire *et al.*, 1994). In-vitro ligand-binding experiments indicate that hsp90 chaperones a high-affinity ligand-binding conformation of the receptor (Pongratz *et al.*, 1992). Consistent with this model, ligand responsiveness of the Ah receptor is severely impaired upon expression in a yeast strain in which hsp90 expression levels are down-regulated to about 5% of wild-type levels (Carver *et al.*, 1994; Whitelaw *et al.*, 1995). Taken together, these data indicate that hsp90 may be critical for folding of a functional, ligand-responsive form of the Ah receptor.

All known ligands of the Ah receptor are of xenobiotic origin, and a physiological receptor ligand has not yet been identified (reviewed by Poland & Knutson, 1982; Poellinger *et al.*, 1992). Given the critical roles of the majority of bHLH factors in general (Jan & Jan, 1993; Weintraub, 1993; Dorschkind, 1994), and bHLH-PAS factors in particular (Nambu *et al.*, 1991; Isaac & Andrew, 1996; Wilk *et al.*, 1996; Tian *et al.*, 1997) for embryonic development, it is plausible that a physiological function (and possibly a ligand-dependent regulatory strategy) of the Ah receptor is to be found in developmental processes. Ah^-/Ah^- mice were found to be viable and fertile, but showed hepatic defects that indicate a role for the Ah receptor in normal liver growth (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). Thus, the exact role of the Ah receptor in mammalian development remains unclear.

4.3.2 *Induction of drug-metabolizing enzymes*

CYP1A1. The induction of *CYP1A1* gene expression and associated enzyme activities by 2,3,7,8-TCDD and other PCDDs is a common result of exposure to these chemicals and is readily demonstrated in laboratory animals and cells in culture. There is relatively low constitutive expression of CYP1A1 in most tissues and cells; however, after treatment with 2,3,7,8-TCDD, the Ah receptor complex rapidly accumulates in the nucleus, and this is accompanied by the sequential induction of CYP1A1 mRNA levels followed by induction of CYP1A1-dependent enzyme activity and immunoreactive

protein (Tukey *et al.*, 1982; Zacharewski *et al.*, 1989; Harris *et al.*, 1990; Pendurthi *et al.*, 1993). The mechanism of this response has been extensively investigated (González & Nebert, 1985; Jones *et al.*, 1985, 1986a,b; Sogawa *et al.*, 1986; Fujisawa-Sehara *et al.*, 1986, 1988; Neuhold *et al.*, 1989) and involves interaction of the Ah receptor complex with functional xenobiotic-responsive enhancers (XREs; also referred to as dioxin-responsive enhancers (DREs)) in the 5′-promoter region of *CYP1A1* genes (Denison *et al.*, 1988; Fujisawa-Sehara *et al.*, 1988; Hapgood *et al.*, 1991). Thus, the Ah receptor functions as a ligand-activated DNA-binding protein directly communicating with its target genes.

Species-, age-, sex- and organ-dependent differences in induction of *CYP1A1* gene expression and dependent enzyme activities have been reported in laboratory animals, mammalian cells in culture, and in humans, and these data have been extensively reviewed (Poland & Knutson, 1982; Whitlock, 1986, 1987; Okey, 1990; Safe, 1990; Whitlock, 1990; Kohn *et al.*, 1993; Whitlock, 1993; Okey *et al.*, 1994; Denison & Whitlock, 1995; Safe, 1995).

The induction of *CYP1A1* gene expression is also influenced by a number of other factors. For example, in wild-type and mutant Hepa-1 cells, treatment with 2,3,7,8-TCDD results in binding of the nuclear Ah receptor complex to enhancer sequences and binding of other proteins to promoter DNA (Watson & Hankinson, 1992; Wu & Whitlock, 1993). The enhancer/promoter sequence in uninduced cells forms a nucleosomal structure; after addition of 2,3,7,8-TCDD, alterations of chromatin structure and nucleosome disruption are observed (Durrin & Whitlock, 1987; Morgan & Whitlock, 1992; Wu & Whitlock, 1992). Induction of *CYP1A1* gene expression and formation of the nuclear Ah receptor complex have also been observed in human keratinocytes and mouse Hepa 1c1c7 cells suspended in solid medium containing methyl cellulose or Percoll solution (Sadek & Allen-Hoffmann, 1994a,b).

Protein kinase C-dependent phosphorylation is important for 2,3,7,8-TCDD activation of the Ah receptor and Arnt proteins (Carrier *et al.*, 1992; Reiners *et al.*, 1992, 1993; Berghard *et al.*, 1993). In human keratinocytes and mouse Hepa-1 cells, phorbol esters and protein kinase C inhibitors blocked 2,3,7,8-TCDD-dependent formation of the transformed Ah receptor complex (Berghard *et al.*, 1993), and similar results were observed in C57BL/6 mice co-treated with 2,3,7,8-TCDD plus phorbol esters (Okino *et al.*, 1992). In contrast, in-vitro transformation of the Ah receptor of guinea-pig, mouse and rat hepatic cytosol is not affected by inhibitors of protein kinase C (Schaefer *et al.*, 1993) and staurosporine, a protein kinase C inhibitor, did not affect nuclear translocation of the Ah receptor in mouse Hepa-1 cells in culture (Singh & Perdew, 1993). Moreover, in MCF-7 human breast cancer cells in which protein kinase C activity was depleted as a result of prolonged treatment with phorbol esters, 2,3,7,8-TCDD caused superinducibility of *CYP1A1* gene expression, and this was accompanied by a two- to three-fold increase in levels of the nuclear Ah receptor complex (Moore *et al.*, 1993). These observations indicate that the role of protein phosphorylation of the Ah receptor complex may vary among different cell lines or target tissues.

Several other factors which inhibit the 2,3,7,8-TCDD-dependent induction of *CYP1A1* and *CYP1A2* gene expression include IL-1β, insulin, EGF, rat hepatocytes (Barker *et al.*, 1992) and TGFβ in squamous carcinoma cells (Hébert *et al.*, 1990b) and human A549 lung cancer cells (Vogel *et al.*, 1994).

CYP1A2. Expression of the *CYP1A2* gene and related enzyme activities is also induced by 2,3,7,8-TCDD and related Ah receptor agonists (Koga *et al.*, 1990; DeVito *et al.*, 1994; Narasimhan *et al.*, 1994; Paroli *et al.*, 1994; De Jongh *et al.*, 1995; Diliberto *et al.*, 1995). In laboratory animals and humans, *CYP1A2* is expressed primarily in liver and is inducible, together with *CYP1A1*, by 2,3,7,8-TCDD in primary cultures of human hepatocytes (Schrenk *et al.*, 1995), whereas in most cell lines, the gene is not expressed (Fagan *et al.*, 1986; Kimura *et al.*, 1986; Silver & Krauter, 1988; Ikeya *et al.*, 1989). CYP1A2 catalyses oxidation of aromatic amines and 17β-oestradiol and may also be important in 4-hydroxylation of tamoxifen (Kupfer *et al.*, 1994). The hydroxylation of 17β-oestradiol by CYP1A2 to catechols has been suggested to play a role in 2,3,7,8-TCDD-induced carcinogenesis (Liehr, 1990; Liehr & Roy, 1990; Lucier *et al.*, 1991; Yager & Liehr, 1996). In addition, PCDDs can bind strongly to this isoenzyme and also function as competitive binding inhibitors (Voornan & Aust, 1987; Poland *et al.*, 1989). Deletion analyses of the CYP1A2 promoter from the human *CYP1A2* gene have revealed XRE core sequences at -2259 to -1970 and -2888 to -2903 (Quattrochi & Tukey, 1989; Quattrochi *et al.*, 1994). Transfected cells with deletion plasmids containing the core (-2888 to -2903) XRE sequence were Ah-receptor responsive in human Hep G2 but not MCF-7 cells, suggesting that *CYP1A2* gene expression is regulated by multiple factors in addition to the Ah receptor complex (Quattrochi *et al.*, 1994).

CYP1B1. 2,3,7,8-TCDD also induces another cytochrome P450, CYP1B1, that occurs in humans and rodents (Sutter *et al.*, 1991; Savas *et al.*, 1994; Shen *et al.*, 1994a,b; Sutter *et al.*, 1994; Bhattacharyya *et al.*, 1995; Walker *et al.*, 1995). CYP1B1 is expressed in a variety of human tissues (Sutter *et al.*, 1994; Hayes *et al.*, 1996) and inducible by 2,3,7,8-TCDD in numerous human cell types (Sutter *et al.*, 1991; Spink *et al.*, 1994; Sutter *et al.*, 1994) and rodent tissues including liver, lung and kidney (Walker *et al.*, 1995). CYP1B1 is active in the metabolism of numerous polycyclic aromatic hydrocarbons and arylamines (Pottenger & Jefcoate, 1990; Pottenger *et al.*, 1991; Otto *et al.*, 1992; Shimada *et al.*, 1996) and can catalyse the 4-hydroxylation of 17β-oestradiol in humans cells (Hayes *et al.*, 1996). Further studies are needed to elucidate the role of CYP1B1 in 2,3,7,8-TCDD-induced toxicity and the mechanisms associated with this induced response.

Aldehyde-3-dehydrogenase. 2,3,7,8-TCDD induces aldehyde-3-dehydrogenase activity (Takimoto *et al.*, 1992; Unkila *et al.*, 1993b; Germolec *et al.*, 1996), and analysis of the promoter region has identified XRE sequences (Hempel *et al.*, 1989; Takimoto *et al.*, 1991; Asman *et al.*, 1993). Deletion analysis of the 5′-flanking region revealed DNA sequences which both enhance and decrease activity of chimeric genes, and an XRE core binding sequence (GCGTG) was also identified (Takimoto *et al.*, 1994).

Glutathione S-*transferase Ya.* 2,3,7,8-TCDD and related compounds induce GST activities and gene expression in laboratory animals and mammalian cells (Paulson *et al.*,

1990; Rushmore *et al.*, 1990; Aoki *et al.*, 1992; Pimental *et al.*, 1993; Rushmore & Pickett, 1993). Analysis of the 5′-promoter region of the rat GST *Ya* gene has revealed several genomic regulatory sequences including an XRE sequence containing the core 5′-GCGTG-3′ sequence which binds the Ah receptor complex. Deletion and mutational analysis studies showed the requirement of XRE for Ah-responsiveness. Pimental *et al.* (1993) have also demonstrated binding of the constitutive protein, C/EBPα, to the XRE and shown that Ah receptor-mediated induction of constructs containing the XRE may involve cooperative protein–protein interactions between C/EBPα and the Ah receptor complex. GSTP-1 is also induced by PCDDs (Aoki *et al.*, 1992, 1993), and this response can be inhibited by glucocorticoids and protein kinase C inhibitors.

Glucuronosyl transferase. UDP-GT activity is also induced by 2,3,7,8-TCDD and other Ah receptor agonists (Owens, 1977), and recent studies with the rat *UGT1A1* gene have identified a functional XRE sequence (-134 to -139) in the 5′-promoter region of this gene (Emi *et al.*, 1995, 1996). 2,3,7,8-TCDD also induced UGT1A1 levels in a human cell line (Abid *et al.*, 1995), although induction in humans *in vivo* has not been proven.

NAD(P)H:quinone oxidoreductase. NAD(P)H:quinone oxidoreductase (or DT diaphorase) activity is also induced by Ah receptor agonists, and the human gene structure, activity and tissue-specific expression have been reported (Shaw *et al.*, 1991; Jaiswal, 1994). The sequence of the 5′-flanking region contains binding sites for several nuclear proteins, and these include an XRE 5′-GCGTG-3′ sequence between -708 and -704; plasmids containing the XRE sequences are inducible by 2,3,7,8-TCDD and β-naphthoflavone in transient assays. The gene is widely expressed in human tissues, but the role of 2,3,7,8-TCDD-induced expression of NAD(P)H:quinone oxidoreductase in the toxic responses elicited by this compound is unknown.

Prostaglandin endoperoxide H synthase-2 (PGHS-2). The *PGHS-2* gene was recently identified as a 2,3,7,8-TCDD-regulated gene in a canine kidney cell line (Kraemer *et al.*, 1996). Its induction by 2,3,7,8-TCDD in human cells has not been investigated.

4.3.3 *Modulation of growth factors, growth factor receptors, transcription factors, lymphokines and related factors*

2,3,7,8-TCDD and related compounds decrease epidermal growth factor (EGF) receptor binding and/or autophosphorylation in several cells or organs, including human keratinocytes in culture (Hudson *et al.*, 1985), mouse hepatoma cells (Kärenlampi *et al.*, 1983), mouse, rat and guinea-pig liver (Madhukar *et al.*, 1984, 1988; Ryan *et al.*, 1989b; Lin *et al.*, 1991a; Sewall *et al.*, 1993), fish liver (Newsted & Giesy, 1993) and rat uterus (Astroff *et al.*, 1990). In contrast, EGF receptor binding was increased in palatal medial epithelial cells from mouse embryo (Abbott *et al.*, 1989). Support for the role of the Ah receptor in mediating down-regulation of the EGF receptor has been supported by structure–activity studies in mice (Ryan *et al.*, 1989b) and the differential responsiveness of congenic mice differing only in the structure of the Ah receptor gene (Lin *et al.*, 1991b). It has been suggested that decreased EGF receptor binding may play a role in the development of hepatocellular carcinomas in female rats treated with 2,3,7,8-TCDD

(Sewall *et al.*, 1993), since development of preneoplastic hepatic lesions and decreased hepatic EGF receptor binding was observed only in unovariectomized 2,3,7,8-TCDD treated animals. 2,3,7,8-TCDD also induces TGFα, IL-1β and TGFβ mRNA levels in some cells (Abbott & Birnbaum, 1990a; Choi *et al.*, 1991; Sutter *et al.*, 1991; Gaido *et al.*, 1992; Döhr *et al.*, 1994; Lee *et al.*, 1996). Expression of PAI-2 is induced in the human keratinocyte cell line SCC-12F (Sutter *et al.*, 1991) and in human hepatocytes in primary culture (Gohl *et al.*, 1996). 2,3,7,8-TCDD has been shown to alter transcription factors involved in growth and differentiation. Both *in vivo* and *in vitro*, 2,3,7,8-TCDD causes a rise in the expression of *ras* and *erbA*, both proto-oncogenes (Matsumura, 1994). In-vitro studies have shown an increase in the expression of c-*fos* and c-*jun*, as well as an increase in AP1 (Puga *et al.*, 1992).

4.3.4 *Modulation of thyroid hormones, vitamin A and retinoids*

Interactions between 2,3,7,8-TCDD and thyroid hormones (T4 and T3) and the effects of altered thyroid hormone levels on the toxicity of 2,3,7,8-TCDD have been extensively studied (see also Section 4.2.1(*d*)). Daily injections of T3 increased survival times of animals treated with 2,3,7,8-TCDD (Neal *et al.*, 1979) and thyroidectomy offered some protection from 2,3,7,8-TCDD-induced mortality and immunotoxicity in rats (Pazdernik & Rozman, 1985; Rozman *et al.*, 1985a, 1987). In mice treated with 2,3,7,8-TCDD plus T3 or T4, there was an increased incidence of cleft palate (Lamb *et al.*, 1986). Decreased serum T4 levels have been observed in some laboratory animals after treatment with 2,3,7,8-TCDD (Bastomsky, 1977; McKinney *et al.*, 1985; Pazdernik & Rozman, 1985; Rozman *et al.*, 1985b; Henry & Gasiewicz, 1987; Jones *et al.*, 1987; Sewall *et al.*, 1995) and this may be related to increased rates of thyroxine glucuronidation. However, in other studies, thyroid hormone levels were unchanged or increased after treatment with 2,3,7,8-TCDD (Potter *et al.*, 1983; McKinney *et al.*, 1985; Potter *et al.*, 1986; Henry & Gasiewicz, 1987). The decrease in serum T4 levels in rats treated with 2,3,7,8-TCDD was preceded by an initial decrease in serum prolactin levels (within 4 h) which were then significantly increased seven days after the initial exposure (Jones *et al.*, 1987). Hydroxylated PCDDs have been found to bind to the thyroid hormone-binding proteins in in-vitro experiments using human TBG (Lans *et al.*, 1993, 1994). For PCDDs, it has not been shown that the observed decreased T 4 levels in in-vivo experiments were caused by these hydroxylated metabolites. In view of the extremely low biotransformation rate of most 2,3,7,8-PCDDs, other mechanisms might be involved (Van den Berg *et al.*, 1994; van Birgelen *et al.*, 1995b).

The similarities between some 2,3,7,8-TCDD-induced toxic responses and hypovitaminosis A, and the synergistic interactions between 2,3,7,8-TCDD- and retinoic acid-induced cleft palate (Abbott & Birnbaum, 1989a,b; Birnbaum *et al.*, 1989) have stimulated several studies on the biochemical responses associated with these interactions. Several studies have reported that 2,3,7,8-TCDD markedly decreases hepatic retinol levels in various laboratory animal species, the guinea-pig being the most sensitive. Modulation of serum retinol levels and of concentrations in other organs was species-dependent (Thunberg *et al.*, 1980; Thunberg & Håkansson, 1983; Rozman *et al.*, 1987;

Brouwer *et al.*, 1989; Håkansson & Hanberg, 1989; Jurek *et al.*, 1990; Pohjanvirta *et al.*, 1990; Håkansson *et al.*, 1991a,b; Hanberg *et al.*, 1996). In 2,3,7,8-TCDD-exposed rats, a pronounced, rapid and long-lasting increase in renal vitamin A was observed. Feeding vitamin A-supplemented diet to male Sprague-Dawley rats did not result in a consistent reduction in 2,3,7,8-TCDD toxicity (Håkansson *et al.*, 1990). Hepatic stellate cells are a major storage site for vitamin A (retinyl ester form) and treatment with 2,3,7,8-TCDD markedly reduces retinol and retinyl palmitate levels in these cells; however, this is not accompanied by decreased cell number or transformation (Hanberg *et al.*, 1996). 2,3,7,8-TCDD also affects retinoic acid metabolism and tissue-specific accumulation of various metabolites (Fiorella *et al.*, 1995), and it has been suggested that 2,3,7,8-TCDD-induced toxicity may be related to altered metabolism of retinoic acid. 2,3,7,8-TCDD also decreases retinoic acid-induced gene expression in mouse embryonic cells (Weston *et al.*, 1995), and both retinoic acid and retinol inhibit 2,3,7,8-TCDD-induced terminal differentiation in human keratinocytes (Berkers *et al.*, 1995). The significance of vitamin A depletion for the chronic toxicity of 2,3,7,8-TCDD is unclear.

4.3.5 *Modulation of protein phosphorylation*

Kinase-dependent protein phosphorylation plays a central role in cell signalling, and 2,3,7,8-TCDD alters both protein phosphorylation patterns and kinase activities. For example, treatment with 2,3,7,8-TCDD stimulates protein kinase C activity in rat splenocytes, hepatocytes, thymocytes, hippocampal cells and rat and guinea-pig hepatic plasma membranes (Bombick *et al.*, 1985; DePetrillo & Kurl, 1993; Wölfle *et al.*, 1993; Zorn *et al.*, 1995; Hanneman *et al.*, 1996). 2,3,7,8-TCDD also induces tyrosine phosphorylation in many of these same cell types (Kramer *et al.*, 1987; Clark *et al.*, 1991c; Ebner *et al.*, 1993; Ma & Babish, 1993; DeVito *et al.*, 1994; Enan & Matsumura, 1994a,b, 1995a,b). Snyder *et al.* (1993) showed that, in B6C3F1 mice, 2,3,7,8-TCDD-induced phosphorylation of proteins was selective for activated B cells. Purified B cells from both DBA/2 (Ah^d/Ah^d) and C57BL/6 (Ah^b/Ah^b) mice demonstrated equivalent enhancement of phosphorylation in response to 2,3,7,8-TCDD. Administration of human γ-interferon produced a reversal of 2,3,7,8-TCDD-induced suppression of in-vitro antibody responses in splenocytes isolated from B6C3F1 mice. Ma and Babish (1993) demonstrated that 2,3,7,8-TCDD enhances phosphorylation of cyclin-dependent kinases (p34cdc2 and p33cdk2) in mouse liver.

4.3.6 *Modulation of biochemical responses associated with glucose metabolism and transport*

In several rodent species, 2,3,7,8-TCDD decreased phosphoenol pyruvate carboxykinase (PEPCK), glucose-6-phosphatase, GGT and pyruvate carboxylase activities, and the reduced enzyme activities were correlated with decreased gluconeogenesis in the treated animals (Górski *et al.*, 1990; Weber *et al.*, 1991b,c; Stahl *et al.*, 1992b, 1993; Sparrow *et al.*, 1994; Fan & Rozman, 1995; Li & Rozman, 1995; Ryu *et al.*, 1995). 2,3,7,8-TCDD also decreased PEPCK mRNA levels in male Sprague-Dawley rats (Stahl *et al.*, 1993) and the decrease in pyruvate carboxylase activity appears to be Ah receptor-

mediated based on studies with congenic Ah-responsive (Ah^b/Ah^b) and less responsive (Ah^d/Ah^d) male C57BL/6 mice (Ryu *et al.*, 1995). A comparison of the effects of 2,3,7,8-TCDD in guinea-pigs (0.3–2.7 μg/kg) and hamsters (900–4600 μg/kg) showed that hepatic PEPCK was decreased in hamsters but not guinea-pigs, suggesting that altered carbohydrate homeostasis does not correlate with species-dependent susceptibility to 2,3,7,8-TCDD-induced lethality (Unkila *et al.*, 1995).

In guinea-pigs, adipose tissue and brain glucose uptake was decreased, whereas in liver, uptake was initially decreased (6–12 h) then increased (24–96 h) (Enan *et al.*, 1992; Enan & Matsumura, 1994a). Decreased glucose transport activity was associated in mice with tissue-specific decreased levels of glucose transporter (Liu & Matsumura, 1995). The relationship between the effects of glucose transport and 2,3,7,8-TCDD-induced toxicity has yet to be determined.

Several studies of rats and rhesus monkeys have shown consistent decreases in serum glucose levels after daily doses of 2,3,7,8-TCDD administered over 30 days (Zinkl *et al.*, 1973) or after a single dose (McConnell *et al.*, 1978b; Gasiewicz *et al.*, 1980; Schiller *et al.*, 1986; Ebner *et al.*, 1988).

4.3.7 *Modulation of oestrogenic responses by PCDDs*

2,3,7,8-TCDD inhibited the following oestrogen-induced responses in the ovariectomized or immature female rodent uterus: uterine weight increase, progesterone receptor levels, peroxidase activity, EGF-receptor binding and mRNA levels, and c-*fos* proto-oncogene mRNA levels (Romkes *et al.*, 1987; Astroff & Safe, 1988; Romkes & Safe, 1988; Umbreit *et al.*, 1988, 1989; Astroff & Safe, 1990; Astroff *et al.*, 1990; Astroff & Safe, 1991; Astroff *et al.*, 1991). In 21 day-old weanling rats, inhibition of oestradiol-induced uterine weight increase was not observed in Sprague-Dawley rats (White *et al.*, 1995). In-vitro studies using human breast cancer cell lines have also demonstrated the negative regulation by 2,3,7,8-TCDD of the following oestradiol-induced responses: cell proliferation, [^{3}H]thymidine uptake, postconfluent focus production, secretion of pS2, cathepsin D, procathepsin D and tissue plasminogen activator activity, progesterone receptor binding, and oestrogen receptor, progesterone receptor, pS2, prolactin receptor and cathepsin D gene expression (Gierthy *et al.*, 1987; Gierthy & Lincoln, 1988; Biegel & Safe, 1990; Krishnan *et al.*, 1992; Krishnan & Safe, 1993; Wang *et al.*, 1993; Harper *et al.*, 1994; Moore *et al.*, 1994; Zacharewski *et al.*, 1994; Krishnan *et al.*, 1995; Lu *et al.*, 1996). Moreover, using oestrogen-responsive promoter-reporter constructs derived from the 5′-regions of the pS2 and cathepsin D genes, 2,3,7,8-TCDD also inhibited E2-induced reporter gene activity in transiently transfected MCF-7 cells (Zacharewski *et al.*, 1994; Krishnan *et al.*, 1995). The role of the Ah receptor in mediating the anti-oestrogenic activities of various structural classes of agonists has been confirmed in several studies (Safe, 1995). Structure–activity studies with several Ah receptor agonists indicated a role of the Ah receptor in negatively modulatory oestrogenic responses (Krishnan *et al.*, 1994; Zacharewski *et al.*, 1994). In fact, interference between oestrogen- and Ah receptor-dependent signalling pathways has recently been proposed to be mediated by physical interaction between these receptor systems (Kharatt & Saatcioglu, 1996).

Expression of a functional nuclear Ah receptor complex is required for ligand-induced anti-oestrogenicity (Moore *et al.*, 1994). Studies of the cathepsin D gene have shown strategically located Ah receptor-binding sites, which may impair oestrogen-receptor function. A similar mechanism may be operative for inhibition of oestradiol-induced pS2 gene expression (Zacharewski *et al.*, 1994). It should be noted that the above oestrogen receptor modulatory effects of PCDDs are tissue-, age- and species-specific (Safe, 1995).

2,3,7,8-TCDD inhibits aromatase (CYP19) activity in the human choriocarcinoma cell line Jeg-3. The EC_{50} value for inhibition was in the same range as that observed for CYP1A1 induction in this cell type (Drenth *et al.*, 1996). This action could be a cause for the anti-oestrogenic effects of PCDDs; these in-vitro results need to be confirmed in in-vivo experiments.

4.3.8 *Role of oxidative stress in the toxicity of PCDDs*

Exposure of rats to an oral dose of 100 µg/kg bw 2,3,7,8-TCDD (a lethal dose) increased lipid peroxidation in hepatic mitochondria (Stohs *et al.*, 1990). Marked increases in hepatic lipid peroxidation were observed in female Sprague-Dawley rats [males not studied] seven days after treatment with 50 µg/kg 2,3,7,8-TCDD (Wahba *et al.*, 1990). The increase in hepatic lipid peroxidation was suppressed by antioxidants such as butylated hydroxyanisole, vitamin A or *d*-α-tocopherol (Stohs *et al.*, 1984). 2,3,7,8-TCDD also enhanced lipid peroxidation in various other tissues (Bagchi *et al.*, 1993) and altered membrane structure and functions. Enhanced lipid peroxidation after 2,3,7,8-TCDD treatment was suggested to arise as a secondary phenomenon in 2,3,7,8-TCDD toxicity, possibly contributing to lethality (Pohjanvirta *et al.*, 1990c). 2,3,7,8-TCDD depleted glutathione levels, altered calcium homeostasis and increased DNA damage in the form of single strand breaks (Stohs *et al.*, 1990). In addition, 2,3,7,8-TCDD enhances the release of TNFα (Alsharif *et al.*, 1994a) and induces production of stress/heat shock protein 90 (hsp90) (Perdew, 1992; Henry & Gasiewicz, 1993; Abbott *et al.*, 1994a). The use of TNF antibody decreases 2,3,7,8-TCDD-induced DNA damage, lipid peroxidation and macrophage activation.

4.3.9 *Cell cycle regulation and apoptosis*

Exposure to 2,3,7,8-TCDD delays G1-S progression in mouse and rat hepatoma cells in a receptor-dependent manner (Göttlicher & Wiebel, 1991; Ma & Whitlock, 1996; Weiss *et al.*, 1996), whereas 2,3,7,8-TCDD-induced enhancement of cell proliferation has been observed in human squamous carcinoma cell lines in a cell confluence-dependent manner (Hébert *et al.*, 1990b).

Tyrosine phosphorylation and the level of expression of two cyclin-dependent kinases which regulate cell cycle progression have been found to be increased in mouse liver after both acute (Ma & Babish, 1993) and subchronic (DeVito *et al.*, 1994) 2,3,7,8-TCDD dosing. Increased cell proliferation does not necessarily determine that cancer will occur, and hence not all chemicals that cause increased cell proliferation cause cancer (Melnick *et al.*, 1992).

Recent studies have also demonstrated that 2,3,7,8-TCDD modulates programmed cell death (apoptosis) *in vivo* and *in vitro*. In Ah-responsive mice deficient in the apoptosis-inducing ligand Fas, 2,3,7,8-TCDD was less toxic to the thymocytes than in Fas-proficient mice (Rhile *et al.*, 1996). In a study in female BALB/cJ mice treated with a single intraperitoneal dose of 30 µg/kg 2,3,7,8-TCDD, however, no indication of thymocyte apoptosis was obtained (Silverstone *et al.*, 1994b).

Stinchcombe *et al.* (1995) reported suppression of apoptosis in preneoplastic hepatocytes in a two-stage initiation–promotion protocol. Inhibition of apoptosis with 1 nM 2,3,7,8-TCDD was also observed in rat hepatocytes in primary culture pretreated with ultraviolet light or 2-acetylaminofluorene (Wörner & Schrenk, 1996). This effect was linked to negative regulation of expression of the *p53* tumour-suppressor gene.

4.4 Reproductive and developmental effects

4.4.1 *Humans*

Most studies on human reproductive effects of PCDDs concern paternal exposure, usually long after a high exposure occurred. A number of studies evaluated reproductive effects in cohorts with high potential for exposure to 2,3,7,8-TCDD or other PCDDs. These studies include the United States Ranch Hand personnel (Wolfe *et al.*, 1995), the Seveso population (Mastroiacovo *et al.*, 1988), workers who manufactured TCP and 2,4,5-T (Townsend *et al.*, 1982; Suskind & Hertzberg, 1984) and pesticide applicators (Smith *et al.*, 1992b). Only the studies of Ranch Hand personnel and of pesticide applicators measured serum 2,3,7,8-TCDD levels, and the quality of the studies varies. Cohorts of Viet Nam veterans other than Ranch Hand personnel (Stellman *et al.*, 1988; Centers for Disease Control Vietnam Experience Study, 1988b) and of Missouri residents (Stockbauer *et al.*, 1988) have not been proven to have high potential for exposure or the exposure was not well defined.

The study populations mentioned below and their exposures are described more fully in Sections 1.3.1, 1.3.2 and 2.2.

(*a*) *Endocrine and gonadal effects*

Total serum testosterone, LH and follicle-stimulating hormone (FSH) were measured in 248 TCP production workers and in 231 non-exposed neighbourhood controls matched for age and race (Egeland *et al.*, 1994). In linear regression analyses, current serum levels of 2,3,7,8-TCDD were positively related to LH and FSH and inversely related to total testosterone levels after adjustment for potential confounders (age, body mass index, diabetes mellitus, current alcohol consumption, race and smoking status). Similar results were obtained by multiple logistic regression, showing stronger adverse effects in higher-exposure groups. The presence of both low testosterone and high LH was not observed in the same individuals, therefore, the authors interpreted their findings as being suggestive of more subtle alterations in gonadotropins and testosterone than of primary gonadal failure due to PCDD exposure.

In a random subsample of 571 men, from a total of 4462 who were examined, Viet Nam veterans (n = 324) had significantly lower sperm concentrations than the 247 non-Viet Nam veterans (64.8 million/mL versus 79.8 million/mL) and a lower proportion of 'normal' sperm heads (57.9% versus 60.8%) (Centers for Disease Control Vietnam Experience Study, 1988a). There was also a doubling of the proportion of men with sperm characteristics (concentration, percentage of motile cells, percentage of morphologically 'normal' cells) below the normal range. Ability to father children was not affected. Differences in semen concentration and morphology could not be related to a particular subgroup of veterans or military occupational specialty, to self-reported combat experiences or to herbicide exposure.

In the 1982 examination, 474 Ranch Hand veterans out of 1045 and 532 of the 1224 comparison veterans were included in the analysis of association between serum 2,3,7,8-TCDD and serum testosterone, FSH, LH, testicular abnormality, sperm count, percentage of abnormal sperm and testicular volume. No pattern of consistent or meaningful associations was seen between 2,3,7,8-TCDD body burden and any of the variables studied, when either categorized (% abnormalities) (Henriksen *et al.*, 1996) or analysed as continuous variables (Henriksen & Michalek, 1996). The authors explain that their study and the previous study by Egeland *et al.* (1994) differ in terms of the exposure circumstances and demographics and conclude that 'the Ranch Hand exposure was not sufficient to exhibit the associations similar to those seen in the industrial workers, who have higher dioxin body burdens and were exposed over a longer period of time than the Ranch Hand veterans.'

(*b*) *Effects on pregnancy* (**Table 63**)

(i) *Studies on Viet Nam veterans (United States and Australia)*

Exposure data relating to these groups are described in Section 1.3.1(*a*)(ii); in general, exposure to PCDDs was low, except in the case of the Ranch Hand Study. In the following studies, exposure to Agent Orange was classified as 'service in Viet Nam'. No direct exposure measurements were made. However, in two studies, exposure indices were based on location of service in Viet Nam. These indices may still misclassify actual exposure to Agent Orange.

Telephone interviews were conducted with 7924 Viet Nam veterans (87% of the eligible) and 7364 non-Viet Nam veterans (84%), randomly selected from the Vietnam-Era United States Army personnel records (Centers for Disease Control Vietnam Experience Study, 1988b). Viet Nam veterans were more likely to report having fathered a pregnancy that ended in a miscarriage than were non-Viet Nam veterans (odds ratio, 1.3; 95% CI, 1.2–1.4). In a birth defects sub-study (1237 Viet Nam veterans and 1045 non-Viet Nam veterans) for which hospital birth records were obtained for all reported births (1791 offspring of Viet Nam veterans and 1575 offspring of non-Viet Nam veterans), no difference in the rates of all birth defects was identified: 72.6 per 1000 births for Viet Nam veterans; 71.1 per 1000 among non-Viet Nam veterans. The rates for major defects were respectively 28.5 per 1000 and 23.5 per 1000. The adjusted odds ratio for total defects among offspring of black veterans was 3.3 (95% CI, 1.5–7.5). The observed

Table 63. Results of studies examining the effect of 2,3,7,8-TCDD on pregnancy outcomes in humans

Reference	Exposed group	Control group	Type of exposure	Data source: exposure/ outcome	Outcome	Outcome in exposed (no.)	Outcome in unexposed (no.)	Odds ratio	CI (95% unless indicated)
Studies of cohorts with high potential exposure									
Wolfe *et al.* (1995)	1006 conceptions among 454 Ranch Hand personnel	1235 conceptions among 570 non-Ranch Hand personnel	Spraying/ handling of Agent Orange	Serum 2,3,7,8-TCDD levels/ medical records	Spontaneous abortion				
					Comparison		172	1	
					Background exposure	57		1.1	0.8–1.5
					Low exposure	56		1.3	1.0–1.7
					High exposure	44		1.0	0.7–1.3
					Stillbirth				
					Comparison		13	1	
					Background exposure	7		1.8	0.7–4.5
					Low exposure	6		1.8	0.7–4.7
					High exposure	1		0.3	0.0–2.3
					Major birth defects				
					Comparison		56	1	
					Background exposure	17		1.1	0.6–1.8
					Low exposure	23		1.7	1.1–2.7
					High exposure	19		1.2	0.8–2.1
					Developmental delays				
					Comparison		71	1	
					Background exposure	24		1.2	0.8–1.8
					Low exposure	26		1.5	1.0–2.3
					High exposure	21		1.1	0.7–1.7
Mastroiacovo *et al.* (1988)	2900 births in zones A, B and R, Seveso, Italy, 1977–82	12 391 births in study area outside zones A, B and R	2,3,7,8-TCDD cloud released from chemical plant accident	2,3,7,8-TCDD soil analysis/ Seveso Congenital Malformations Registry	Total birth defects	137	605	1.0	0.8–1.1[a]
					Multiple birth defects	10	38	1.1	0.6–2.0[a]
					Syndromes	5	29	0.7	0.3–1.6[a]
					Major birth defects	67	343	0.8	0.7–1.0[a]
					Minor birth defects	70	262	1.1	0.9–1.4[a]

Table 63 (contd)

Reference	Exposed group	Control group	Type of exposure	Data source: exposure/ outcome	Outcome	Outcome in exposed (no.)	Outcome in unexposed (no.)	Odds ratio	CI (95% unless indicated)
Studies of cohorts with high potential exposure (contd)									
Townsend *et al.* (1982)	Male chemical workers exposed to any PCDDs	Unexposed workers	Chlorophenol and 2,4,5-T production	Company records/ interview	Total conceptuses	737	2031	–[b]	
					All fetal deaths	100	246	1.0	0.8–1.4
					Stillbirth	15	33	1.1	0.5–2.1
					Spontaneous abortions	85	213	1.0	0.8–1.4
					Infant deaths	9	39	0.6	0.3–1.4
					Health defects	52	155	0.9	0.6–1.2
					Congenital malformations	30	87	0.9	0.5–1.4
Suskind & Hertzberg (1984)	189 male chemical workers exposed to 2,4,5-T processes	155 male workers in the same plant not exposed to 2,4,5-T processes	2,4,5-T process	Interview, personal work records/ interview	Pregnancies	655	429		
					Miscarriages	69	51	0.9[c]	NS
					Stillbirths	11	5	1.4[c]	NS
					Dead in 4 weeks	17	6	1.8[c]	NS
					Birth defects	18	11	1.1[c]	NS
Smith *et al.* (1982b)	548 male pesticide applicators who sprayed 2,4,5-T and other pesticides	441 agricultural contractors	Spraying of 2,4,5-T	Mailed survey/ mailed survey	Total pregnancies	486	401		
					Congenital defect	13	9	1.2	0.6–2.5[a]
					Miscarriage	43	40	0.9	0.6–1.3[a]
					Stillbirth	3	0	–	–
Studies of cohorts with potential for low or undefined exposure									
Stockbauer *et al.* (1988)	402 births to exposed mothers	804 births to unexposed mothers (matched on maternal age and race, hospital and year of birth, plurality)	Contact with soil sprayed with 2,3,7,8-TCDD for dust control	EPA soil analyses for 2,3,7,8-TCDD/ vital statistics and hospital records	Birth defects - all	17	42	0.8	0.4–1.5
					Major birth defects	15	35	0.8	0.4–1.7
					Multiple birth defects	2	11	0.3	0.0–1.7
					Fetal deaths	4	5	1.6	0.3–7.4
					Infant deaths	5	5	2.0	0.5–8.7
					Perinatal deaths	6	9	1.3	0.4–4.2
					Low birth weight	27	36	1.6	0.9–2.8
					Very low birth weight	1	4	0.5	0.0–5.1

Table 63 (contd)

Reference	Exposed group	Control group	Type of exposure	Data source: exposure/ outcome	Outcome	Outcome in exposed (no.)	Outcome in unexposed (no.)	Odds ratio	CI (95% unless indicated)
Studies of cohorts with potential for low or undefined exposure (contd)									
CDC (1988b)	7294 Viet Nam veterans	7364 non-Viet Nam veterans	Viet Nam military service	Military records/self reports	Total birth defects	826	590	1.3	1.2–1.4
					Spontaneous abortion			1.3	1.2–1.4
					Low birth weight	716	655[d]	1.1	0.8–1.4
					Childhood cancer	25	17	1.5	0.8–2.8
CDC (1988b)	1791 offspring of Viet Nam veterans	1575 offspring of non-Viet Nam veterans	Viet Nam military service	Military records/self report and hospital records verification	Total birth defects	130	112	1.0	0.8–1.4
					Major birth defects	51	37	1.1	0.7–1.8
					Minor birth defects	58	54	1.0	0.7–1.5
					Suspected birth defects	21	21	0.9	0.5–1.7
Stellman *et al.* (1988)	2858 Viet Nam veterans	3933 non-Viet Nam veterans	Viet Nam military service	Survey/ survey	Difficulty conceiving	349	363	1.3	$p < 0.01$
					Spontaneous abortion	231	195	1.4	$p < 0.01$
Case–control studies									
Erickson *et al.* (1984)	7133 infants from the Metropolitan Atlanta Congenital Defects Program	4246 infants from Georgia Vital Statistics Records	Viet Nam military service	Self-reported and Exposure Opportunity Index/Birth defects registry and vital statistics	Total birth defects (96 subcategories also examined)	428	268	1.0	0.8–1.1
					Spina bifida	NR	NR	1.1	
					Cleft lip without cleft palate	NR	NR	1.1	
Donovan *et al.* (1984)	8517 infants born with congenital anomalies (Australia, 1966–79)	8517 infants without anomalies matched by hospital, period of birth, age of mother, hospital payment category	Past paternal military service in Viet Nam	Military records/ hospital records	Congenital anomalies	127	123	1.0	0.8–1.3

Table 63 (contd)

Reference	Exposed group	Control group	Type of exposure	Data source: exposure/ outcome	Outcome	Outcome in exposed (no.)	Outcome in unexposed (no.)	Odds ratio	CI (95% unless indicated)
Case–control studies (contd)									
Aschengrau & Monson (1989)	201 spontaneous abortion cases at Boston Hospital for Women	1119 full-term births at Boston Hospital for Women	Viet Nam military service	Military records/ hospital records	Spontaneous abortion	10	60	0.9	0.4–1.9
Aschengrau & Monson (1990)	966 infants with late adverse pregnancy outcomes at Boston Hospital for Women	998 normal term infants at Boston Hospital for Women	Viet Nam military service	Military records/ hospital records	Total birth defects	55	146[c]	1.1	0.7–1.8
					≥ 1 major malformation	18	45	1.7	0.8–3.5
					Minor malformation	11	32	0.9	0.4–2.3
					Stillbirths	5	5	3.2	0.7–14.5
					Neonatal deaths	3	9	1.1	0.2–4.5
Ha *et al.* (1996)	87 cases of gestational trophoblastic disease at Ho Chi Minh City Gynaecology Hospital	87 surgical controls matched for age and last residence	Cumulative exposure to Agent Orange in the environment	Residence history and US military records/ hospital admissions	Gestational trophoblastic disease (hydatidiform mole or choriocarcinoma)				
					Background exposure	–	–	1.0	
					Highest cumulative exposure	–	–	0.7	0.2–1.8

NS, not stated; NR, not reported

[a] 90% confidence interval

[b] Adjusted for mother's age at time of birth, birth control methods, labour and delivery complications, medical conditions and mediations during pregnancy, smoking and alcohol use during pregnancy, high rob risk and gravidity

[c] Estimated from published results

[d] Referent category: non-Viet Nam veterans

number of cases of central nervous system defects (26) among babies of Viet Nam veterans was within the expected range (18.3–32.4) based on national rates (Birth Defects Monitoring Program) or Metropolitan Atlanta race-specific rates, whereas the observed number (12) among non-Viet Nam veterans was lower than expected (17.0–30.2).

Verified conceptions and births fathered during or after service in south-east Asia in relation to serum 2,3,7,8-TCDD levels were compared in 454 Ranch Hand veterans and 570 air force veterans not involved in Ranch Hand (Wolfe *et al.*, 1995). For the Ranch Hand veterans, three categories of exposure were defined according to the extrapolated initial 2,3,7,8-TCDD level at the time of conception: background, low (≤ 110 ng/kg serum fat) and high (> 110 ng/kg serum fat). There was no significant elevation in risk for spontaneous abortions, stillbirths, major birth defects or delays in development after adjustment for several covariates. [The Working Group noted that, of the studies of pregnancy outcomes, this study presents the best-quality data with biological measurement of exposure and medical reports of reproductive outcomes. However, measurements were made up to 25 years after exposure ended and extrapolation to the initial 2,3,7,8-TCDD level at the time of conception may be imprecise. The power of the study for detecting an increase in the rate of specific birth defects is limited.]

A cross-sectional survey of the health status of Viet Nam veterans was conducted in a random sample of members of the American Legion who served in the United States armed forces during 1961–75 (Stellman *et al.*, 1988). Return rates of the self-administered mail questionnaire ranged from 52.5% to 64.1%. Among the respondents, 42% had served in south-east Asia. Of a total of 3046 live births and miscarriages, 2215 were fathered by veterans who served in south-east Asia; the potential exposure of these veterans to Agent Orange was ranked. The self-reported miscarriage rate among wives of Viet Nam veterans was 7.6% versus 5.8% among wives of non-Viet Nam veterans ($p < 0.01$) and increased with Agent Orange exposure index (7.3% for low exposure, 9.6% for high exposure). In a multivariate model, Agent Orange exposure remained a significant predictor of the rate of miscarriage. [The Working Group noted the low participation rate and low reliability of miscarriage information from male partners. The miscarriage rates reported (5.8% for non-Viet Nam veterans) are in fact very low.]

A case–control study (Erickson *et al.*, 1984) compared the Viet Nam experience of fathers of 7133 babies born with a serious structural congenital malformation with 4246 babies born without defects. Seventy per cent of eligible mothers and 56.3% of eligible fathers completed an interview. An 'exposure opportunity index' (EOI) score was assigned to Viet Nam veterans potentially exposed to Agent Orange. The risk of Viet Nam veterans fathering babies with birth defects was not increased (odds ratio, 1.0; 95% CI, 0.8–1.1); however, there was a slight increase for spina bifida (odds ratio, 1.1; $p > 0.05$), cleft lip with or without cleft palate (odds ratio, 1.1; $p < 0.05$) and congenital neoplasms with the higher EOI scores.

A case–control study was conducted in Australia including 8517 infants born with congenital anomalies between 1966 and 1979 and the same number of matched control children born without an anomaly (Donovan *et al.*, 1984). Viet Nam veterans comprised

127 fathers of cases and 123 fathers of controls, giving an overall odds ratio among veterans for fathering a malformed infant of 1.0 (95% CI, 0.8–1.3). No significant difference was observed for the numbers of discordant pairs for anomalies of the central nervous system, cardiac anomalies and chromosomal anomalies. No other group was more frequent among cases.

A case–control study was conducted in Boston Hospital for Women among 201 women admitted for spontaneous abortion (< 28 weeks of gestation) and 1119 controls (Aschengrau & Monson, 1989). The adjusted odds ratio for spontaneous abortion among Viet Nam veterans' wives was 0.9 (95% CI, 0.4–1.9) and 0.7 (95% CI, 0.5–1.2) for non-Viet Nam veterans' wives, using a group with no known military service as a reference. Odds ratios for early spontaneous abortion (< 13 weeks of gestation) were 1.2 (95% CI, 0.6–2.8) and 0.7 (95% CI, 0.4–1.2) for Viet Nam and non-Viet Nam veterans' wives, respectively.

In a second case–control study conducted at Boston Hospital (Aschengrau & Monson, 1990), 857 cases of congenital anomaly, 61 cases of stillbirth, 48 cases of neonatal death and 998 normal controls were identified. For Viet Nam veterans, the relative risk of fathering an infant with one or more major malformations was slightly elevated when compared with non-Viet Nam veterans (RR, 1.7; 95% CI, 0.8–3.5) and with non-veterans (RR, 2.2; 95% CI, 1.2–4.0). Fathers of babies with major malformations served more often in the Marine Corps and had longer durations of Viet Nam service. No particular type of defect was reported.

(ii) *Environmental studies*

The Seveso accident has provided a unique opportunity to evaluate the effect of environmental contamination on reproductive outcomes.

The Seveso Congenital Malformations Registry examined all live births and stillbirths that occurred from 1 January 1977 to 31 December 1982 to women who were residents of zones A, B and R and non-ABR (control area) in July 1976 (Mastroiacovo *et al.*, 1988). A total of 15 291 births occurred, out of which 742 malformed were identified (48.5/1000 births). Twenty-six births were recorded in the most highly contaminated area (Zone A); none had any major structural defect and two infants had mild defects. The frequencies of major defects observed in the area of low contamination (Zone B) or very low contamination (Zone R) were 29.9/1000 and 22.1/1000, respectively, compared to 27.7/1000 in the control area. No specific subgroup of malformations was seen in excess in the contaminated areas. The authors did not rule out the possibility that an increase in spontaneous abortions could have obscured an increase in malformations seen at birth. Due to the small number of exposed pregnancies, this study had insufficient power to show a low and specific teratogenic risk increase. Examination of 30 induced abortions which occurred just after the accident did not disclose any gross developmental abnormalities or chromosomal aberrations (Rehder *et al.*, 1978).

Spontaneous abortions which occurred in Cesano, Seveso, Desio, Meda and seven other cities in the area were identified by reports to the County Medical Officer and searching among admission/discharge hospital forms between July 1976 and December 1977 (Bisanti *et al.*, 1980). Rates of spontaneous abortion (per number of pregnancies,

excluding induced abortions) increased in the fourth trimester of 1976 (21.3%) in the 'most exposed' cities of Cesano and Seveso, compared with 13.9% in Desio and Meda, and 14.0% in the other seven cities ($p < 0.05$). It decreased in the following trimesters. When pregnancies were regrouped according to exposure zone, spontaneous abortion rates were always higher in Zone B than in other zones after the last trimester of 1976. [The Working Group noted that these results are hard to interpret because ascertainment bias cannot be ruled out. Inadequate details on the procedure are given to evaluate this potential problem.]

Data on births in Zone A between April 1977 (nine months after the accident) and December 1984 showed a significant excess of female births: 48 females versus 26 males ($p < 0.001$) (Mocarelli *et al.*, 1996). This ratio declined (64 females versus 60 males) in the years 1985 to 1994 and was no longer significant. Parents with an excess of female offspring were reported to have had a high 2,3,7,8-TCDD serum concentration in 1976. This observed change in the sex ratio in the years following the accident, if it is meaningful, could have several possible explanations (change in parental hormone concentrations, selective male miscarriages, mutations) in relation to high 2,3,7,8-TCDD exposure. [The Working Group noted that the number of reported births in Zone A between 1977 and 1984 (74) is much higher than the 26 births reported by Mastroiacovo *et al.* (1988) in Zone A between 1977 and 1982.]

Exposure to 2,3,7,8-TCDD occurred in eastern Missouri, after contaminated oil was sprayed for dust control in 1971. A total of 402 births (i.e., any product of conception with a gestational age of 20 weeks or longer) were identified between 1972 and 1982 among women who had potential exposure to PCDDs, based on proximity of residence to a location of known contamination (Stockbauer *et al.*, 1988); 804 unexposed births among Missouri residents were selected for comparison. Increased but not statistically significant risk ratios were observed for infant, fetal or perinatal death and low birth weight. Birth defects were not increased; however, the power of this study, as computed by the authors, for detecting a doubling in the risk of total birth defects was low at 34%.

Four ecological studies have evaluated the relationship between reproductive outcomes and the potential for environmental exposure to 2,3,7,8-TCDD. These studies have analysed correlations between annual rates of birth defects and usage of 2,4,5-T in corresponding geographical areas having different levels of aerial spraying. As in all ecological studies of this kind, it is not possible to extrapolate results to an individual level. These studies failed to show consistent patterns of birth defects or malformations (Field & Kerr, 1979; Nelson *et al.*, 1979; Thomas, 1980; Hanify *et al.*, 1981).

A summary of methods and findings of several investigations conducted in Viet Nam by Vietnamese researchers, on the reproductive effects of Agent Orange sprayings, was published by Constable and Hatch (1985) and Sterling and Arundel (1986). All these studies showed evidence of an increased risk for adverse reproductive outcomes such as abortions, stillbirths, congenital malformations (especially of the central nervous system and oral clefts) or molar pregnancies in relation to Agent Orange sprayings, both among southern Vietnamese populations and to wives' of veterans from the north. Although many of the results are striking, details on the methods used are lacking and there is no

reassurance that potential selection bias, reporting bias or confounding which are major problems in the type of studies presented, were avoided.

A case–control study on gestational trophoblastic disease (molar pregnancy or choriocarcinoma) was conducted in 1990, in the Obstetrics and Gynaecology Hospital in Ho Chi Minh City (Ha *et al.*, 1996). A total of 87 cases and 87 surgical controls matched for age and area of residence (two strata: Ho Chi Minh City, province) were interviewed at the hospital. All had been married. Cumulative exposure to Agent Orange was estimated from history of residence since the sprayings and from data on spraying missions from the United States military records. There was no difference in past exposure to Agent Orange between cases and controls. Negative findings could be explained by misclassification of exposures or by the decrease in exposure since the end of the sprayings. The 2,3,7,8-TCDD levels however remain elevated today in Vietnamese blood and tissue (Schecter *et al.*, 1995).

(iii) *Occupational studies*

A study was conducted among wives of chlorophenol production workers who were potentially exposed to PCDDs and unexposed controls (Townsend *et al.*, 1982). Information on reproductive outcomes was obtained by interview from 370 and 345 wives of exposed and unexposed workers, respectively: 737 pregnancies occurred after potential paternal exposure, whereas 2031 pregnancies were considered to be unexposed. Odds ratios were 1.1 (95% CI, 0.5–2.1) for stillbirths, 1.0 (95% CI, 0.8–1.4) for spontaneous abortions and 0.9 (95% CI, 0.5–1.4) for congenital malformations. No specific pattern of malformations or increasing risk of unfavourable outcome with length of paternal exposure (12 months or less, more than 12 months) was seen. [The Working Group noted that this study assumed the effect of paternal exposure to PCDDs to be an irreparable event, so that every subsequent pregnancy was considered to be exposed, whatever the time since last exposure. No figures were presented according to this last variable and no detail was given on the actual level of exposure to PCDDs in the plant.]

In New Zealand, the rates of various outcomes among wives of men who sprayed 2,4,5-T at some time during the two-year period preceding birth outcome (486 pregnancies) were compared with rates among wives of agricultural contractors who did not spray pesticides during the corresponding period (401 pregnancies) (Smith *et al.*, 1982b). Relative risks were 0.9 (90% CI, 0.6–1.3) for miscarriage and 1.2 (90% CI 0.6-2.5) for congenital defect. Three stillbirths (7.0 /1000 live births) were reported in the exposed group and none in the unexposed group. The authors concluded, however, that small increases in risk, especially for individual defects, could not be ruled out. The nine pesticide applicators had been chosen because they had the longest durations of exposure to phenoxy herbicides. The average exposure of the cohort would have therefore been much lower.

Information on reproductive factors and birth defects was obtained from 189 PCDD-exposed herbicide production workers in West Virginia, USA, and 155 unexposed controls, but was not confirmed by physician or hospital records. Among 655 pregnancies in the exposed population and 429 in the controls, the rates of miscarriages,

stillbirths or birth defects and the patterns of birth defects were similar (Suskind & Hertzberg, 1984).

4.4.2 *Experimental systems*

Developmental toxicity has been observed at lower 2,3,7,8-TCDD exposure levels than those producing male and female adult reproductive toxicity in various animals.

(*a*) *Developmental effects*

(i) *General embryo- and fetotoxicity*

There are numerous reviews concerning aspects of the developmental effects of PCDDs and related compounds in a range of vertebrate species (Couture *et al.*, 1990a; Birnbaum, 1991; Peterson *et al.*, 1993; Battershill, 1994; Sauer *et al.*, 1994; Birnbaum, 1995b; Brouwer *et al.*, 1995; Lindstrom *et al.*, 1995; Abbott, 1996; Birnbaum, 1996; Birnbaum & Abbott, 1997).

At dose levels below those where overt toxicity occurred in the dam, growth retardation was detected in the offspring. Thymic and splenic atrophy were also noted. Subcutaneous oedema was observed in several species and gastrointestinal haemorrhage in others. Higher levels of exposure resulted in fetal deaths and resorptions. One of the key observations has been that the dose levels associated with fetal toxicity are similar across species, regardless of the dose associated with adult lethality. For example, Han/Wistar (Kuopio) rats, that are uniquely resistant to 2,3,7,8-TCDD-induced lethality in the adult, exhibit developmental toxicity similar to that of Long-Evans (Turku) rats that are extremely susceptible to the lethal effects of 2,3,7,8-TCDD (Huuskonen *et al.*, 1994). Fetotoxicity occurs after approximately the same dose to the dam of both rats and hamsters, although the adult LD_{50} varies by over a factor of 100 (Olson *et al.*, 1990).

(ii) *Teratogenic effects*

Cleft palate and hydronephrosis

Exposure of pregnant mice to 2,3,7,8-TCDD and related compounds caused a pathognomonic syndrome of effects in the offspring at doses (e.g., a subcutaneous dose of 0.3 µg/kg/day during gestation days 6–15) that result in no overt toxicity to the mother (Couture *et al.*, 1990a). Either divided doses throughout organogenesis or a single higher dose resulted in a similar spectrum of structural malformations in the pups consisting of clefting of the secondary palate and hydronephrosis. The peak period of sensitivity for induction of palatal clefting was gestational days 11–12; exposure on gestational day 14 or later cannot induce cleft palate since fusion has already occurred. A clear window of sensitivity was not seen for hydronephrosis. The same dose (3–24 pg/kg) was associated with an identical incidence and severity of the renal lesion whenever treatment occurred during gestational days 6–12 (Couture *et al.*, 1990b). Hydronephrosis also resulted from lactational exposure only (dose 3–12 µg/kg given on gestation day 6), although this was less efficient than transplacental exposure (Couture-Haws *et al.*, 1991). Hydronephrosis was also induced at doses below those which induced palatal clefting. At low doses, mild hydronephrosis was observed, predominantly in the right kidney. At higher doses, both

the incidence and severity of response increased in both kidneys. Hydronephrosis was often accompanied by hydroureter. The cause of both conditions was inappropriate proliferation of the ureteric epithelium resulting in a narrowing and blockage of the lumen (Abbott *et al.*, 1987). Urine produced by the fetal kidney was blocked from being eliminated, leading to destruction of the renal parenchyma.

Inappropriate epithelial cell proliferation also appears to play a major role in palatal clefting. 2,3,7,8-TCDD blocked fusion of the opposing palatal shelves in mice (Pratt *et al.*, 1984), although the shelves did make contact. During normal development, the medial epithelium transforms into mesenchyme (Fitchett & Hay, 1989; Shuler *et al.*, 1992). This epithelial–mesenchymal differentiation was blocked by 2,3,7,8-TCDD. The medial epithelium continued to proliferate and under the influence of 2,3,7,8-TCDD, transformed into an oral-like stratified squamous epithelium complete with desmosomes, tonofilaments and keratins (Abbott & Birnbaum, 1989a).

No species other than the mouse shows similar responses at non-toxic doses (reviewed in Couture *et al.*, 1990a). For example, cleft palate has been reported in rats exposed during organogenesis, but the dose needed to produce clefting also results in fetotoxicity, fetal wastage and maternal toxicity. Although increased incidence of hydronephrosis has been reported, in no case was the incidence statistically significant. Renal abnormalities have been seen in hamsters, but these also occurred only at doses where fetotoxicity was evident.

Although the majority of studies examining the mechanism of PCDD teratogenicity in mice have been conducted with 2,3,7,8-TCDD, there have been many investigations of the induction of cleft palate and hydronephrosis by other PCDD-like compounds (see cited reviews). Several higher chlorinated PCDDs cause the same spectrum of birth defects as 2,3,7,8-TCDD (Birnbaum, 1991).

Other developmental effects

Gastrointestinal haemorrhage has been observed in guinea-pigs and rats. Prenatal exposure of mice to either 2,3,7,8-TCDD or 2,3,4,7,8-PeCDF resulted in haemorrhage of embryonic blood into the maternal circulation because of rupture of the embryo-maternal vascular barrier (Khera, 1992).

While the majority of experimental studies with PCDDs have focused on exposure during organogenesis, treatment earlier in gestation has been noted to result in fetotoxicity (Giavini *et al.*, 1982). In addition, however, recent studies have indicated that PCDDs can accelerate differentiation of the preimplantation embryo (Blankenship *et al.*, 1993). The Ah receptor has been demonstrated to be present in the developing embryo from the eight-cell stage (Peters & Wiley, 1995).

The developing teeth also appear to be a target for PCDD-induced effects. Neonatal exposure of BALB/c mice to 150 µg/kg 2,3,7,8-TCDD by intraperitoneal injection leads to accelerated tooth eruption in mice (Madhukar *et al.*, 1984). Impaired dentin and enamel formation has been observed in the continuously growing incisors of young male Han/Wistar rats (1000 µg/kg intraperitoneal injection) (Alaluusua *et al.*, 1993) following 2,3,7,8-TCDD exposure.

The developing immune system is also a target for PCDDs (reviewed in Birnbaum, 1995a). Exposure during organogenesis results in lymphoid atrophy. Mice fail to survive because of the induction of cleft palate. To avoid this effect, mice can be treated on gestation day 14, when the palate has already fused. Under these conditions, 2,3,7,8-TCDD accelerated the differentiation of prothymocytes in the bone marrow (Fine *et al.*, 1989, 1990a,b). This suggests that thymic atrophy may be a result of altered differentiation of cells from the bone marrow. The ratio of T-lymphocyte subsets is altered in the pups as well. Holladay *et al.* (1991) also observed changes in the surface markers of lymphocytes following prenatal exposure. Exposure of rats on gestational day 15, which is developmentally slightly earlier than gestation day 14 in the mouse, leads to similar changes in T-cell subset ratios in the rat offspring (Gehrs & Smialowicz, 1994). This is associated with permanent suppression of delayed-type hypersensitivity in the rat offspring (Gehrs *et al.*, 1995), an immunological measure highly correlated with altered host resistance and increased disease sensitivity. The dose levels associated with permanent immune suppression in the developing rat are even lower than those needed in the prenatally exposed mouse. This is in contrast to the apparent resistance in adult rats to immunosuppression following exposure to PCDDs (Smialowicz *et al.*, 1994).

(iii) *Role of growth factors and hormones*

Further information on this topic is presented in Sections 4.3 and 4.6.

Alterations in proliferation and differentiation play a role in cleft palate, hydronephrosis and immunological developmental effects. While reactive oxygen species may participate in some of the teratogenic effects of 2,3,7,8-TCDD (Hassoun *et al.*, 1995), changes in various growth factors and receptors are correlated with palatal clefting. During normal development, levels of EGF decrease and TGFα increases during palatal fusion. 2,3,7,8-TCDD has little effect on EGF, but does block the increase in TGFα in the developing palate (Abbott & Birnbaum, 1990a, 1991). The EGF receptor, which is normally present in the epithelium, decreases during palatal fusion (Abbott & Birnbaum, 1989a). This decrease is blocked by 2,3,7,8-TCDD (Abbott & Birnbaum, 1989b; Abbott *et al.*, 1994b). Whether this is a compensatory increase in the receptor level due to the decrease in the presence of its ligands remains to be determined. However, these changes probably play a role in the continued proliferation of the medial epithelium resulting in cleft palate due to 2,3,7,8-TCDD. Several members of the TGFβ family also respond to 2,3,7,8-TCDD. While these growth factors frequently inhibit epithelial cell proliferation, they are often stimulatory to cells of mesenchymal origin. 2,3,7,8-TCDD causes an increase in TGFβ1 and TGFβ2 in the medial epithelium (Abbott & Birnbaum, 1990a, 1991; Abbott *et al.*, 1994b). These growth factors also increase in the underlying mesenchyme in response to 2,3,7,8-TCDD.

Cleft palate can also be induced by exposure to glucocorticoids; however, the mechanism is distinct from that of 2,3,7,8-TCDD. Glucocorticoids cause growth inhibition of the palatal shelves, resulting in small shelves which fail to make contact and thus cannot fuse (Pratt, 1985). Co-treatment of mice with hydrocortisone and 2,3,7,8-TCDD leads to a synergistic increase in the incidence of cleft palate (Birnbaum *et al.*, 1986). The size of the cleft suggests that growth inhibition plays a major role in this clefting. The changes

in EGF, TGFα and β and the EGF receptor also suggest that the effects resemble those seen following exposure to hydrocortisone (Abbott, 1995). Sensitivity to glucocorticoid-induced cleft palate is related to the numbers of glucocorticoid receptors. 2,3,7,8-TCDD exposure causes an increase in the numbers of glucocorticoid receptors in the developing palate (Abbott *et al.*, 1994c), suggesting that the synergism of hydrocortisone-induced cleft palate by 2,3,7,8-TCDD may be associated with an increase in the number of steroid receptors. However, there is an additional level of complexity in this interaction, since hydrocortisone has been shown to increase the expression of the Ah receptor in the palate (Abbott *et al.*, 1994c). The Ah receptor is required for 2,3,7,8-TCDD induction of cleft palate, as well as for all other well studied responses (reviewed in Birnbaum, 1994a). 2,3,7,8-TCDD causes a decrease in the level of Ah receptor expression in the palate at the time of fusion (Abbott *et al.*, 1994d). Hydrocortisone blocks this decrease, suggesting that the synergistic induction of cleft palate by 2,3,7,8-TCDD and glucocorticoids may involve interactions of multiple receptor and growth factor systems.

2,3,7,8-TCDD can also interact synergistically with retinoic acid in the induction of cleft palate (Birnbaum *et al.*, 1989). In contrast to the interaction with hydrocortisone, the retinoid/2,3,7,8-TCDD combination results in effects on growth factors resembling those seen with 2,3,7,8-TCDD (Abbott & Birnbaum, 1989b). It is not yet known whether retinoic acid up-regulates the Ah receptor in the palate, as has been demonstrated for the glucocorticoid receptor. However, 2,3,7,8-TCDD exposure can block a retinoid-induced increase in the retinoic acid receptor (Weston *et al.*, 1995) in cultured mouse embryonic palatal mesenchyme cells. Wanner and co-workers (1995) have also shown that retinoic acid can suppress the differentiation-induced increase in the Ah receptor in cultured keratinocytes. Thus, there appears to be potential for cross-talk between the Ah receptor and receptors in the steroid family.

While the majority of studies examining 2,3,7,8-TCDD-induced changes in growth factor and hormone receptors have concentrated on the developing palate, changes have also been observed in the developing urinary tract. An increase in EGF receptors in the ureteric epithelium in response to PCDDs is associated with the induction of hydronephrosis (Abbott & Birnbaum, 1990b). This is similar to that observed in the enhanced proliferation of the medial epithelium in the palate. However, while PCDDs decreased Ah receptor expression in the developing palate at the time of fusion (Abbott *et al.*, 1994d), there were no detectable changes in the levels of Ah receptor in the developing urinary tract at the same time (Bryant *et al.*, 1995). 2,3,7,8-TCDD exposure on gestation day 10 leads to reduced levels of EGF in the ureteric epithelial cells, while TGFα remains unchanged (Bryant *et al.*, 1996). No other growth factors or receptors have been examined in this target tissue. However, neither retinoids nor glucocorticoids cause hydronephrosis.

(*b*) *Functional developmental toxicity*

(i) *Male reproductive system effects*

In a series of studies, Mably *et al.* (1992a,b,c) exposed pregnant Holtzman rats on gestational day 15 to 2,3,7,8-TCDD at oral doses ranging from 0.064 to 1.0 μg/kg. They focused on the effects upon male offspring because of the decrease in circulating

androgens observed in highly exposed adult male rats (Moore *et al.*, 1985). There was no effect on anogenital distance in the PCDD-treated pups at birth and on postnatal day 4 when corrected for body weight differences with the controls. At all times when they were measured (32–120 days of age), the weights of the accessory sex organs of the prenatally exposed male offspring were decreased in a dose-related fashion and both testicular and epididymal sperm counts were permanently reduced. The decrease in epididymal sperm count was observed at the lowest maternal dose tested (0.064 μg/kg) on day 120 and at most earlier times. When these male rats were mated around 70 and 120 days of age with control females, there was no significant effect upon fertility or survival and growth of the offspring.

Male sexual behaviour was also altered. The males took longer to mount receptive females, had more difficulty in achieving intromission and took more thrusts to achieve ejaculation (Gray *et al.*, 1995a). Mably *et al.* (1992c) reported a decrease in circulating androgen levels in the male offspring at birth. However, later studies from the same laboratory were not able to replicate these findings (Roman *et al.*, 1995). Feminization of sexual behaviour was also reported. When male pups prenatally exposed to PCDDs were treated with oestrogens and progesterone following castration, they demonstrated an increased lordotic response as compared to controls. The sexually dimorphic nuclei of the preoptic area of the hypothalamus were also examined to see if there was 'demasculinization', but no change was observed (Bjerke *et al.*, 1994).

Effects due to prenatal exposure to 2,3,7,8-TCDD were investigated in another laboratory (reviewed in Gray *et al.*, 1995b) using Long-Evans rats and Syrian hamsters. Both male and female offspring were studied. Rats were dosed with 1 μg/kg bw 2,3,7,8-TCDD by gastric instillation on gestational day 8 or 15 in order to determine if certain sensitive developmental effects would have been missed by exposing only towards the end of organogenesis. Hamsters were treated on gestational day 11, which is developmentally similar to gestational day 15 in the rat (Gray & Ostby, 1995; Gray *et al.*, 1995a). Dose–response studies were also conducted in rats, with doses of 0.8, 0.2, and 0.05 μg/kg maternal weight 2,3,7,8-TCDD (Gray *et al.*, 1995b). In general, the results obtained with Long-Evans rats were similar to those with Holtzman rats described above: prenatal exposure resulted in decreased sperm counts, decreased accessory sex organ weights and altered male mating behaviour. However, there were several significant differences. The decrease in anogenital distance was associated with a decrease in body weight (Gray *et al.*, 1995a). While the decrease in testicular sperm was statistically significant, it was quite small (~6%) and unlikely to explain the much larger decrease in epididymal (~35%) and ejaculated (~60%) sperm counts. No feminization of sexual behaviour was seen, and there was no reduction in serum testosterone level or in ventral prostate weight.

Although the male rat pups in both of these studies appeared to become aroused as readily as controls, they had more difficulty in achieving intromission and ejaculation. Whether this was associated with subtle changes in the penis remains to be determined. However, Bjerke & Peterson (1994) have observed a decrease in the weight of the glans penis. Gray *et al.* (1995a), in confirmation of the results of Roman *et al.* (1995), failed to

observed any change in androgen status, including the lack of change in the number or affinity of androgen receptors. However, the two studies contrast in that no feminization of behaviour was observed in the Long-Evans rats (Gray *et al.*, 1995b). In the male hamster, there was no effect on the weight of testes or on serum testosterone level, although there was a reduction in epididymal and ejaculated sperm (Gray *et al.*, 1995a). The delay in puberty, however, as measured by preputial separation, appears to be a consistent finding among the two strains of rats and the hamster.

A significant decrease in epididymal sperm count was seen in the Long-Evans rats at a maternal dose of 0.2 μg/kg 2,3,7,8-TCDD and the ejaculated sperm count was reduced by 25% at a 0.05 μg/kg dose (Gray *et al.*, 1995b). Few other adverse effects were observed in the male Long-Evans offspring below 0.2 μg/kg. Premature eye opening, an effect previously seen in mice (Madhukar *et al.*, 1984), was seen at 0.05 μg/kg. Prenatal exposure was more effective on gestational day 15 than on gestational day 8 in terms of male reproductive effects. This suggests that the window of sensitivity for the male effects occurs late in organogenesis. Cross-fostering studies by Bjerke and Peterson (1994), and earlier studies by Khera and Ruddick (1973), indicated that all of the male reproductive effects can be induced prenatally. The only exception is the feminization of mating behaviour, already noted as a weak, if not species- and strain-specific, response (Brouwer *et al.*,1995).

Exposure of Syrian hamsters on gestational day 11 to 2 μg/kg 2,3,7,8-TCDD (a dose over 1000 times lower than the adult LD_{50}) resulted in effects similar to those observed in rats and, in spite of a decrease in seminal vesicle weight, there were no effects on male sexual behaviour or on testis weight. Puberty was delayed, and epididymal and ejaculated sperm count were permanently decreased (Gray *et al.*, 1995a,b).

(ii) *Female reproductive system effects*

Multiple developmental effects were observed in female rats prenatally exposed to 1 μg/kg 2,3,7,8-TCDD on gestation day 8 or gestation day 15 (Gray & Ostby, 1995) and in female hamsters prenatally exposed to 2 μg/kg 2,3,7,8-TCDD on gestation day 11 (Gray *et al.*, 1995b). Vaginal opening was delayed in both species. In rats, two structural abnormalities were seen: a persistent vaginal thread across the normal vaginal opening, and clefting of the external genitalia in the female pups. In young adult female rats, there was no change in oestrous cyclicity, suggesting a normal hormonal profile. The changes in the external genitalia occurred at a higher incidence following exposure on gestational day 15 as compared to gestational day 8. In contrast, exposure early in organogenesis was associated with premature reproductive senescence in the female pups, many of whom stopped oestrous cycling before six months of age.

Prenatal exposure of hamsters resulted in similar effects (Gray *et al.*, 1995b). In some of the hamster pups, vaginal opening could not be easily detected; it was delayed in all of the others. Clefting of the external genitalia was present in all of the female hamster pups. Fertility was also decreased in the female hamster offspring, probably due to the structural problems in the external genitalia, since no effect on cyclicity was observed.

(iii) *Central nervous system effects*

Although most of the effects of prenatal exposure to 2,3,7,8-TCDD appear to directly target the developing genitourinary system, there is some evidence for involvement of the central nervous system. Gordon *et al.* (1995, 1996) have demonstrated that prenatal 2,3,7,8-TCDD exposure permanently depresses core body temperature in both rats and hamsters. Rats were examined at 18 months of age following prenatal exposure on gestational day 15 to 1 μg/kg 2,3,7,8-TCDD and hamsters at one year of age following exposure of the dam to 2 μg/kg on gestational day 11. The set point for body temperature is in the hypothalamus. The treated animals can still respond to a cold or heat stress by raising or lowering their body temperature as required, but their body temperature is always lower than that of controls.

(iv) *Persistence of effects*

A key finding of these functional developmental toxicity studies is the permanent nature of the responses following prenatal exposure. This is in contrast to some of the biochemical responses, such as induction of hepatic cytochrome P450 content and EROD activity, which returned to control levels by 120 days of age (Mably *et al.*, 1992a).

(v) *Hormonal effects*

Many of the developmental effects of 2,3,7,8-TCDD, such as clefting of the external genitalia, resemble those seen with high doses of oestrogens (Vannier & Raynaud, 1980). Endometriosis also requires the presence of oestrogens and, while low doses of 2,3,7,8-TCDD (Cummings *et al.*, 1996) and related compounds (Johnson *et al.*, 1996) promote the growth of endometriotic lesions in rodent models, high doses which cause ovarian atrophy, and thus reduced levels of oestrogens, are less effective.

Prenatal exposure to 0.025 or 0.1 μg/kg per day 2,3,7,8-TCDD on gestational days 10 to 16 of pregnant rats results in moderately depressed plasma T4 levels at weaning (Seo *et al.*, 1995). While the pups had lowered T4 levels, no effects on thyroid status were observed in the dams. This may have been associated with an increase in peripheral T4 metabolism in the pups (Morse *et al.*, 1993).

(vi) *Neurobehavioural effects*

Hearing deficits in rats, due to an increase in the auditory threshold, can be induced by exposure to a single dose of 2,3,7,8-TCDD of 0.3, 1.3 or 10 μg/kg on gestational day 19 (Goldey *et al.*, 1995, 1996). This response can be partially blocked by the addition of exogenous thyroxine, suggesting a role for a depression in circulating thyroid hormones in this response. Late gestational and lactational exposure of rats to 2,3,7,8-TCDD can result in changes in locomotor activity and rearing behaviour (Thiel *et al.*, 1994). Prenatal and lactational exposure of rhesus monkeys to 2,3,7,8-TCDD also causes changes in object learning (Schantz & Bowman, 1989).

(*c*) *Reproductive effects*

Reproductive effects have been reviewed by Allen *et al.* (1979), Morrissey and Schwetz (1989) and Theobald and Peterson (1994).

Multi-generation studies have been used to assess reproductive effects in both sexes. In a three-generation study in Sprague-Dawley rats, Murray *et al.* (1979) noted that exposure to a diet providing doses of up to 0.1 μg/kg bw per day 2,3,7,8-TCDD resulted in impaired reproductive capacity in rats as indicated by reductions in fertility and litter size. While they reported a maximal no-effect level of 1 ng/kg bw per day, a different method of statistical analysis indicated a lowest observed effect level of 1 ng/kg bw per day (Nisbet & Paxton, 1982). [The Working Group noted that no sex ratio data were provided.]

(i) *Male reproductive system*

2,3,7,8-TCDD causes a loss of germ cells, degeneration of both spermatocytes and mature spermatozoa within the seminiferous tubules, and a reduction in the number of tubules with mature spermatozoa. The lowest observed effect level for decreased spermatogenesis in Sprague-Dawley rats was 1 μg/kg per day in a 13-week study (Kociba *et al.*, 1976). This dose was associated with a depression in body weight gain and food consumption, indicating that effects on spermatogenesis occur only under conditions of overt toxicity. It was suggested that these adverse effects on the male reproductive system might be due to decreases in plasma testosterone and dihydrotestosterone concentration. A single dose of 15 μg/kg 2,3,7,8-TCDD caused a significant decrease in circulating testosterone levels within one day (Moore *et al.*, 1985). The decrease in circulating androgens appears to be due to decreased testicular responsiveness to LH and enhanced sensitivity of the pituitary to feedback inhibition by androgens and oestrogens (Moore *et al.*, 1989; Bookstaff *et al.*, 1990a,b; Kleeman *et al.*, 1990; Moore *et al.*, 1991).

The Leydig cell is the primary site of steroidogenesis in the testis. At intraperitoneal 2,3,7,8-TCDD doses of 12.5–50.0 μg/kg bw, there was a dose-dependent reduction in Leydig cell volume in Harlan/Sprague-Dawley rats, due to both fewer cells and reduced size of the individual cells (Johnson *et al.*, 1992c, 1994). This could play a role in the androgenic deficiency observed. Wilker *et al.* (1995) have shown that the effects on the Leydig cells can be prevented by treatment with human chorionic gonadotropic hormone (hCG) which had been previously shown to block the decrease in circulating testosterone levels (Ruangwies *et al.*, 1991).

Under normal conditions, reduction in circulating androgens would cause an increase in plasma LH, leading to a compensatory increase in testosterone biosynthesis by the Leydig cells. In adult male rats treated with toxic doses of 2,3,7,8-TCDD (50 μg/kg bw), in which the serum levels of androgens are decreased, the pituitary feedback regulation is impaired (Moore *et al.*, 1989; Bookstaff *et al.*, 1990a,b; Kleeman *et al.*, 1990).

(ii) *Female reproductive toxicity*

Treatment of the adult female with PCDDs can lead to reduced fertility, reduced litter size (Murray *et al.*, 1979), effects on ovarian cycling and overt ovarian toxicity. In adult rats, higher levels of exposure (1 μg/kg per day for 13 weeks) led to anovulation and suppression of the oestrous cycle (Kociba *et al.*, 1976).

Several reproductive studies have also been conducted in rhesus monkeys exposed to 2,3,7,8-TCDD in the diet (Allen *et al.*, 1977, 1979; Barsotti *et al.*, 1979). Females

exposed to 500 ng/kg in the diet for nine months showed overt signs of maternal toxicity, including death. Only one of eight monkeys at this dose was able to carry an infant to term. In contrast to the higher dose, no overt effect on maternal health was seen at 50 ng/kg in the diet (Allen *et al.*, 1979).

When female rhesus monkeys were exposed to 5 or 25 ng/kg of 2,3,7,8-TCDD in the diet (Bowman *et al.*, 1989a; Schantz & Bowman, 1989), reproductive performance was not impaired in the low-dose group, in which seven out of eight monkeys gave birth to live offspring. In contrast, only one of eight females gave birth to a live infant in the 25-ng/kg group. These studies suggest that fetotoxicity can occur at doses below those causing maternal toxicity. Exposure of monkeys during early pregnancy to single or divided doses of 1 μg/kg bw 2,3,7,8-TCDD resulted in only three of 16 monkeys having live offspring, with most of the fetal loss being due to abortions (McNulty, 1984, 1985).

Anovulation and suppression of the oestrous cycle indicate ovarian dysfunction, and occur in both adult rats and monkeys following high doses of 2,3,7,8-TCDD causing overt toxicity (Kociba *et al.*, 1976; Allen *et al.*, 1979; Barsotti *et al.*, 1979). Li *et al.* (1995b) demonstrated that exposure of adult female Sprague-Dawley rats to a single dose of 10 μg/kg 2,3,7,8-TCDD resulted in a decrease in the number of ova ovulated per female, a decrease in the number of females ovulating and an increase in time spent in oestrous concomitant with a decrease in pro-oestrous and dioestrous. These effects were dose-dependent (Li *et al.*, 1995c), but were statistically significant only at doses that caused a loss in body weight (≥ 10 μg/kg).

Rhesus monkeys were exposed to 0, 5, or 25 ng/kg 2,3,7,8-TCDD in their diet for four years and then held for up to an additional 10 years, when laparascopic surgery was performed. Both the incidence and severity of endometriosis were increased in a dose-dependent manner (Rier *et al.*, 1993). These findings have been supported by results from surgically induced endometriosis in both rats and mice (Cummings *et al.*, 1996). In both species, treatment with 2,3,7,8-TCDD (1.3 or 10 μg/kg bw given five times over a 16-week period) led to an increase in the size of endometriotic cysts in a dose-dependent fashion. 1,3,6,8-TCDD (2 or 20 mg/kg bw) did not enhance the growth of the endometriotic cysts (Johnson *et al.*, 1996). The highest dose of 2,3,7,8-TCDD (five times 10 μg/kg) was associated with ovarian atrophy and a decrease in the endometriotic response. This is not unexpected, since promotion of endometriosis requires oestrogen (Cummings & Metcalf, 1995). Studies with rhesus monkeys demonstrated that high levels of exposure to 2,3,7,8-TCDD result in a reduction in serum 17β-oestradiol levels (Barsotti *et al.*, 1979). This could be due to increased metabolism of oestrogen due to induction of hepatic microsomal enzymes.

Thus, while oestrogen is required for PCDD-induced promotion of endometriosis, the decrease in circulating oestradiol levels suggests an antioestrogenic effect of 2,3,7,8-TCDD. However, no effects on circulating oestradiol levels were seen in pregnant rats treated with 2,3,7,8-TCDD (Shiverick & Muther, 1983) or in mice (DeVito *et al.*, 1992). Another possibility is a direct effect on gonadal tissue, as suggested more recently by Li *et al.* (1995b,c). A third possibility is an alteration in target tissue responsiveness.

4.5 Genetic and related effects (see also Appendix 3 and **Table 64** for references)

Genetic effects of 2,3,7,8-TCDD have been reviewed (Kociba, 1984; Shu *et al.*, 1987). Indirect genetic effects are discussed in Section 4.6.

4.5.1 *Humans*

In 19 women [age unspecified] exposed to 2,3,7,8-TCDD after the Seveso accident and 16 control women aged 17–37 years, abortions were induced between weeks 8 and 16 of gestation in the exposed group and between weeks 8 and 13 in the control group. Cytogenetic studies were carried out on maternal peripheral lymphocytes, placental and umbilical cord tissues and fetal tissues. Significant increases (16.8% versus 5.5% and 1.51% versus 0.96%, respectively) in the frequencies of aberrant cells and in the average number of aberrations per damaged cell were found only in the fetal tissues in the group of exposed pregnancies (Tenchini *et al.*, 1983). [The Working Group noted the absence of differences in other tissues and of data on 2,3,7,8-TCDD concentrations in the fetuses; there was no indication of the zone in which the parents lived.]

In 27 male subjects aged 54–88 years (average age, 65.3 years) who were potentially exposed to 2,3,7,8-TCDD after the BASF accident in 1953 (see Section 1.3.1(*a*)(i)) and with a current blood concentration of 2,3,7,8-TCDD on a lipid basis exceeding 40 ng/kg, and 28 controls aged 53–93 years (average age, 65.1 years) without known exposure to 2,3,7,8-TCDD and with blood concentrations of less than 10 ng/kg, no statistically significant difference was found in the frequencies of chromosomal aberrations or sister chromatid exchange in peripheral lymphocytes in 1991 (Zober *et al.*, 1993).

4.5.2 *Experimental systems*

2,7-DCDD did not induce mutations in *Salmonella typhimurium* in either the presence or absence of an exogenous metabolic system in the only reported study. 2,7-DCDD did not transform C3H 10T1/2 cells or initiate transformation in these cells subsequently treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Continuous exposure to 2,7-DCDD did not promote cell transformation when the same cells were initiated by treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).

2,3,7,8-TCDD did not induce mutations in *S. typhimurium* in either the presence or absence of an exogenous metabolic system.

2,3,7,8-TCDD produced conflicting results in tests for mutation in mouse lymphoma cells.

Single treatments with 2,3,7,8-TCDD did not induce transformation of C3H 10T1/2 cells or initiate the process of transformation in cultures subsequently exposed to TPA, whereas continuous treatment with low concentrations enhanced transformation of the same cells pretreated with MNNG. Neoplastic transformation was also observed in human epidermal keratinocytes immortalized by adenovirus 12–simian virus 40 (Ad12–SV40) but not in primary human epithelial keratinocytes.

Inhibition of gap-junctional intercellular communication was observed in mouse hepatoma cells and rat hepatocytes in primary culture, but not in Chinese hamster cells or

Table 64. Genetic and related effects of PCDDs

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
2,7-Dichlorodibenzo-*para*-dioxin				
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	128	Mortelmans *et al.* (1984)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	128	Mortelmans *et al.* (1984)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	128	Mortelmans *et al.* (1984)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	128	Mortelmans *et al.* (1984)
TCM, Cell transformation, C3H 10T1/2 mouse cells	–[f,g]	NT	5	Abernethy & Boreiko (1987)
2,3,7,8-TCDD				
SA0, *Salmonella typhimurium* TA100, reverse mutation	NT	–	10	Geiger & Neal (1981)
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	385	Mortelmans *et al.* (1984)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	NT	–	10	Geiger & Neal (1981)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	385	Mortelmans *et al.* (1984)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	385	Mortelmans *et al.* (1984)
SA7, *Salmonella typhimurium* TA1537, reverse mutation	–	–	10	Geiger & Neal (1981)
SA8, *Salmonella typhimurium* TA1538, reverse mutation	0	–	10	Geiger & Neal (1981)
SA9, *Salmonella typhimurium* TA98, reverse mutation	0	–	10	Geiger & Neal (1981)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	385	Mortelmans *et al.* (1984)

Table 64 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
2,3,7,8-TCDD (contd)				
G51, Gene mutation, mouse lymphoma L5178Y cells, methotrexate or thymidine selection	+		0.1	Rogers *et al.* (1982)
G51, Gene mutation, mouse lymphoma L5178Y cells, thioguanine selection	(+)		0.5	Rogers *et al.* (1982)
G51, Gene mutation, mouse lymphoma L5178Y cells, ouabain or AraC selection	–		0.5	Rogers *et al.* (1982)
G5T, Gene mutation, mouse lymphoma L5178Y cells, *tk* locus	–	–	1	McGregor *et al.* (1991)
TCM, Cell transformation, C3H 10T1/2 mouse cells	–	NT	1.6	Abernethy *et al.* (1985)
UIH, Unscheduled DNA synthesis, human mammary epithelial cells *in vitro*	–	NT	0.003	Eldridge *et al.* (1992)
SHL, Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	0.0004	Nagayama *et al.* (1995a)
MIH, Micronucleus test, human lymphocytes *in vitro*	+	NT	0.0004	Nagayama *et al.* (1993)
TIH, Cell transformation, immortalized human keratinocytes *in vitro*	+	NT	0.00004	Yang *et al.* (1992)
TIH, Cell transformation, primary human keratinocytes *in vitro*	–	NT	0.001	Yang *et al.* (1992)
HMA, Host-mediated assay, peritoneal macrophages in mouse	+[c]		0.0004 × 1 ip	Massa *et al.* (1992)
DVA, DNA strand breaks, rat liver *in vivo*	+		0.025 × 1 po	Wahba *et al.* (1989)
DVA, DNA strand breaks, rat peritoneal lavage cells *in vivo*	+		0.025 × 1 po	Alsharif *et al.* (1994b)
MST, Mouse spot test	–[d]		0.003 × 1 ip	Fahrig (1993)
SVA, Sister chromatid exchange, rat lymphocytes *in vivo*	–[e]		0.03 × 1 po	Lundgren *et al.* (1986)
SVA, Sister chromatid exchange, C57BL/6J and DBA/2J mouse bone marrow *in vivo*	–		0.15 × 1 ip	Meyne *et al.* (1985)
MVM, Micronucleus test, C57BL/6J and DBA/2J mouse bone marrow *in vivo*	–		0.15 × 1 ip	Meyne *et al.* (1985)

Table 64 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
2,3,7,8-TCDD (contd)				
CBA, Chromosomal aberrations, C57BL/6J and DBA/2J mouse bone marrow *in vivo*	–		0.15 × 1 ip	Meyne *et al.* (1985)
SLH, Sister chromatid exchange, human lymphocytes *in vivo*	–		NG	Zober *et al.* (1993)
CLH, Chromosomal aberrations, human lymphocytes *in vivo*	–		(Seveso)	Tenchini *et al.* (1983)
CLH, Chromosomal aberrations, human lymphocytes *in vivo*	–		NG	Zober *et al.* (1993)
CLH, Chromosomal aberrations, human lymphocytes *in vivo*	–		(Seveso)	Reggiani (1980)
CVH, Chromosomal aberrations, human placental and umbilical cord tissues *in vivo*	–		(Seveso)	Tenchini *et al.* (1983)
CVH, Chromosomal aberrations, human fetal tissues *in vivo*	?		(Seveso)	Tenchini *et al.* (1983)
BVD, Binding (covalent) to DNA, mouse liver *in vivo*	–		0.1 × 1 ip	Turteltaub *et al.* (1990)
ICR, Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*	–	NT	0.003	Lincoln *et al.* (1987)
ICR, Inhibition of intercellular communication, C3H 10T1/2 mouse fibroblasts *in vitro*	–	NT	0.00003	Boreiko *et al.* (1989)
ICR, Inhibition of intercellular communication, mouse hepatoma cells (Hepa1c1c7) *in vitro*	+	NT	0.00003	De Haan *et al.* (1994)
ICR, Inhibition of intercellular communication, rat hepatocytes *in vitro*	+	NT	0.000000003	Baker *et al.* (1995)
ICR, Inhibition of intercellular communication (^{3}H]-uridine exchange), C3H 10T1/2 mouse cells	–	NT	0.01	Boreiko *et al.* (1989)
Octachlorodibenzo-*para*-dioxin				
SA0, *Salmonella typhimurium* TA100, reverse mutation	–	–	385	Zeiger *et al.* (1988)
SA5, *Salmonella typhimurium* TA1535, reverse mutation	–	–	385	Zeiger *et al.* (1988)
SA9, *Salmonella typhimurium* TA98, reverse mutation	–	–	385	Zeiger *et al.* (1988)

Table 64 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Octachlorodibenzo-*para*-dioxin (contd)				
SAS, *Salmonella typhimurium* TA97, reverse mutation	–	–	385	Zeiger *et al.* (1988)
Mixtures of PCDDs, PCDFs and PCBs				
SHL, Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	0.0004	Nagayama *et al.* (1994)
MST, Mouse spot test	–[d]		0.128 × 1 ip	Fahrig (1993)
SLH, Sister chromatid exchange, human lymphocytes *in vivo*	–		NG	Lundgren *et al.* (1988)
CLH, Chromosomal aberrations, human lymphocytes *in vivo*	–		NG	Lundgren *et al.* (1988)
BHD, Binding (covalent) to DNA, human placenta *in vivo*	–		NG	Gallagher *et al.* (1994)
1,2,3,6,7,8-Hexachlorodibenzo-*para*-dioxin				
TCM, Cell transformation, C3H 10T1/2 mouse cells	–	NT	0.39	Abernethy & Boreiko (1987)
1,2,3,7,8,9-Hexachlorodibenzo-*para*-dioxin				
TCM, Cell transformation, C3H 10T1/2 mouse cells	–	NT	0.39	Abernethy & Boreiko (1987)

[a] +, positive; (+), weak positive; –, negative; NT, not tested; ?, inconclusive

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL; in-vivo tests, mg/kg bw/day; NG, not given

[c] Administred with 12-*O*-tetradecanoyl phorbol-13-acetate

[d] Co-treatment with ethylnitrosourea enhanced ENU activity two-fold

[e] α-Naphthoflavone-induced sister chromatid exchange was enhanced in lymphocyte cultures from TCDD-treated rats compared to controls

[f] Initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

[g] Subsequently exposed to 12-*O*-tetradecanoyl phorbol-13-acetate

murine fibroblasts, as measured by a metabolic cooperation assay or dye transfer. These results are discussed in Section 4.6.

Unscheduled DNA synthesis was not induced in normal human mammary epithelial cells. Increased formation of sister chromatid exchange and micronuclei was found in human lymphocytes *in vitro* in the presence or absence of α-naphthoflavone.

In a host-mediated assay, 2,3,7,8-TCDD elicited a dose-dependent response in cell-transforming potential on peritoneal macrophages which was seven times that of the 2,3,7,8-tetrabromo analogue.

In studies *in vivo*, positive results were reported for DNA single-strand breaks in rat liver and rat peritoneal lavage cells and for sister chromatid exchange frequency in rat lymphocytes in the presence but not in the absence of α-naphthoflavone. Use of a highly sensitive accelerator mass spectrometry approach did not reveal adduct formation following very low-level exposure in mouse liver.

Mutagenic and recombinogenic effects were observed only in combination with *N*-ethyl-*N*-nitrosourea in the spot test with mice.

A method capable of detecting one DNA adduct in 10^{11} nucleotides did not show binding of 2,3,7,8-TCDD to DNA in liver of mice dosed *in vivo*.

Changes in DNA I (indigenous)-compound formation were studied in Sprague-Dawley rats treated orally by gastric instillation with 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD or 1,2,4,7,8-PeCDD (1 and 5 μg/kg bw in corn oil per week for four weeks). There were significant reductions in female, but not male rat hepatic I-compound formation after treatment with the two compounds substituted in all four lateral positions, whereas 1,2,4,7,8-PeCDD was inactive in this respect. No I-compound changes were observed in renal DNA following treatment with any of the compounds (Randerath *et al.*, 1988, 1990).

1,2,3,6,7,8- and 1,2,3,7,8,9-HxCDD did not transform C3H 10T1/2 cells or initiate transformation in the same cells subsequently treated with TPA. Continuous exposure to either of these two hexachlorinated congeners promoted cell transformation when the same cells were initiated by treatment with MNNG.

OCDD was not mutagenic in *S. typhimurium* (Zeiger *et al.*, 1988).

Mutations in tumours

DNA was extracted and analysed for activating mutations in H-*ras* codon 61 arising in hepatocellular adenomas and carcinomas of male and female B6C3F1 and C57BL/6 mice treated with a single intraperitoneal dose of vinyl carbamate (0.005 μM/g bw in saline) or vehicle and then gastric instillation doses of 2,3,7,8-TCDD (2.5 μg/kg bw in corn oil) every two weeks for 52 weeks. Another group received only the vinyl carbamate treatment. Of 45 tumours from B6C3F1 mice treated with 2,3,7,8-TCDD alone, only 23 (51%) had H-*ras* codon 61 mutations and 70% of these were C→A transitions in the first base. The pattern was similar to that found in spontaneous tumours of this strain and contrasted with the combined vinyl carbamate plus 2,3,7,8-TCDD and the vinyl carbamate alone-treated groups. In these two groups, respectively, there were A→T transitions in the second base in 39/53 (74%) and 17/27 (63%) of the tumours

containing activating mutations. Similar results were obtained for the C57BL/6 mouse strain. Thus, 2,3,7,8-TCDD treatment did not change the mutational spectrum arising within H-*ras* codon 61 of tumours arising either without treatment or as a result of treatment with vinyl carbamate (Watson *et al.*, 1995).

Mixture of PCDDs and/or PCDFs

Mixtures of PCDDs did not enhance mutagenic or recombinogenic effects, but in combination with *N*-ethyl-*N*-nitrosourea, positive effects were obtained in the spot test with mice.

Mixtures of PCDDs, PCDFs and coplanar PCBs increased the frequency of sister chromatid exchange formation in human lymphocytes *in vitro* in the presence or absence of α-naphthoflavone.

4.6 Mechanisms of carcinogenicity

4.6.1 *Introduction*

In lifetime bioassays for cancer in rodents, 2,3,7,8-TCDD is a multisite carcinogen in both sexes of all species tested and causes tumours at sites distant from the point of administration (see Section 3).

2,3,7,8-TCDD is the most toxic of the PCDD and PCDF congeners. PCDDs and PCDFs exhibit a similar rank order of potency across species and within different cell types for numerous biochemical and biological responses. This is consistent with a similar mechanism of action via the Ah receptor. Binding to the Ah receptor is necessary but not sufficient for the expression of toxicity for this entire class of chemicals. For this reason, 2,3,7,8-TCDD and all PCDDs/PCDFs will be considered together regarding potential mechanisms of carcinogenicity (Poland & Knutson, 1982; Goldstein & Safe, 1989).

It is likely that the dose of 2,3,7,8-TCDD determines the mechanism of toxicity and carcinogenicity. At high doses, many acute effects are seen that have not been demonstrated in chronic dosing studies utilizing lower doses more relevant to human environmental exposures. Acute and chronic dosing regimens that yield equivalent body burdens may also produce different pathologies by different mechanisms. For these reasons, only effects seen at doses well below the lethal dose will be considered here in relation to the mechanism of carcinogenesis of 2,3,7,8-TCDD (see Sections 4.3, 4.4 and 4.5).

4.6.2 *General issues regarding mechanisms of carcinogenesis*

(*a*) *Carcinogenesis is a multistep process*

Fundamentally there are three ways in which a compound such as a PCDD can influence the process of carcinogenesis (Barrett, 1993):

(i) it may induce a heritable mutation (initiation);

(ii) it may induce a heritable epigenetic change in a critical gene(s);

(iii) it may increase the clonal expansion of a cell possessing a heritable alteration in a critical gene(s).

(*b*) *Genotoxicity*

2,3,7,8-TCDD is considered a 'non-genotoxic' substance (see Section 4.5).

(*c*) *Ah receptor*

It has been proposed that the broad spectrum of biological responses associated with exposure to 2,3,7,8-TCDD is due to alteration in expression of 2,3,7,8-TCDD-regulated genes mediated by the Ah receptor (Section 4.3). However, even with the same receptor and the same ligand, there are both qualitative and quantitative differences between species. Even though Ah receptor activation is likely to be required for the carcinogenicity of 2,3,7,8-TCDD, its precise role in this process remains unclear.

In analogy to the mouse, two forms of Ah receptor exhibiting about a 4–5-fold difference in binding affinity for 2,3,7,8-TCDD (K_d, ~ 0.4 nM and ~ 2 nM, respectively) have been described in humans (Ema *et al.*, 1994).

(*d*) *Effects of 2,3,7,8-TCDD on gene expression*

Induction of expression of genes driven by the XRE element (recognized by the Ah receptor), e.g., CYP1A1 and CYP1A2, represents a useful marker for 2,3,7,8-TCDD effects. The broad spectrum of effects of 2,3,7,8-TCDD on hormone and growth factor systems, cytokines and other signal transduction pathways indicates that this substance is a powerful growth dysregulator (see Sections 4.3 and 4.4).

(*e*) *Oxidative damage*

In a series of studies by Stohs and colleagues, single treatment of rats or mice with high doses (≥ 50 µg/kg) resulted in increased superoxide anion production by peritoneal lavage cells, lipid peroxidation and DNA single-strand breaks (Stohs *et al.*, 1990; Alsharif *et al.*, 1994b). The relevance of these high dose studies to the carcinogenicity of 2,3,7,8-TCDD is questionable.

In-vitro studies showed that the promotion of transformation of C3H mouse fibroblasts by low non-cytotoxic concentrations of 2,3,7,8-TCDD (1.5 pM) was inhibited by the antioxidants mannitol and vitamins C and A (Wölfle & Marquardt, 1996). Promotion of cellular transformation is a well documented effect of 2,3,7,8-TCDD, as discussed below, and this study provides evidence for an oxidative-stress mechanism for this effect. Another study showed an Ah receptor-dependent formation of 8-hydroxydeoxyguanine (8-OH-dG) adducts in DNA following treatment of the mouse Hepa1c1c7 cell line with 500 pM 2,3,7,8-TCDD for 48 h (Park *et al.*, 1996).

Production of oxidative damage by 2,3,7,8-TCDD is consistent across several different experimental systems, both *in vivo* and *in vitro*. The requirement in rats for ovarian hormones in the mechanism of tumour promotion by 2,3,7,8-TCDD (Lucier *et al.*, 1991) is associated with a 2–3-fold higher level of 8-OH-dG DNA adduct formation in intact compared with ovariectomized rats (Tritscher *et al.*, 1996). It has been suggested that this increase in 8-OH-dG DNA adducts is a result of a production of genotoxic metabolites via redox cycling of catechol oestrogens, although there may be

alternative sources of reactive oxygen species. The role of these adducts in carcinogenicity has yet to be established.

(f) Cell transformation

In the mouse C3H 10T1/2 embryonic fibroblast cell transformation system (which contains a functional Ah receptor (Okey *et al.*, 1983)), significant increases in foci formation in cells pre-initiated with MNNG were observed following exposure to non-cytotoxic doses of 2,3,7,8-TCDD (4–4000 pM 2,3,7,8-TCDD) in a dose-dependent pattern (Abernethy *et al.*, 1985). 2,3,7,8-TCDD also gave positive results in an in-vitro transformation assay using rat tracheal epithelial cells only after prior initiation of the cells with MNNG and at concentrations of at least 300 pM continuously for seven days (Tanaka *et al.*, 1989). These data support the observations that 2,3,7,8-TCDD acts as a tumour promoter *in vivo*.

Virally immortalized human foreskin epidermal keratinocytes treated with 100 pM 2,3,7,8-TCDD were transformed, as shown by colony formation in soft agar, foci formation, an increased maximal cell density and a 100% incidence of squamous-cell carcinomas in nude mice when injected with 1×10^7 cells compared with an incidence of zero with cells exposed to 0.1% dimethyl sulfoxide (Yang *et al.*, 1992). Maximal induction of neoplastic transformation occurred at 1 nM, whereas induction of aryl hydrocarbon hydroxylase activity was maximal at 30 nM. Neoplastic transformation of human cells occurs at a concentration similar to that needed for rodent cell transformation.

(g) Cell proliferation and tumour promotion

Since 2,3,7,8-TCDD is not directly genotoxic, it either could be acting to'promote' the development of tumours from previously initiated cells and/or may be causing mutations via an indirect mechanism.

In a rat tumour initiation–promotion protocol experiment, hepatic cell proliferation as measured by BrdU incorporation (labelling index) was decreased at a dose of 3.5 ng/kg 2,3,7,8-TCDD per day and increased an average of three-fold at 125 ng/kg 2,3,7,8-TCDD per day, after 30 weeks of 2,3,7,8-TCDD treatment (Maronpot *et al.*, 1993). Significant interindividual variation was observed in labelling index, with approximately half of the animals exhibiting significantly higher labelling indices than similarly treated animals. Preneoplastic GSTP-positive foci were elevated only at 125 ng/kg per day. Cell proliferation was significantly stronger in initiated than in non-initiated rats. In another study by Stinchcombe *et al.* (1995), 2,3,7,8-TCDD only marginally affected DNA synthesis in GSTP-positive liver foci after treatment with a dose 100 ng/kg 2,3,7,8-TCDD per day for 115 days. However, in this study, apoptosis in foci was markedly reduced.

(h) Suppression of immune surveillance

Effects on the immune system can have significant effects on the disease process and the manifestation of toxicity in other organ systems. This is particularly relevant to cancer, where a primary effect of 2,3,7,8-TCDD on immune function could secondarily

aid in the progression and development of malignancy by allowing genetically altered cells to escape immune surveillance. There is a wealth of information concerning immune suppression in laboratory animals, but results on immune function in human studies are inconsistent (see Sections 4.2.1 and 4.2.2(*b*)).

4.6.3 *Tissue-specific mechanisms of carcinogenicity of 2,3,7,8-TCDD*

(*a*) *Liver*

(i) *Sex differences in carcinogenicity in the liver*

Female rats appear to be more sensitive than male rats to the hepatocarcinogenic effects of 2,3,7,8-TCDD. This sex difference has not been observed in mice. Furthermore, sex differences in carcinogenic responsiveness were not observed in either rats or mice given a mixture of 1,2,3,6,7,8-HxCDD and 1,2,3,7,8,9-HxCDD (see Section 3).

(ii) *Possible role of ovarian hormones in tumorigenesis*

Sex-dependent cell proliferation and altered hepatic foci formation were observed in the livers of intact but not ovariectomized rats in a 2,3,7,8-TCDD tumour promotion study, an effect which was not attributable to differences in liver concentrations. This suggests a possible role of ovarian hormones, presumably oestrogens. On the basis of these results, an indirect genotoxic mechanism of tumour promotion by TCDD was suggested. This involves induction of cytochromes P450 which metabolize 17β-oestradiol to catechols (Lucier *et al.*, 1991). These catechols (e.g., 2-hydroxyoestradiol and 4-hydroxyoestradiol) can be converted to semiquinone intermediates, possibly forming reactive singlet oxygen species (Liehr & Roy, 1990), subsequently leading to an increase in oxidative stress or DNA damage (Liehr, 1990; Yager & Liehr, 1996).

Another hypothesis for the role of ovarian hormones in the mechanism of liver tumour development is an alteration in the signal transduction pathway for oestrogens. Hepatic oestrogen receptor complex level and binding capacity are down-regulated in rats after in-vivo exposure to 2,3,7,8-TCDD (Romkes *et al.*, 1987; Romkes & Safe, 1988; Harris *et al.*, 1990; Clark *et al.*, 1991a; Zacharewski *et al.*, 1991, 1992, 1994).

(iii) *Effects on epidermal growth factor receptor*

2,3,7,8-TCDD decreases the amount of detectable plasma membrane EGF receptor in liver *in vivo* and keratinocytes *in vitro* (Madhukar *et al.*, 1984; Hudson *et al.*, 1985; Astroff *et al.*, 1990; Choi *et al.*, 1991; Lin *et al.*, 1991a; Sewall *et al.*, 1993, 1995). In one study, however, changes in cell proliferation occurred in populations of hepatocytes different from those in which there was induction of CYP1A1/CYP1A2 (Fox *et al.*, 1993), while down-regulation of the EGF receptor occurs throughout the liver (Sewall *et al.*, 1993).

In a tumour initiation–promotion protocol, there was no effect of 2,3,7,8-TCDD on the EGF receptor in ovariectomized rats paralleling the tumour incidence data, even though CYP1A1 and CYP1A2 induction profiles were similar. The maximal decrease in EGF receptor binding was three-fold at a dose of 125 ng/kg per day and an ED_{50} of 10 ng/kg per day (1.5 μg 2,3,7,8-TCDD/kg liver fat). EGF receptor effects occur at lower

doses than induction of cell proliferation or foci, consistent with this effect being a contributing factor in the hepatocarcinogenic action of TCDD. Decreased EGF receptor binding may be indicative of increased EGF expression and thus a mechanistically plausible predictor of increased cell proliferation (Clark *et al.*, 1991a; Lucier *et al.*, 1991; Sewall *et al.*, 1993).

(iv) *Cellular localization*

Induction of CYP1A1 and CYP1A2 protein following chronic exposure to 2,3,7,8-TCDD exhibited a dose-dependent increase in acinar zones 2 and 3 (Tritscher *et al.*, 1992). In contrast, changes in cell proliferation following 2,3,7,8-TCDD exposure do not show an acinar-dependent pattern and in one study occurred in different populations of hepatocytes than induction of CYP1A1/CYP1A2 (Fox *et al.*, 1993). In addition, down-regulation of the EGF receptor (Sewall *et al.*, 1993) and expression of the Ah receptor are observed throughout the liver. This may imply that there is a differential sensitivity of hepatocytes to 2,3,7,8-TCDD induction.

(v) *Alterations in gap-junctional communication by 2,3,7,8-TCDD*

Whether inhibition of gap-junctional intercellular communication (GJIC) has a causal role in the mechanism of carcinogenesis for 2,3,7,8-TCDD, or is a response that occurs as a result of other events during tumour promotion is unknown.

Alterations in connexin (Cx) expression and alterations in GJIC have been observed following exposure to 2,3,7,8-TCDD *in vitro* and *in vivo* in rodents. Treatment of Fischer 344 rats (that had been initiated orally with 10 mg/kg NDEA) with 100 ng/kg 2,3,7,8-TCDD per day for eight months resulted in a significant increase in altered hepatic foci. In these foci, there was decreased expression of Cx32, increased Cx26 expression, and a complete absence of Cx43 expression (Neveu *et al.*, 1994). 2,3,7,8-TCDD inhibited GJIC in rat hepatocytes grown in primary culture in a time-, dose- and Ah receptor-dependent manner at doses of 2,3,7,8-TCDD from 10^{-12} to 10^{-8} M, indicating that inhibition of GJIC is a highly sensitive response to 2,3,7,8-TCDD (Baker *et al.*, 1995). Cx32 expression was decreased, while Cx26 was unaffected.

Alteration of GJIC has also been seen with other PCDDs and PCDFs. Inhibition of GJIC by PCDD and PCDFs correlated well with their potency to induce CYP1A1 (as measured by EROD activity in Hepa1c1c7 cells (De Haan *et al.*, 1996). The concentration required for half-maximal response (EC_{50}) for both responses for all congeners was $\leq$ 1 nM and the dose–response curves were parallel. These same authors previously demonstrated that this effect was Ah receptor-dependent (De Haan *et al.*, 1994). 2,3,7,8-TCDD had no effect on GJIC in mouse V79 or C3H 10T1/2 cells (Lincoln *et al.*, 1987; Boreiko *et al.*, 1989).

(vi) *Cytotoxicity as mechanism for hepatic lesions*

Hepatocellular toxicity has been observed in rat carcinogenicity experiments (see Section 3) and might play a role in carcinogenesis.

(*b*) *Other target tissues*

2,3,7,8-TCDD-induced carcinogenesis has been reported in a number of other tissues including lung, nasal ethnoturbinates, thyroid, lymphoid tissues, skin and tongue (see Section 3). Ah receptor expression and receptor-dependent responses have been observed in many of these tissues (see Section 4.3) and may play a role in carcinogenesis. A study in congenic strains of mice and using a number of isomers has indicated a role for the Ah receptor in PCDD-induced skin papilloma (Poland *et al.*, 1982).

In the case of thyroid carcinogenesis, an indirect mechanism has been proposed involving enhanced metabolism of thyroid hormones in the liver (see Section 4.3) (Hill *et al.*, 1989; Kohn *et al.*, 1996).

4.6.4 *Mechanisms for reduced cancer incidence following 2,3,7,8-TCDD exposure*

Two mechanisms have been proposed to explain negative trends in cancer incidence. Firstly, reductions in tumour incidence could be due to alterations in body weight (known since Tannenbaum, 1940) as a result of 2,3,7,8-TCDD exposure. Secondly, 2,3,7,8-TCDD disrupts the endocrine homeostasis, and may thereby reduce the incidence of hormone-dependent cancers such as mammary and uterine cancers.