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Short-Term Toxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Laboratory Animals: Effects, Mechanisms, and Animal Models*

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I. Introduction

TCDD‡ (fig. 1) is the most potent congener of the halogenated Ahs. This class of toxicologically important environmental contaminants includes polychlorinated (or polybrominated) dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and azo(xy)benzenes. Every subgroup contains a large number of congeners displaying widely varying potencies. The compounds share structural similarity and, except for some nondioxin-like polychlorinated biphenyls, are believed to have a common mode of action. Although this review will be confined to TCDD alone, it should be borne in mind that the findings are largely applicable to other TCDD-like halogenated Ahs as well.

A. Seveso Accident

TCDD was first associated with human illness in the mid-1950s (Kimmig and Schultz, 1957). In the next decade, researchers showed growing interest in the compound whose peculiar properties gradually began to emerge. In the early 1970s in the United States, there was publicity and concern regarding TCDD because several defoliants used in the Vietnam war contained high levels of TCDD as an impurity and because of an accident in Missouri that frighteningly demonstrated the toxic potency of TCDD. The accident stemmed from the spraying of horse arenas with waste oil contaminated

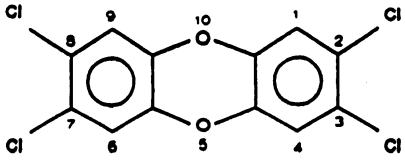

FIG. 1. Structural formula of TCDD. The molecular weight of TCDD is 322.0.

‡ Abbreviations: Ah, aromatic hydrocarbon; EGF, epidermal growth factor; 5-HT, 5-hydroxytryptamine, serotonin; H/W, Han/Wistar (Kuopio); L-E, Long-Evans (Turku AB); LH, luteinizing hormone; PEPCK, phospho*enol*pyruvate carboxykinase; S-D, Sprague-Dawley; T_3, 3,3′,5-triiodothyronine; T_4, thyroxine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transforming growth factor; TNF, tumor necrosis factor-α; UDPGT, uridine diphosphate glucuronosyltransferase; LD_{50}, median lethal dose; ED_{50}, median effective dose.

with TCDD, which resulted in the death of at least 65 horses, several dogs and cats, hundreds of birds, and numerous rodents (Carter et al., 1975; Long and Hanson, 1983; Kimbrough, 1984). Nevertheless, it was not until 1976 that TCDD gained worldwide fame among laymen. This was due to a serious explosion at a chemical plant producing trichlorophenol in Seveso, Italy. The chemical process used in the synthesis of trichlorophenol creates TCDD as a byproduct. Although explosions had previously occurred at several other trichlorophenol plants, they had not led to the exposure of the general public to TCDD (Holmstedt, 1980). In Seveso, however, a toxic cloud containing an estimated 300 to 2000 g of TCDD escaped from the plant and eventually covered an area of about 2.8 km^2. As a result, thousands of both domestic and wild animals died, a large number of inhabitants had to be evacuated, and the worst contaminated areas had to be perpetually isolated (for an overview, see Homberger et al., 1979). In the long run, the Seveso disaster turned out to be a powerful impetus for research concerning the diverse facets of TCDD.

B. Sources

Although industrial accidents still pose a hazard, the principal sources of exposure to the general public are now much less dramatic. By far the most important direct source of TCDD for humans appears to be food, especially dairy products, meat, and fish (Beck et al., 1987, 1992; Connett and Webster, 1987; Hattemer-Frey and Travis, 1989; Jones and Bennett, 1989; Fries and Paustenbach, 1990; Svensson et al., 1991; Travis and Hattemer-Frey, 1991; Henry et al., 1992; Schecter et al., 1992). This is not surprising in view of the known ability of TCDD to bioaccumulate in the food chain (Norris, 1981; Kenaga and Norris, 1983; Branson et al., 1985; Adams et al., 1986; Geyer et al., 1986; Mehrle et al., 1988; Opperhuizen and Sijm, 1990). Among humans, a particular risk group may be newborn infants, who can get higher amounts of TCDD from breast milk (per kg body weight) than adults can get from food (Lindström, 1988; Yrjänheikki, 1989; Koppe et al., 1991; Kello and Yrjänheikki, 1992).

TCDD is not manufactured for any purpose other than scientific research. It arises as an unwanted impurity or byproduct in a variety of chemical and technical processes. For example, the herbicide 2,4,5-trichlorophenoxyacetic acid and the antiseptic hexachlorophene typically contain low concentrations of TCDD (Rappe et al., 1979; Kriebel, 1981). TCDD, among other dioxins, has been detected in fly ash from municipal waste and metal reclamation incinerators (Olie et al., 1977; Hryhorczuk et al., 1981; Wakimoto and Tatsukawa, 1985; Benfenati et al., 1986; Hagenmaier, 1986; Öberg and Allhammar, 1989; Zitko, 1989; de Jong et al., 1993), and recent findings have underscored the importance of various thermal processes, especially iron ore sintering, as a source of dioxins (Fiedler, 1993; Lahl, 1993). Fly ash can also catalyze the formation of TCDD and more chlorinated dioxins from the common wood preservative, pentachlorophenol, at temperatures of 300 to 400°C (Karasek and Dickson, 1987). Transformer fires can lead to the formation of dioxins, including small quantities of TCDD, from chlorobenzenes (Buser, 1985; Hutzinger et al., 1985). The combustion of leaded gasoline in car engines appears to give rise to TCDD and related substances (Marklund et al., 1987), which may, in part, account for the observed ten-fold higher levels of TCDD in urban than in rural air (Jones and Bennett, 1989). Finally, chlorine bleaching of paper is responsible for the occurrence of TCDD in pulp and paper mill sludge (Kuehl et al. 1987; Clement et al., 1989). Low concentrations (<10 pg/g) have also been found in paper products (Beck et al., 1988, 1989; Wiberg et al., 1989).

C. Physicochemical Properties

TCDD is physicochemically stable and inert. Its water solubility is extremely low, on the order of 8 to 19 ng/liter at room temperature (Adams and Blaine, 1986; Marple et al., 1986b). The octanol-water partition coefficient has been determined or estimated to be about 9×10^5 to 4×10^6 (log K_{ow} = 5.95 to 6.64) (Marple et al., 1986a; Jackson et al., 1993). The solubility in organic solvents is not remarkable, being 10, 110, 370, and 570 mg/liter for methanol, acetone, chloroform, and benzene, respectively (Schroy et al., 1985a). TCDD is virtually nonvolatile with a vapor pressure of 1 to 2×10^{-7} Pa at 25°C (Schroy et al., 1985a; Podoll et al., 1986). The melting point has been reported to be approximately 305°C (Boer et al., 1972; Pohland and Yang, 1972). There are no direct measurements of the boiling point; however, the estimated values range from 421 to 447°C (Schroy et al., 1985b; Rordorf, 1986). TCDD shows considerable thermal stability: temperatures >800°C are needed for its complete degradation (Kearney et al., 1973; Stehl et al. 1973).

D. Toxicological Characterization

Toxicologically, TCDD possesses several properties that make it unique among all xenobiotics. One of the most intriguing and perplexing features is a specific intracellular receptor (see section II.F) which occurs in a variety of species, but the physiological role, if any, is still a mystery. This receptor mediates at least part of the actions of TCDD, the best known of which at the molecular level is induction of cytochromes CYP1A1 and CYP1A2 in the liver. With regard to induction ability of these monooxygenases, TCDD is the most potent compound known.

Extreme potency is typical of the toxic properties of TCDD as well. This is especially true for the acute lethality of TCDD in certain animal species such as the guinea pig, in which the LD_{50} value is lower for TCDD than for any other synthetic compound. However, there are exceptionally wide interspecies, interstrain, and even intersubstrain differences in susceptibility to TCDD lethality (see section II.A). Another peculiarity is the delayed appearance of mortality; irrespective of dose, death does not ensue earlier than at least 1 week after exposure. Before death, the exposed animals undergo a rapid and impressive loss of body weight designated "wasting syndrome," which is a notably rare manifestation of acute toxicity among chemical compounds.

One of the most exhaustively examined facets of TCDD toxicity is immune suppression (see section II.G). Again, TCDD appears to exert a highly specific effect on both B- and T-lymphocytes, along with associated structures such as the thymus. TCDD is a potent carcinogen as well, if assessed on the basis of absolute doses needed to elicit the response (Van Miller et al., 1977; Kociba et al., 1978; Della Porta et al., 1987; Rao et al., 1988a). On the other hand, if assessed relative to toxic doses, the tumorigenic potential of TCDD may not be so remarkable (Kociba et al., 1978). TCDD is a specific teratogen, in particular in mice, producing cleft palate and hydronephrosis (Courtney and Moore, 1971; Moore et al., 1973; Abbott et al., 1987; Birnbaum et al., 1989; Couture et al., 1990b; Olson and McGarrigle, 1992; Huuskonen et al., 1994). In several species, it is also lethal to embryos or fetuses (Sparschu et al., 1971; Neubert et al., 1973; Allen et al., 1979; Murray et al., 1979; Giavini et al., 1982; McNulty, 1985; Olson et al., 1990). With repeated dosing, TCDD affects both the synthesis and the degradation of heme, leading to porphyria and icterus, respectively (Goldstein et al., 1973, 1982; Zinkl et al., 1973; Kociba et al., 1976; Cantoni et al., 1981; Sweeney et al., 1984). Finally, a plethora of health effects have been associated with exposure to TCDD in humans, ranging from mood alterations (Levy, 1988) to diabetes (Wolfe et al., 1992) and cancer (Zober et al., 1990; Fingerhut et al., 1991; Bertazzi et al., 1993). Yet, compelling evidence exists almost solely for TCDD-induced chloracne (May, 1973, 1982; Oliver, 1975; Pocchiari et al., 1979; Assennato et al., 1989).

More than a decade has passed since the comprehensive review of the toxicity of TCDD and related chemicals

by Poland and Knutson (1982) appeared. During this period, other overviews have been published, emphasizing especially the carcinogenicity (Kociba, 1984, 1991; Sielken, 1987; Huff et al., 1991, 1994; Greim et al., 1992; Lucier et al., 1993), mutagenicity (Giri, 1986), developmental and reproductive toxicity (Pratt et al., 1984b; Couture et al., 1990a; Birnbaum, 1991; Peterson et al., 1993), immunotoxicity (Holsapple et al., 1991a,b; Vos et al., 1991), or human toxicity (Abel, 1987; Skene et al., 1989; Mocarelli et al., 1991; Johnson, 1992, 1993) of TCDD. The molecular mechanism of the Ah receptor-mediated enzyme induction by TCDD has also been dealt with extensively (Goldstein and Hardwick, 1984; Whitlock et al., 1984, 1989; Greenlee and Neal, 1985; Durrin et al., 1987; Whitlock, 1987, 1989, 1990, 1993; Safe, 1988; Nebert and Jones, 1989; Denison, 1991; Landers and Bunce, 1991; Swanson and Bradfield, 1993). However, there has been a conspicuous paucity of reviews addressing the short-term toxicity of TCDD. The reviews concerning the wasting syndrome by Peterson and coworkers (1984a,b) and by the present authors (Tuomisto and Pohjanvirta, 1991), and the more or less polemic papers by Rozman (1989) and Greim and Rozman (1987), have been the only ones in this important field. The objective of this review is to attempt to bridge the existing gap.

II. Short-Term Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Laboratory Animals

A. Acute Lethality

1. General features. Acute lethality is usually defined as mortality within 14 days after a single dose. TCDD does not readily fit into this time frame, because its lethality is characteristically delayed. In general, death ensues 1 to 6 weeks after exposure (Greig et al., 1973; Gupta et al., 1973; see also table 1). With the possible exceptions of the hamster and the rabbit, it is almost invariably preceded by a dramatic loss of up to >50% of body weight, which is known as wasting syndrome (see below). Another characteristic phenomenon of TCDD is the wide inter- and intraspecies differences in LD_{50} values (table 1). This feature has been taken advantage of in developing animal models for mechanistic studies of TCDD toxicity. The models will be described in detail later (section V), but three points are worth noting. First, at the species level, the guinea pig and the hamster represent the two extremes in susceptibility. Second, two inbred mouse strains, C57BL/6 and DBA/2, differ in this respect by a factor of 5 to 15 (for congenic substrains of C57BL/6, C57BL/$6^{b/b}$, and C57BL/$6^{d/d}$, the difference is approximately 20-fold). Third, there is an even greater (about 1000-fold) disparity between two rat strains, L-E and H/W; the latter strain may actually be the most TCDD-resistant laboratory animal. The sensitivity of a given animal to TCDD is also dependent on age and sex (table 1). Young rats may be more susceptible than adults, but gender-related sensitivity varies among strains of rat.

No systematic phylogenetic study of TCDD susceptibility has been performed so far. TCDD appears to lack microbicidal activity (Arthur and Frea, 1988). Likewise, plants, snails, worms, and insects seem to be relatively insensitive to the acute lethality of TCDD (reviewed by Kenaga and Norris, 1983; Cooper, 1989), but the evidence is still scanty. American bullfrogs are also strikingly resistant (intraperitoneal injections of up to 500 and 1000 μg/kg to adults and tadpoles, respectively, were virtually without effect) (Beatty et al., 1976). Fish, in contrast, have proved to be highly susceptible (single intraperitoneal LD_{50} levels of 3 to 16 μg/kg for the species tested), although there are some species and strain differences in the manifestations of toxicity (Miller et al., 1973; Kleeman et al., 1988; Spitsbergen et al., 1988a,b). As is the case in mammals, fish embryos may be more sensitive than adult fish to TCDD (Wisk and Cooper, 1990).

2. Cause of death. The ultimate cause of death in TCDD intoxication is not certain. In light of the wasting syndrome (see section II.B), an obvious factor could be exhaustion of energy stores, especially fat, in the body. This may often be of crucial importance, because pair-fed controls (see section II.B.2) usually die at approximately the same time as TCDD-exposed animals (Peterson et al., 1984b; Kelling et al., 1985; Weber et al., 1991b). On the other hand, it cannot clearly account for TCDD lethality in all cases, because rats and guinea pigs maintained with total parenteral nutrition, which prevented body weight loss, still died of TCDD intoxication (Gasiewicz et al., 1980; Huang Lu et al., 1986). Rozman and associates emphasized the role of progressive hypoglycemia (Gorski and Rozman, 1987; Gorski et al., 1990) in this respect. Hypoglycemia is indeed a common finding in TCDD-treated laboratory animals such as rats, mice, and rabbits (Zinkl et al., 1973; Potter et al., 1983; Chapman and Schiller, 1985; Gorski and Rozman, 1987; Ebner et al., 1988; Gorski et al., 1990). In the most TCDD-susceptible species, the guinea pig, however, serum glucose values remain within the normal range until the preagonal state (Gasiewicz and Neal, 1979; Bak et al., 1982; Brewster and Matsumura, 1988). Furthermore, although control rats pair fed to their counterparts treated with a lethal dose of TCDD were able to maintain virtual euglycemia (Gorski et al., 1990), their mortality and time course to death did not differ from those of the TCDD-dosed rats (Weber et al., 1991b).

Compromised function of the heart has also been suggested to be a critical factor, but the findings are somewhat contradictory, even within the same species. For example, Kelling et al. (1987a) reported that isolated atria from male S-D rats treated with 100 μg/kg of TCDD 7 days previously, displayed increased responsiveness to the chronotropic and inotropic effects of (−)-isoprotere-

TABLE 1
Acute lethality of TCDD to various species and (sub)strains

Species/strain (sex)	Route	LD_{50} (μg/kg)	Time of death (days postexposure)	Follow-up (days)	Body weight loss* (%)	Reference
Guinea pig/Hartley	po†	2.0	>5	30	50	McConnell et al., 1978b
(male)		0.6–2.1	5–42	NP	NP	Schwetz et al., 1973
Mink (male)	po	4.2	7–17	28	31	Hochstein et al., 1988
Chicken	po	<25	12–21	NP	NP	Greig et al., 1973
Ring-necked pheasant (female)	ip	~25	3–7 wk	49‡	25–40	Nosek et al., 1992a
Rhesus monkey (female)	po	~70	14–34	42–47	13–38	McConnell et al., 1978a
Rat/L-E (male)	po	17.7	13–35	42	35	Pohjanvirta et al., 1993a
(Female)	po	9.8	13–34	42	26	
(Male)	ip	~10	15–23	48–49	39	Tuomisto and Pohjanvirta, 1987 (and unpublished data)
Rat/Sherman, Spartan (male)	po	22	9–27	NP	NP	Schwetz et al., 1973
(Female)		45	13–43			
Rat/S-D (male)	ip	60	NP	20	NP	Beatty et al., 1978
(Female)		25				
(Weanling male)		25				
Rat/Fischer, Harlan (male)	po	340	28§	30	43	Walden and Schiller, 1985
Rat/H/W (male)	po	>7200	29, 37	42	46	Pohjanvirta et al., 1993a
(Female)	po	>7200	17–42	42	34	
(Male)	ip	>3000	23–34	39–48	46	Pohjanvirta et al., 1987; Pohjanvirta and Tuomisto, 1987
Mouse/C57BL/6 (male)	po	182	24§	30	25	Chapman and Schiller, 1985
DBA/2 (male)		2570	21§		33	
$B6D2F_1$ (male)		296	25§		34	
Mouse/C57BL/6	ip	132	NP	NP	NP	Neal et al., 1982
DBA/2		620				
$B6D2F_1$		300				
Rabbit/New Zealand White (mixed)	po	115	6–39	NP	NP	Schwetz et al., 1973
	derm.	275	12–22	22	NP	
	ip	~50	7–10	10–20	11	Brewster et al., 1988
Golden Syrian hamster (male)	po	1157	2–47	50	1‖	Olson et al., 1980b
	po	5051	9–43	55	NP	Henck et al., 1981
(mixed)	ip	>3000	1–32	50	NP	Olson et al., 1980b

* Of animals that died.
† Abbreviations: po, oral; ip, intraperitoneal; derm., dermal; NP, not provided.
‡ Data from the first 7 weeks only.
§ Mean time to death.
‖ Data from five animals.

nol, whereas Hermansky et al. (1988) observed decreased sensitivity to the positive inotropic effect of the β_1-agonist in intact female S-D rats 6 days after a dose of 120 μg/kg of TCDD. In male guinea pigs, a dose of 10 μg/kg diminished isoproterenol responsiveness at 5 days (Canga et al., 1988) and a ten-fold lower dose at 10 and 20 days (Brewster et al., 1987). The basal force of atrial muscle contraction was found to be enhanced by TCDD in the rat (Kelling et al., 1987a), whereas TCDD-treated guinea pigs first exhibited elevated (at 10 days) and then decreased (at 20 days) basal force of atrial muscle contraction compared with controls fed ad libitum. In the

most recent study of chick embryo hearts, no alteration in cardiac function under basal conditions was detected. Nevertheless, ventricular responses to various inotropic stimuli (including isoproterenol) were impaired by TCDD, probably by a mechanism involving disturbed intracellular calcium processing (Canga et al., 1993).

A fourth proposed cause of death is endotoxic shock, based on the finding that TCDD-dosed mice display endotoxin hypersensitivity (Vos et al., 1978; Rosenthal et al., 1989). As will be discussed later (section II.G.2), it is possible that the aggravated endotoxin toxicity is applicable to only mice, and this phenomenon may not occur in rats. At least a contributing factor during the terminal phase in rats, however, seems to be lipid peroxidation, because antioxidant treatment has been shown to reduce mortality and/or to prolong survival time in rats, sometimes in the absence of an effect on the initial wasting (Stohs et al., 1984; Hassan et al., 1985a, 1987; Pohjanvirta et al., 1990b). In any case, a most important point to note is that the pathological changes found in organs and tissues after TCDD administration (see section II.C.1) usually cannot account for the high mortality, with the possible exception of severe liver necrosis that occurs in the rabbit (Kimbrough, 1983).

B. Wasting Syndrome

1. Characterization. Mammals exposed to a lethal dose of TCDD usually exhibit a prominent loss of body weight prior to death (table 1). A high sublethal dose, in turn, brings about persistently (≥6 months) stunted growth (Seefeld et al., 1984b; Pohjanvirta and Tuomisto, 1990a,b; R. Pohjanvirta and J. Tuomisto, unpublished data). Collectively, these effects of TCDD on body weight have been designated the wasting syndrome. It is worth emphasizing here that such a drastic impact on body weight after a single exposure is quite uncommon among chemical compounds, having been described only (except for the halogenated Ahs) in the case of some perfluorinated fatty acids (Andersen et al., 1981; George and Andersen, 1986) and cobalt protoporphyrin (Galbraith and Kappas, 1989). Although early studies provided inconsistent data concerning the influence of TCDD on feed consumption, it has now become clear from experiments with pair-fed rats, mice, hamsters, and guinea pigs that the decline in body weight results mainly, if not entirely, from hypophagia (Seefeld and Peterson, 1983; Seefeld et al., 1984a; Kelling et al., 1985; Christian et al., 1986a; Gasiewicz et al., 1986b; Potter et al., 1986a). Because TCDD increases feed spillage dose dependently (Seefeld et al., 1984a), it has been difficult to correctly measure feed intake; therefore, to obtain reliable results, metabolic cages must be used or at least all spilled feed in ordinary cages must be carefully collected.

2. Problems with pair feeding. Most of the studies of pathogenetic aspects of the wasting syndrome have been performed on rats. Because the marked change in body weight alone is bound to severely disturb the intermediary metabolism, the usual practice has been to incorporate into the study design a group of pair-fed control rats that are given exactly the same amount of feed as their TCDD-treated counterparts consumed the day before. Although this procedure broadly obviates the problem, it is a crude way to simulate the metabolic effects of TCDD and has at least two major drawbacks.

The first stems from the fact that control rats receiving the feed restriction regimen are extremely hungry and will devour their feed. This means that the daily feeding pattern differs completely in TCDD-treated rats and pair-fed control rats. In an attempt to maintain the physiological feeding rhythm of pair-fed rats, some researchers have divided the daily amount of feed into two or three portions spaced appropriately throughout the day (Gorski and Rozman, 1987; Gorski et al., 1988c) or have habituated both TCDD-treated and pair-fed control rats to a schedule (Christian et al., 1986a). However, no investigators have taken into account the finding that TCDD disrupts the normal circadian feeding rhythm (see section II.E.1.a).

The second weakness is due to the phase shift of the experiment with pair-fed control rats. Because these will be treated with a 1-day delay, compared with the TCDD-dosed animals, ambient conditions may not be identical, which may influence the levels of parameters highly responsive to external factors, such as concentrations of certain hormones. These reservations should be kept in mind when assessing metabolic results obtained with TCDD.

3. Absorption of nutrients. Basically, the characteristic wasting of TCDD intoxication could result from either reduced energy intake or induced energy expenditure. As mentioned above, suppressed food consumption has unequivocally proved to be the primary factor underlying the loss of body weight. This will be further discussed in section II. E.1.A. As to other possible contributing factors, the percentage of food energy absorbed remained unaffected by TCDD (Seefeld and Peterson, 1984; Potter et al., 1986a), indicating that there is no gross malabsorption involved, although slight modifications in the absorption of certain nutrients have been described. After TCDD exposure, lipid absorption was augmented without the concomitant stimulation of intestinal apoprotein synthesis typical in control rats (Schiller et al., 1984). Although Schiller et al. (1984) found no influence of TCDD on rates of amino acid or monosaccharide absorption, both Ball and Chhabra (1981) and Richter et al. (1992) reported that TCDD moderately (≤30%) inhibited the active absorption of glucose in the small intestine. Moreover, Ball and Chhabra (1981) observed that active absorption of leucine was also impaired. The absorption of water, sodium, calcium, or organic anions was not interfered with by TCDD, but the transport

rates for potassium and iron were elevated (Manis and Apap, 1979; Richter et al., 1992). These partially contradictory findings do not give much evidence for a crucial role of absorption deficits as the cause of the wasting syndrome.

4. Energy expenditure. The main routes of energy expenditure, basal metabolic rate, nonshivering thermogenesis, and locomotion, have also been explored in relation to TCDD treatment in rats. The basal metabolic rate (as assessed by oxygen consumption at rest) decreased dose and time dependently after TCDD exposure compared with controls fed at libitum (Seefeld et al., 1984a; Potter et al., 1986a,b; Rozman and Greim, 1986b). However, the pair-fed control rats behaved similarly to the TCDD-treated animals (Potter et al., 1986a,b). The effect of slight TCDD on body temperature may depend on age, because slight hypothermia (compared with both ad libitum-fed and pair-fed controls) has been detected in young (80–150 g) rats housed at room temperature (Potter et al., 1983; Jones et al., 1987b), but sexually mature animals were able to maintain their body temperatures within the control range at various ambient temperatures (Potter et al., 1986b; Rozman and Greim, 1986b). In any case, there are no reports of hyperthermia following TCDD administration.

The dominant (and probably the exclusive) effector organ of nonshivering thermogenesis in rodents is brown adipose tissue (for reviews, see Nicholls and Locke, 1984; Cannon and Nedergaard, 1985; Himms-Hagen, 1985). Its possible involvement in the TCDD-induced wasting syndrome has been examined by Rozman and associates, who found that both a sublethal and a lethal dose of TCDD caused morphological alterations in the interscapular brown fat (Rozman et al., 1986a,b, 1987b; see section II.C.1.c), but there was no activation of thermogenesis. On the contrary, the thermogenic response of the tissue to noradrenaline was attenuated 8 days after a lethal dose (125 μg/kg) of TCDD, coupled with unchanged thermogenic capacity (Weber et al., 1987b).

Locomotor activity has traditionally been measured in special cages during certain (relatively brief) periods. In the case of TCDD, this approach has provided slightly conflicting results. For example, Seefeld et al. (1984a), housing rats at 27°C, observed a gradual, dose-dependent depression of motility in TCDD-treated rats compared with control S-D rats fed ad libitum; this effect was progressive until death if the dose was lethal. However, Potter et al. (1986b), who assessed motor activity only on day 7 postexposure, failed to find any effect at all in comparison with control S-D rats fed ad libitum, regardless of whether the rats were kept at an ambient temperature of 15 or 27°C. The only divergent group were the pair-fed controls, which increased their activity at 15°C. Recently, a new method for continuous monitoring of all movements (including shivering) of laboratory animals in their home cages was described (Räisänen et al., 1992). The most exhaustive and thorough study carried out so far of the influence of TCDD on muscular activity was based on this new method and covered the entire period of first 9 days postexposure in L-E and H/W rats (see section V.C). The results revealed progressively reduced motor activity in both TCDD-treated (50 μg/kg; 100% lethal dose) and pair-fed L-E rats from day 4 on, whereas there was no change in the TCDD-resistant H/W rats (Tuomisto et al., 1993b).

5. Intermediary metabolism. Thus, the available data do not point to increased energy expenditure in TCDD-treated rats. Nevertheless, there is evidence to indicate an altered mode of intermediary metabolism after TCDD exposure (table 2). It was recently reported that TCDD was able to inhibit cellular respiration and uncouple oxidative phosphorylation in beef heart mitochondria in vitro (Nohl, 1989; Nohl et al., 1989). The relevance of this finding to the wasting syndrome in rodents is, however, questionable, because most in vivo studies that have evaluated either the function (Lucier et al., 1973; Courtney et al., 1978; Neal et al., 1979) or morphology (Fowler et al., 1973; Jones and Butler, 1974; Schecter et al., 1985) of rat liver mitochondria have failed to detect any marked abnormalities attributable to TCDD. An exception to this rule is the demonstration by Stohs et al. (1990b) of time-related decreases in membrane fluidity and nonprotein sulfhydryl, as well as NADPH content, in conjunction with increases in calcium content and lipid peroxidation, in hepatic mitochondria from TCDD-exposed rats versus controls fed ad libitum. It is also pertinent to note in this context that TCDD has been shown to exert opposite effects on cholesterol movement in bovine and rat adrenal mitochondria (see section II.D.2).

On the other hand, TCDD may bring about a shift in the proportional use of endogenous fuels for energy. When maintained with carbohydrate-rich diets, TCDD-treated rats had lower respiratory quotients than did pair-fed animals 2 to 3 weeks after exposure (Potter et al., 1986a; Muzi et al., 1989), indicating greater combustion of fat for energy production. Rats exposed to a lethal dose of TCDD also appeared to mobilize their peripheral adipose stores and accumulate substantial amounts of fat in the liver, whereas this did not happen in pair-fed controls (Pohjanvirta et al., 1990b).

In contrast to the evident augmentation of fat utilization, TCDD was shown to impair gluconeogenesis from alanine (Gorski et al., 1990), probably by inhibiting two key enzymes of gluconeogenesis: PEPCK and pyruvate carboxylase (Weber et al., 1991a; Stahl et al., 1992a, 1993a). Concurrently, there was no change in the activity of the key glycolytic enzyme activity, pyruvate kinase (Weber et al., 1991a). Hepatic glycogenolysis may also be inhibited by TCDD. Christian et al. (1986b) reported that TCDD-treated rats accumulated glycogen in their livers during the first 6 days postexposure, and Al-Bayati and Stohs (1991) detected depressed hepatic glycogen

TABLE 2

Comparison of changes in some parameters related to intermediary metabolism in TCDD-treated adult male S-D rats and their pair-fed controls

Parameter	Dose (μg/kg TCDD)	Time (days postexposure)	TCDD vs. ad libitum-fed control	TCDD vs. pair-fed control	Pair-fed control vs. ad libitum-fed control	Reference
Serum glucose	45	7	↓*	↓	⇄	Potter et al., 1983
Plasma lactate	75	6	⇄	⇄	⇄	Christian et al., 1986b
Liver glycogen	75	6	↑↑↑	↑↑↑	⇄	Christian et al., 1986b
Plasma free fatty acids	75	6	⇄†	(↓)	(↑↑)	Christian et al., 1986b
Liver free fatty acids‡	50	13	↑↑↑			Albro et al., 1978
Plasma triglycerides	75	6	(↑↑)§	(↑↑↑)	(↓↓)	Christian et al., 1986b
Liver triglycerides	75	6	↑↑↑	↑↑↑	⇄	Christian et al., 1986b
Plasma ketone bodies	75	6	⇄	(↓↓)	(↑↑)	Christian et al., 1986b
Serum insulin	45	7	↓↓↓	⇄	↓↓↓	Potter et al., 1983
Serum glucagon	45	7	⇄			Potter et al., 1983
Serum somatostatin	45	7	⇄			Potter et al., 1983
Plasma corticosterone	25	8	(↑↑↑)‖	↑↑↑	⇄	Gorski et al., 1988c
Plasma growth hormone	50	7	⇄	⇄	⇄	Moore et al., 1989
Plasma adrenocorticotropic hormone	50	7	⇄	⇄	⇄	Moore et al., 1989
Serum T_4	45	7	↓↓↓	↓↓↓	⇄	Potter et al., 1983
Serum T_3	45	7	⇄¶	⇄	⇄	Potter et al., 1983

* The arrows show the direction and magnitude (one arrow, <25%; two, 25 to 50%, three, >50%) of statistically significant ($P < 0.05$) changes observed; ⇄, no difference; arrow in parentheses, not statistically significant.

† Different results have been obtained in some studies (see II.B.5.)

‡ Female Fischer rats.

§ Unaltered triglyceride levels (vs. ad libitum-fed controls) in various rat strains have also been reported (Poli et al., 1980; Pohjanvirta et al., 1989a). Schiller et al. (1984) found three-fold elevated levels (vs. ad libitum-fed controls) in male Fischer rats 7 days after a dose of 52 μg/kg.

‖ Different results have been obtained in some studies (see section II.D.2.a).

¶ Different results have been obtained in some studies (see section II.D.4).

phosphorylase activities in rats treated with TCDD 3 or 7 days earlier. Likewise, Gasiewicz et al. (1986b) recorded a dose-dependent increase in hepatocellular glycogen content in TCDD-exposed hamsters. Impeded gluconeogenetic capacity might be related to the finding that TCDD-treated rats survived longer receiving a high-carbohydrate rather than a high-fat diet (Muzi et al., 1987, 1989). Furthermore, elimination of [^{14}C]glucose-derived radioactivity was retarded in TCDD-dosed rats (Weber et al., 1987a), whereas the exhalation of $^{14}CO_2$, following administration of [^{14}C]palmitic acid, was promoted (Rozman and Greim, 1986a). Finally, a tissue-specific effect of TCDD on glucose uptake in guinea pigs (decrease in white adipose tissue, pancreas, and brain; increase in liver) was recently disclosed (Enan et al., 1992a,b).

To make the matter less straightforward, other findings contrast with the convergence of the forgoing data pointing to increased fat combustion in TCDD-exposed animals. Christian et al. (1986b) found evidence of reduced fatty acid β-oxidation (and induced esterification) in the livers of male S-D rats treated with 75 μg/kg TCDD versus pair-fed controls. Tomaszewski et al. (1988) reported unaltered mitochondrial and peroxisomal β-oxidation in the livers of male Fischer 344 rats exposed to 160 μg/kg TCDD versus controls fed ad libitum. Lakshman et al. (1991) concluded that β-oxidation was operating normally in TCDD-treated (20 μg/kg) male Wistar rats but that there was a diversion of the acetyl-coenzyme A generated from the tricarboxylic acid cycle to the ketogenic pathway. However, plasma ketone bodies do not usually increase in response to TCDD treatment (Christian et al., 1986b; Pohjanvirta et al., 1990b), although acetone may be an exception (Bagchi et al., 1993). Moreover, guinea pigs exposed to a toxic dose of TCDD (2 μg/kg) 7 days earlier were able to metabolize orally administered [^{14}C]glucose,[^{14}C]alanine, and [^{14}C]oleate to $^{14}CO_2$ as effectively as their pair-fed controls (Neal et al., 1979). Finally, when rats treated with a lethal dose of TCDD were maintained with total parenteral nutrition, they showed hypoglycemia and increased concentrations of serum lipids despite continuous infusion of a liquid diet containing approximately 70% of the caloric intake as dextrose (Gasiewicz et al., 1980).

It seems clear that acute exposure to TCDD can result in relatively large changes in parameters related to lipid metabolism (see also tables 2 and 5). For example, in rats the clearance rate of chylomicra triglycerides was decreased by TCDD; this was coupled with marked shifts in chylomicra apoprotein composition (decrease in apoprotein A-I, increase in apoprotein E; Schiller et al., 1984). TCDD also characteristically brings about marked alterations in plasma levels of triglycerides, free fatty acids, and cholesterol. Nevertheless, the significance of these shifts to the wasting syndrome and TCDD lethality is unclear because of a number of inconsistencies in the findings. First, there are qualitative differences in the

effects without any obvious relation to species-specific susceptibility to TCDD. During the first 10 days after exposure, guinea pigs and rabbits always respond to TCDD with hypertriglyceridemia (Gasiewicz and Neal, 1979; Swift et al., 1981; Brewster and Matsumura, 1984; Brewster et al., 1988), which is a frequent finding in rats as well (Schiller et al., 1984, 1985; Christian et al., 1986b; Tomaszewski et al., 1988). On the other hand, minks, which have almost the same LD_{50} value for TCDD as do guinea pigs, develop hypotriglyceridemia (Brewster, 1985), as may C57BL/6 mice, which have an intermediate sensitivity (Chapman and Schiller, 1985). The highly resistant hamsters tend to show elevated triglyceride levels a few days after TCDD exposure, which then gradually descend below control values by day 20 (Olson et al., 1980b; Brewster, 1985; R. Pohjanvirta and J. Tuomisto, unpublished data).

Second, the findings are sometimes at variance within a given species. Hepatic de novo fatty acid synthesis was reported to be stimulated in S-D rats 8 days after a dose of 125 μg/kg (Gorski et al., 1988e) but decreased in Wistar rats 7 days after doses of 1 to 50 μg/kg (Lakshman et al., 1988, 1989), as compared with pair-fed controls. The latter effect was subsequently confirmed by demonstrating that TCDD inhibits the activity of a key enzyme in the synthesis of fatty acids, acetyl-coenzyme A carboxylase, by 65% (McKim et al., 1991). A similar discrepancy occurs in findings concerning cholesterol synthesis after TCDD administration. The synthetic rate was increased in S-D rats treated with 10 μg/kg TCDD (Edwards, 1984) but was inhibited in Wistar rats exposed to the same dose of TCDD (Lakshman et al., 1988, 1989).

Third, even if the direction of alteration in a given parameter is consistent, the correlation with TCDD lethality may be poor. The serum concentration of free fatty acids was observed to increase at a lethal dose (50 μg/kg) in L-E rats versus controls fed ad libitum, whereas nonlethal doses did not affect this parameter in L-E (5 μg/kg), H/W (5 to 500 μg/kg), S-D (75 μg/kg), or Fischer (60 μg/kg) rats (Schiller et al., 1985; Christian et al., 1986b; Pohjanvirta et al., 1989a). However, a low dose in the nonlethal range (20 μg/kg) was capable of increasing the level of free fatty acids in Wistar rats (Lakshman et al., 1991). Both total cholesterol and high-density lipoprotein-associated cholesterol were found to be elevated in sera of L-E and H/W rats regardless of the TCDD dose (5 to 500 μg/kg) (Pohjanvirta et al., 1989a).

Fourth, pharmacological intervention with drugs that alter lipid metabolism has produced discrepant results. Both diet-induced experimental hyperlipidemia (Marinovich et al., 1983) and treatment with a hypolipidemic agent, di(2-ethylhexyl)phthalate (Tomaszewski et al., 1988), partially protected rats from TCDD lethality, whereas another hypolipidemic substance, 4-aminopyrazolo-[3,4-*d*]-pyrimidine, shortened the mean time to death (Schiller et al., 1986).

Despite the qualitative differences among species in the shifts caused by TCDD in lipid metabolism, in both rats and guinea pigs the rapid decline in body weight after lethal or near-lethal TCDD doses results mainly from loss of body lipid and in pair-fed controls is of the same magnitude as in TCDD-treated animals (Kelling et al., 1985; Potter et al., 1986a; Pohjanvirta et al., 1990b). On the other hand, at sublethal doses in animals with permanently lower body weight the proportional composition of the carcass in TCDD-exposed rats is similar to that of the controls fed ad libitum (Seefeld et al., 1984b). In guinea pigs, the shrinkage of white adipose tissue appears to be due to rapid, grave, and persistent inhibition of lipoprotein lipase activity by TCDD (Brewster and Matsumura, 1984, 1988; Brewster, 1985). In rats treated with 25 μg/kg TCDD, however, there was no reduction in lipoprotein lipase activity in the adipose tissue (Brewster, 1985). Although a lethal dose might have led to a different outcome, it is possible that different mechanisms are at work in rats and guinea pigs but that the final result is the same.

One such mechanism in rats may be related to inositol metabolism. Inositol deficiency elicits adipose lipolysis and hepatic fat accumulation very similar to that seen in lethally TCDD-intoxicated rats, presumably in response to decreased hypothalamic concentrations of inositol (Hayashi et al., 1978). A recent study revealed that TCDD significantly reduces inositol levels in various regions of the brain, including the hypothalamus (Pohjanvirta et al., 1994a). Furthermore, the lipolysis associated with inositol deficiency appears to be mediated through activation of the sympathetic nervous system (Hayashi et al., 1978). In TCDD-exposed rats, propranolol treatment on day 10 attenuated, but did not totally abolish, the increase in free fatty acids in the plasma (R. Pohjanvirta and J. Tuomisto, unpublished data).

Protein metabolism may be less affected by TCDD than fat or carbohydrate metabolism. Although statistically significant alterations (preponderantly increases) were observed in the plasma concentrations of several amino acids in TCDD-treated (75 μg/kg) S-D rats, the alterations in both gluco- and ketogenic amino acids were inconsistent (Christian et al., 1986b). These findings were recently corroborated in L-E rats (R. Pohjanvirta and J. Tuomisto, unpublished data). An exception to this general scheme is tryptophan, which appears to be affected by TCDD in a specific manner (see section II.E.3.c). Urinary urea and ammonia secretions were similar in TCDD-treated and pair-fed rats up to day 8 after exposure (Christian et al., 1986b; Potter et al., 1986a). Thereafter, in TCDD-dosed rats there was a tendency toward increased ammonia excretion (Potter et al., 1986a). The toxicity of TCDD was not markedly influenced by the concentration of protein in the diet (van Logten et al., 1981; Muzi et al., 1987).

6. Role of altered intermediary metabolism in wasting syndrome. The contribution to the wasting syndrome of TCDD-provoked changes in intermediary metabolism is still obscure but does not seem to be a major determinant. Some investigators have found a slightly or moderately higher rate of body weight decline in TCDD-treated rats than in their pair-fed counterparts (Gorski et al., 1988c; Rozman, 1989), whereas others have recorded a similar or even identical pattern of body weight alterations in the two groups (Peterson et al., 1984b; Kelling et al., 1985; Moore et al., 1985; Christian et al., 1986a; Pohjanvirta et al., 1990b). A possible reason for the variation in outcome is the conspicuous feed spillage behavior of animals treated with TCDD. If this is not rigorously controlled, pair-fed controls will be overfed relative to TCDD-exposed animals. Another important factor may be the relative toxicity of the administered dose of TCDD, i.e., whether the exposure is lethal or sublethal. During the actual body weight loss phase of TCDD intoxication (usually the first 1 to 2 weeks), there is often little, if any, difference in body weights between TCDD-treated and pair-fed rats, but when body weight restabilizes (at sublethal doses), pair-fed rats tend to permanently retain a 3 to 4% higher level (Seefeld and Peterson, 1984).

7. Relationship between wasting syndrome and 2,3,7,8-tetrachlorodibenzo-p-dioxin lethality. At first sight, it would seem clear that such a drastic impact on body weight, which invariably accompanies lethal TCDD intoxication, should be fatal by itself. Indeed, this has proved to be the case in several studies in which a similar time course to death and similar mortality rate in TCDD-treated and pair-fed animals were reported (Peterson et al., 1984b; Kelling et al., 1985; Weber et al., 1991b). The view is further supported by findings from a study assessing the contribution of inhibited gluconeogenesis by TCDD to its lethality. When pair-fed to lethally TCDD-exposed rats (125 μg/kg), both untreated controls (euglycemic) and rats treated with a sublethal dose of 25 μg/kg (suffering from inhibited gluconeogenesis) exhibited the same mortality rate and the same time course to death as their lethally intoxicated counterparts (Weber et al., 1991b).

However, the issue is ultimately more complicated. In some cases the pair-fed controls died far sooner than the TCDD-dosed animals and obviously from a different cause, i.e., severe gastrointestinal bleeding (Pohjanvirta et al., 1990b). TCDD-treated rats seem to be protected against gastrointestinal ulceration and mucosal atrophy created by inanition, at least in part because of substantially elevated concentrations of gastrin (Mably et al., 1990; Theobald et al., 1990, 1991), which acts as a mucoprotectant (Johnson, 1988). TCDD may exert a disinhibitory effect on gastrin release by reducing the gastric concentration of somatostatin (Potter et al., 1983). It is also possible that TCDD blunts responsiveness to stress (Christian and Peterson, 1983). The fate of pair-fed rats is further dependent on factors such as type of cage. Pair-fed L-E rats die earlier from gastrointestinal bleeding in cages that prevent them from engaging in coprophagia and pica than in solid-bottom cages with suitable bedding material (R. Pohjanvirta and J. Tuomisto, unpublished data). The contribution of wasting to TCDD lethality may also vary among species and (sub)strains, albeit wasting is always a major determinant (Kelling et al., 1985).

What would then happen if hypophagia and the concomitant loss of body weight were prevented in TCDD-treated animals? Bearing in mind the findings in pair-fed animals, a logical guess might be that such a procedure should abolish TCDD lethality. Surprisingly enough, however, this is not the case. When TCDD-exposed rats and control rats (strain unspecified) or guinea pigs were maintained with total parenteral nutrition leading to an equal gain in body weight (rats) or at least maintenance of original body weight (guinea pigs), no alleviating effect on TCDD lethality was detected (Gasiewicz et al., 1980; Huang Lu et al., 1986). Although the forced feeding exacerbated some detrimental effects of TCDD in the rat (e.g., liver lesion), this was not observed in the guinea pig. Recently, we replicated the rat study in L-E rats with permanent gastric cannulas (R. Pohjanvirta and J. Tuomisto, unpublished data). The rats were fed a nutritionally complete liquid diet that was administered with a peristaltic pump at predetermined times mimicking the normal circadian feeding rhythm. No wasting syndrome was recorded in rats receiving a dose of TCDD that would usually be lethal. In spite of this, all of the TCDD-dosed rats died at approximately the same time after exposure that is typical for free-feeding L-E rats. Thus, it is evident that TCDD possesses at least two lethal mechanisms (which need not be independent of each other). One of them is associated with the wasting syndrome and is operative if the exposed animals have free access to feed. The other is normally latent and becomes manifest only if the TCDD-treated animals are forced to consume more energy than they would voluntarily. The wasting syndrome has also occasionally been dissociated from the lethal action of TCDD in free-feeding rats (Rozman, 1984a). The biochemical nature of these mechanisms, as well as their mutual relationship, remains to be elucidated.

C. Histopathology, Hematology, and Clinical Chemistry

1. Histopathology. The morphological lesions elicited by TCDD and related compounds have previously been reviewed extensively (Vos, 1978; McConnell and Moore, 1979; Kimbrough, 1983). Therefore, we will present only a summary of the findings along with a brief description of changes in tissues that are most pertinent to the mechanistic hypotheses described in section VI (liver and brown adipose tissue), to immune suppression (sec-

tion II.G; thymus), or to endocrine imbalances (section II.D; pancreas, gonads, and the pituitary, adrenal, thyroid, and pineal glands).

As pointed out by Poland and Knutson (1982) and as is also evident from table 3, TCDD predominantly affects epithelial tissues, inducing proliferative, metaplastic, or atrophic changes. At some sites, however, cells of (ecto-)mesenchymal origin are the preferential targets. For example, in murine bone marrow the mesenchymal stem cells were considered to be primarily affected by TCDD, not the epithelial cells of the feeder layer (Luster et al., 1985). Likewise, in rat incisors the ectomesenchymal odontoblasts turned out to be more vulnerable to TCDD than were the epithelial ameloblasts (Alaluusua et al., 1993).

a. THYMUS. Thymic atrophy is one of the most uniform and consistent findings in TCDD-exposed mammals. It consists of depletion of small immature cortical thymocytes to the extent that it becomes almost impossible to make a distinction between cortex and medulla (Gupta et al., 1973; Vos and Moore, 1974; Vos et al., 1974; McConnell et al., 1978b; Pohjanvirta et al., 1989a; Kerkvliet and Brauner, 1990). Although both pyknotic (Buu-Hoï et al., 1972b) and scattered necrotic lymphocytes (McConnell et al., 1978b) have been reported, frank necrosis is not a typical feature of the lesion. Major (immuno)histological and ultrastructural changes indicative of an altered state of differentiation have recently been described in thymic epithelial cells (De Waal et al., 1992, 1993). Four underlying mechanisms have been implicated in the pathogenesis of thymus atrophy by TCDD: inability of thymic epithelial cells to support the maturation process of T-lymphocyte precursors (Greenlee et al., 1984, 1985a; Lundberg et al., 1990), enhanced apoptosis (McConkey et al., 1988; McConkey and Orrenius, 1989), blocked or delayed thymocyte maturation (Holladay et al., 1991; Blaylock et al., 1992), and impaired thymic seeding by prothymocytes (Fine et al., 1990a,b). The stimulation of protein-tyrosine kinase activities by TCDD is likely to play a part in the process of atrophy (Bombick et al., 1988). An indirect mechanism through increased levels of circulating corticosterone has also been suggested (Gorski et al., 1987). However, adrenalectomy or hypophysectomy does not prevent the impact of TCDD on the thymus (van Logten et al., 1980), the cellular targets for TCDD and glucocorticoids are distinct (Fine et al., 1990b), and TCDD-induced atrophy is likewise detectable in rats treated repeatedly with a high dose of dexamethasone (Pohjanvirta et al., 1988b). Transplantation experiments have revealed that thymic atrophy is reversible within 3 weeks (Van Loveren et al., 1991).

b. LIVER. TCDD affects the liver most severely in the rabbit, which displays extensive necrosis (Kimbrough, 1983). In other species, such as the rat, mouse, and (to a lesser extent) guinea pig and hamster, the predominant features are hepatocellular hypertrophy, multinucleate hepatocytes (in rats and hamsters), steatosis, and inflam-

TABLE 3
*Histopathological alterations caused by TCDD**

	Rhesus monkey	Guinea pig	Cow	Rat	Mouse	Rabbit	Chicken	Hamster
Hyperplasia and/or metaplasia								
Gastric mucosa	++	0	+	0	0			0
Intestinal mucosa	+							++
Urinary tract	++	++	++	0	0			
Bile duct and/or gall bladder	++		+		++			
Lung: focal alveolar				++				
Skin	++		+†	0	0	++		
Pancreas								+‡
Hypoplasia or atrophy								
Thymus	+	+	+	+	+		+	+
Bone marrow	+	+			+§		+	
Testicle	+	+		+	+		+	
Other								
Hepatomegaly	+	+		+	+	+	+	+
Liver necrosis	0	0		+	+	++	+	0
Edema	+	0		0	+		++	+
Tooth lesion				+‖				
Brown fat lesion				+¶				
Toxic polyneuropathy				++#				

* Symbols: 0, lesion not observed; +, lesion observed (with number of + signs denoting severity); blank, no evidence reported in literature. Adapted from Poland and Knutson (1982; with permission granted by Annual Reviews Inc.) as modified by McConnell (1984).

† Different from those in other species.

‡ Rao et al., 1988b.

§ Luster et al., 1985.

‖ Alaluusua et al., 1993.

¶ Rozman et al. 1986a, b; Rozman et al., 1987b.

Grehl et al., 1993.

matory cell infiltration, often accompanied by scattered focal necrosis with a preferentially centrilobular location (Greig et al., 1973; Jones and Butler, 1974; Vos et al., 1974; Turner and Collins, 1983; Gasiewicz et al., 1986b; Pohjanvirta et al., 1989a). Electron microscopy reveals proliferation of smooth and, frequently, rough endoplasmic reticula (Fowler et al., 1973; Jones and Butler, 1974; Olson et al., 1980b). Plasma membrane abnormalities have also been detected by electron microscopy and histochemistry (Jones and Butler, 1974; Jones, 1975). Absolute and/or relative liver weight is consistently increased for extended periods (Olson et al., 1980b; Chapman and Schiller, 1985; Pohjanvirta et al., 1990a). At least in the rat, however, the initial liver swelling changes to liver atrophy between days 8 and 16 after lethal doses are given (Pohjanvirta et al., 1989a, 1990a,b).

c. BROWN ADIPOSE TISSUE. TCDD-provoked histological changes in the brown adipose tissue of male S-D rats have been described by Rozman and his colleagues. After either a sublethal or a lethal dose of TCDD, the interscapular brown fat underwent a similar course of morphological events. Initially, the number of lipid droplets decreased and concomitantly the individual droplets enlarged. This was followed by progressive lipid depletion starting at 4 days postexposure. There seemed to be parallel alterations in glycogen with a short time lag. During days 8 to 14, the cells shrank, the intercellular spaces widened, the mitochondria swelled and became disorganized, and lysosomal activity was enhanced (Rozman et al., 1986a,b, 1987b).

d. PANCREAS, THYROID, PITUITARY, ADRENALS. Few studies have detected histological evidence of damage in these endocrinologically important organs after exposure to TCDD, in spite of marked deviations in the respective hormone levels. However, Rozman et al. (1986a) reported changes in pancreatic and thyroid architecture 7 days after a dose of 150 μg/kg to male S-D rats. In the pancreas, the cells of the islets of Langerhans failed to stain normally and exhibited cytoplasmic and nuclear hypertrophy. In the thyroid, the follicles became distended, which was accompanied by flattening of the follicular epithelium, suggesting diminished activity. The alterations in both tissues were more pronounced 14 days after the lethal dose. By contrast, Gupta et al. (1973) found that TCDD noticeably reduced the amount of follicular colloid in the thyroid glands of rats treated repeatedly with a sublethal dose of TCDD. In addition, the epithelial lining was disorganized, was exfoliated, or had papillary projections into the lumen of the follicle; this suggested high functional activity. Bastomsky (1977) observed that TCDD increased the weight of the rat thyroid, but he did not examine its morphology. The conflicting findings may be attributable to disparities in the severity of the TCDD exposure, with sublethal doses tending to stimulate the thyroid tissue and lethal doses depressing it. The changes may also be dependent on time such that initial suppression could be followed by increased activity. However, wide interindividual variation in thyroid architecture, regardless of TCDD dose, has been noted in rats (Potter et al., 1986b), which probably contributes to the more usual lack of differences among dosage groups after either sublethal or lethal doses (Potter et al., 1986b; Pohjanvirta et al., 1989a).

As for the pituitary gland in rats, Gorski et al. (1988b) recorded a delayed atrophying effect of TCDD, mainly on the chromophobe cells. The impact was discernible on day 32 after exposure and was more severe at a lethal than at a sublethal dose. Vos and Moore (1974) noted less intense staining in acidophilic cells of the anterior lobe in rat pups exposed to 5 μg/kg TCDD postnatally on days 0, 7, and 14 and killed on day 25. More recently, Pohjanvirta et al. (1993b) found incidentally that TCDD decreased pituitary weight in adult rats as an early effect, developing by day 4. Because the decrease occurred in both L-E and H/W rats and only later in feed-restricted L-E rats, it appeared to be a primary response. Unfortunately, histological examination was not performed.

Gupta et al. (1973) described lesions in the adrenal gland of guinea pigs that had died or become moribund from TCDD intoxication. These comprised atrophy of the zona glomerulosa and mitotic figures along with loss of lipid vacuoles in the zona fasciculata, accompanied by medullary hemorrhage. Similar findings were made in S-D rats by Gorski et al. (1988c). The zona fasciculata was widened at the expense of the zona glomerulosa, concomitantly with hyperplasia in the zona reticulosa and hyperplasia as well as hypertrophy in the medulla. These changes were progressive from day 8 onward at a lethal dose (125 μg/kg) but did not occur at a sublethal dose (25 μg/kg) until the end of the study (day 32).

e. GONADS. TCDD does not appear to exert any severe effect on the ovaries. Nevertheless, a diminished number of corpora lutea were noticed 2 weeks after a single exposure to 10 or 20 μg/kg in C57BL/6 mice (Silbergeld and Mattison, 1987) and 13 weeks after continuous daily exposure to 1 μg/kg TCDD in S-D rats (Kociba et al., 1976). In the latter study, cytoplasmic foaminess and nuclear hyperchromatism in the interstitial gland cells of the ovarian stroma were also reported at the same dose level.

At high doses of TCDD, degeneration of the seminiferous components of the testicles was detected in guinea pigs, C57BL/6 mice, and Wistar rats (McConnell et al., 1978b; Chahoud et al., 1989; Birnbaum et al., 1990). Recent studies of Wistar rats and marmoset monkeys by Neubert and associates (Rune et al., 1991a,b; Chahoud et al., 1992) revealed that a single dose of 3 μg/kg was sufficient to result in decreased intercellular contact between Sertoli cells or between Sertoli and neighboring germ cells, along with sloughing off of immature spermatids into the tubular lumen, 7 days after exposure. However, when testicles from S-D rats treated with a

single dose of 12.5 to 50.0 μg/kg TCDD 4 weeks earlier were evaluated both qualitatively and quantitatively, Johnson et al. (1992b) found a dose-dependent reduction in the volume of Leydig cells without appreciable changes in spermatogenetic indices.

2. Hematology and clinical chemistry. The most important findings for guinea pigs, rats, mice, rhesus monkeys, and hamsters have been compiled in tables 4 and 5.

a. HEMATOLOGY AND DERAILMENTS IN THE SYNTHESIS AND CATABOLISM OF HEME. A relatively consistent feature across species (except for the guinea pig) appears to be a shift in the proportional numbers of leukocytes with a trend toward an increase in neutrophils at the expense of lymphocytes. This is the classical manifestation of stress. Although in most cases this shift occurred either dose dependently or at doses of approximately the LD_{50} level, it cannot be totally due to the wasting syndrome, because it was also (transiently) discernible in hamsters with negligible or no loss of body weight (R. Pohjanvirta and J. Tuomisto, unpublished data). However, it may be related to the endocrine imbalances produced by TCDD (especially corticosterone; see section II.D).

Another common alteration is thrombocytopenia, which displays a distinct dose dependence in rats (Zinkl et al., 1973). The etiological factors underlying this condition are as yet unresolved. Weissberg and Zinkl (1973) found a normal number of bone marrow megakaryocytes in female CD rats given daily oral doses of 10 μg/kg TCDD for 14 days, which suggests that platelet production remained unaffected. In addition, no fibrinogen degradation products were detected in serum, which seemed to rule out the possibility of increased platelet consumption due to disseminated intravascular coagulation. It should be noted, however, that both thrombus formation in various tissues and tail tip necrosis have been reported in rats (male or mixed sex) given high doses of TCDD (Gupta et al., 1973; Pohjanvirta et al., 1987). As for platelet function, Weissberg and Zinkl (1973) observed unaltered bleeding time and velocity of platelet aggregation but diminished clot reaction in their TCDD-treated rats. A perplexing finding was prolonged prothrombin consumption time concurrent with normal prothrombin time, possibly suggesting deficiency or inactivation of coagulation factor VII. In a recent study, Bouwman et al. (1992), using a much lower exposure (1 μg/kg by single injection), failed to find any effect of TCDD on the activity of this or two other vitamin K-dependent factors (II and X) in germ-free WAG/Rij rats. In conventionally raised rats of the same strain, even a ten-fold lower dose of TCDD reduced the levels of factor VII in females but increased them in males; analogous trends were detected for factor II (Bouwman, 1994). More sensitive analytical methods were used in the latter experiments, possibly accounting for the different outcomes in the two studies. The gender dependence of the shift cannot, however, be explained in the same way and warrants further research.

The increased values for hemoglobin, hematocrit, and red blood cells in TCDD-treated rats may result from hemoconcentration in the early phases of intoxication. Alternatively, they might also reflect a gender-specific pattern of responses, because female rats were used in the studies cited in table 4. In subchronic experiments, TCDD treatment (25 μg/kg/week for 6 weeks in mice; 0.1 or 1.0 μg/kg, 5 days/week for 13 weeks in rats) depressed hemoglobin concentration in male C57BL/6 mice and male S-D rats but tended to elevate it in the respective female animals (Vos et al., 1974; Kociba et al., 1976).

TABLE 4

*General hematological trends in five laboratory animal species in response to TCDD**

	Guinea pig (Hartley; female)†	Rat (CD; female)†,‡	Mouse			Rhesus monkey (female)‖	Syrian hamster (male)¶
			CD-1 (female)†	C57BL/6$^{b/b}$ (male)§	C57BL/6$^{d/d}$ (male)§		
Leukocytes (total)	↓	⇄	↓	⇄	⇄	↑	⇄
Neutrophils	⇄ or ↓	↑		↑	↑	↑	⇄ or ↑
Lymphocytes	↓	⇄	↓	⇄ or ↓	⇄ or ↓	↓	⇄ or ↓
Monocytes		⇄		⇄	⇄	⇄	
Eosinophils		⇄		⇄	⇄	⇄	⇄
Hemoglobin	⇄	↑		⇄	↓	⇄	⇄
Packed cell volume	⇄	↑		⇄	↓	↓	⇄
Erythrocytes	⇄	↑	⇄	⇄	↓	⇄	⇄
Platelets	↓	↓		⇄ or ↑	⇄	↓	⇄
Reticulocytes		⇄ or ↑		⇄	⇄		
Megakaryocytes#		⇄		⇄	⇄		

* Symbols: ↑, increase; ↓, decrease; ⇄, unaltered; blank, not determined.
† Zinkl et al., 1973.
‡ Weissberg and Zinkl, 1973.
§ Birnbaum et al., 1990.
‖ McConnell et al., 1978a.
¶ Our own unpublished data.
# Bone marrow smears.

Two heme-related phenomena usually not seen in the early stages after TCDD exposure, but characteristic of its prolonged toxicity in some rodents, are porphyria and jaundice. The porphyrinogenic effect of TCDD has been detected in mice (Goldstein et al., 1973; Sweeney et al., 1984) and rats (Kociba et al., 1976; Cantoni et al., 1981). It results in an accumulation of porphyrins (mostly uroporphyrin), particularly in the liver (Goldstein et al., 1973; Cantoni et al., 1987). The predominant mechanism seems to be suppressed activity of uroporphyrinogen decarboxylase (Jones and Sweeney, 1977; Smith et al., 1985; Cantoni et al., 1987), possibly contributed to by (presumably secondary) induction of δ-aminolevulinic acid synthetase (Goldstein et al., 1973, 1982) and auxiliary factors (Lambrecht et al., 1988). Moreover, iron deficiency (Jones et al., 1981) and supplementation of the diet with the antioxidant butylated hydroxyanisole (Sweeney et al., 1984) was demonstrated to delay or mitigate TCDD-induced porphyria, pointing to involvement of free radicals in its pathogenesis.

TCDD has been shown to elevate serum total and/or direct bilirubin concentrations following subacute (Zinkl et al., 1973) and subchronic (Kociba et al., 1976) dosing in rats. With regard to single exposure, earlier studies suggested that this alteration might emerge more rapidly in hamsters (within 3 days; Olson et al., 1980b) than in rats, in which it was not detectable until 10 days after exposure (Buu-Hoï et al., 1972a; Greig et al., 1973). Recent studies of L-E rats, however, revealed that both total and direct bilirubin were progressively elevated with time, and the departure from control values was already statistically significant on day 1 (Unkila et al., 1994b). The increase in serum bilirubin may be due to diminished clearance (Choe and Yang, 1983; Yang et al., 1983b) or augmented formation (Mitchell et al., 1990) of bilirubin in the liver by TCDD. On the other hand, in congenitally jaundiced Gunn rats a single dose of TCDD reduced serum bilirubin levels by inducing hepatic bilirubin catabolism (Cohen et al., 1986).

b. CLINICAL CHEMISTRY. The morphologically observable liver damage after TCDD exposure (see section II.C.1.b) is reflected in serum activities of the liver-specific transaminases (alanine aminotransferase, aspartate aminotransferase) and alkaline phosphatase. The milder effect of TCDD on the liver of hamsters and guinea pigs, as compared with rats and mice, probably accounts for their differential transaminase responses. The increases in alanine aminotransferase and aspartate aminotransferase appear to be closely associated with necrotic changes in the liver, because there was no departure from control levels in these enzyme activities in sera from lethally intoxicated L-E rats that displayed all of the other typical features of TCDD-induced hepatic lesion, except for necrotic foci (Pohjanvirta et al., 1989a, 1990b).

A noteworthy aspect of the clinical chemistry trends depicted in table 5 is the striking scarcity of alterations in the most TCDD-susceptible species, the guinea pig, which has also been corroborated in other studies [Gasiewicz and Neal, 1979; Bak et al., 1982 (although the former researchers reported dehydration-related changes and an initial decrease followed by an increase in blood urea nitrogen)]. In all cases, there has been a consistent and pronounced elevation in cholesterol and triglycerides. This suggests that TCDD may have a more selective impact on lipid metabolism in guinea pigs than in other laboratory animals. Regarding the mouse, it should be noted that Chapman and Schiller (1985) found dose-dependent reductions in serum triglycerides and glucose in both C57BL/6 and DBA/2 mice in response to TCDD exposure. The major difference in the experimental design between that study and the one cited in table 5 (Birnbaum et al., 1990) was that Chapman and Schiller's mice fasted overnight before termination, whereas Birnbaum's group used ad libitum-fed animals. In contrast to the unaltered levels of total cholesterol reported in Wistar rats (table 5; Buu-Hoï et al., 1972a), several studies of various rat strains have detected elevations in serum (or plasma) cholesterol after exposure to TCDD (Zinkl et al., 1973; Poli et al., 1980; Walden and Schiller, 1985; DiBartolomeis et al., 1986a; Pohjanvirta et al., 1989a). On the other hand, the increase in blood urea nitrogen recorded by Buu-Hoï et al. (1972a) may have been due to the supralethal dose used (10,000 μg/kg). When lower doses have been administered to rats, no changes have usually been found in this parameter (Zinkl et al., 1973; R. Pohjanvirta and J. Tuomisto, unpublished data).

D. Endocrine Imbalances

Most studies dealing with the influence of TCDD on the endocrine status in the body have used rats as subjects. Actually, information concerning other species is still extremely sparse and patchy. In view of the wide variability in clinical chemistry responses among species, one should be cautious about generalizing the findings until more data are available.

1. Pituitary gland. a. ANTERIOR PITUITARY HORMONES. TCDD was reported to affect circadian profiles of plasma prolactin in male S-D rats (Jones et al., 1987b; Russell et al., 1988). As few as 4 h after a dose of 50 μg/kg, compared with pair-fed or ad libitum-fed controls, there was a decrease in prolactin concentration, which could be reversed by pimozide, a dopamine receptor antagonist. At 7 days, the opposite was true for the overall daily secretion of prolactin; the peak concentrations also exhibited temporal shifts. Furthermore, TCDD diminished (by up to 88%) the responsiveness of ornithine decarboxylase to prolactin stimulation in various tissues (cf. section II.I.3). In contrast to these results, Moore et al. (1989) discovered a dose-related decrease (versus ad libitum-fed and pair-fed controls) in plasma prolactin 7 days after TCDD exposure. They also examined the time

TABLE 5

*General trends in selected serum clinical chemistry values induced by TCDD in five laboratory animal species**

	Guinea pig (Hartley; male)†	Rat (Wistar; both sexes)‡	Mouse		Rhesus monkey (female)‖	Syrian hamster (male)¶
			C57BL/6$^{b/b}$ (male)§	C57BL/6$^{d/d}$ (male)§		
Sodium	⇄	↓			⇄	⇄
Potassium		⇄			⇄	⇄
Calcium	⇄				⇄ or ↓	⇄
Chloride	⇄	⇄			⇄	↓
Aspartate aminotransferase	⇄	↑			↑	⇄
Alanine aminotransferase	⇄	↑	↑	↑		⇄
Alkaline phosphatase	⇄	⇄			⇄	↑
Urea nitrogen	⇄	↑			⇄ or ↑	↓ or ↑
Creatinine	⇄					⇄
Total protein	⇄	↓			↓	⇄ or ↑
Albumin	⇄				↓	↓
Glucose	⇄	↓	⇄	↓	↓	⇄
Triglycerides	↑	↑	⇄	⇄	↑	⇄ or ↓
Cholesterol	↑	⇄	↓	↓	↓	↑

* The symbols are as in Table 4.
† Huang Lu et al., 1986.
‡ Buu-Hoï et al., 1972a (see also Table 2).
§ Birnbaum et al., 1990.
‖ McConnell et al., 1978a.
¶ Olson et al., 1980b.

course at 50 μg/kg during the first 7 days posttreatment and recorded an increase on day 3 with no other statistically significant differences. Because both research groups used male S-D rats (although of somewhat different ages), at present the discrepancy in the findings precludes a clear view of the influence of TCDD on plasma prolactin. In addition, Russell et al. (1988) found alterations in hypothalamic dopamine turnover, which has not been corroborated in subsequent studies (see section II.E.3.a). However, the responsiveness of cells to prolactin appears to be substantially reduced by TCDD.

Data reported in the literature are also inconsistent with regard to adrenocorticotropin. Whereas Moore et al. (1989) failed to detect any alterations in the plasma levels of this hormone during the first week after dosing and DiBartolomeis et al. (1987) found no changes on day 13, Piper and coworkers (Bestervelt et al., 1993a) observed consistent 1.5- to 3.0-fold elevations during days 1 to 14 after exposure; in all these studies male S-D rats were treated with 50 μg/kg TCDD. Piper's group also reported a 4.7-fold increase in pituitary adrenocorticotropin levels on day 2 (Bestervelt et al., 1990). They further demonstrated that both in vivo and in vitro exposure to TCDD was capable of diminishing the bioactivity of the adrenocorticotropin secreted by the anterior pituitary cells (Bestervelt et al., 1993a,b).

Adrenocorticotropin originates from a large precursor molecule, pro-opiomelanocortin. Another compound arising from this molecule and secreted by the pituitary gland is β-endorphin. A recent study disclosed a differential β-endorphin response to TCDD between L-E and H/W rats (Pohjanvirta et al., 1993b). A dose of 50 μg/kg rapidly and persistently reduced plasma β-endorphin concentration by 24 to 37% in L-E rats but had no discernible effect on that parameter in H/W rats. Concurrently, pituitary β-endorphin concentration remained at the control level in both strains. The decrease in plasma β-endorphin in L-E rats coupled with the above-mentioned increased secretion of functionally improper adrenocorticotropin in S-D rats collectively suggest that TCDD may interfere with peptide processing in the anterior pituitary gland.

No statistically significant effects of TCDD on plasma growth hormone or follicle-stimulating hormone have been observed in adult male S-D rats (Gorski et al., 1988b; Moore et al., 1989). Concentrations of thyroid-stimulating hormone have frequently been found to be promoted by TCDD, whether assessed by radioimmunoassays (Potter et al., 1986b; Pohjanvirta et al., 1989a) or a bioassay (Bastomsky, 1977). This upward shift could represent the response to a lowered level of circulating T_4 (see section II.D.4). In hamsters, however, a sublethal dose of 100 μg/kg TCDD elevated the levels of thyroid-stimulating hormone (measured on days 2, 7, and 21) as well as those of T_4 (throughout the 53-day experiment) and T_3 (on days 2 and 7; Henry and Gasiewicz, 1987), suggesting that TCDD either enhances the secretion of thyroid-stimulating hormone (directly or via the hypothalamic releasing hormone) or diminishes the potency of thyroid hormones for feedback inhibition (cf. below). The increase detected in rats tended to level off at high doses of TCDD, with reduced intake of feed exerting a counteracting force (Potter et al., 1986b). There are also reports of unchanged serum levels of

thyroid-stimulating hormone in TCDD-treated rats (Gorski and Rozman, 1987; Henry and Gasiewicz, 1987; Gorski et al., 1988b).

In addition to prolactin, LH provides another example of how TCDD may alter the sensitivity of cells to endocrine factors. Although serum LH concentration may decrease in the early phases of TCDD intoxication (Ruangwises et al., 1991), it returns to the control level by day 7 (Moore et al., 1989; Ruangwises et al., 1991). At that time, however, serum testosterone reaches its nadir (see section II.D.5.b). Thus, TCDD seems to decrease testicular responsiveness to LH. Furthermore, untreated rats would normally react to the diminished feedback inhibition by testosterone by markedly increasing their LH secretion. The reason for the striking lack of such a response in TCDD-treated rats has been elegantly elucidated by Peterson and his associates (Bookstaff et al., 1990a,b). They demonstrated that TCDD did not prevent plasma LH from increasing in castrated male rats; nor did TCDD affect pituitary content of LH in castrated rats with testosterone implants. These findings indicated that the synthesizing capacity of the pituitary remained intact. The clearance rate of exogenous LH was also similar in TCDD-treated and control rats. However, the ability of testosterone and its two metabolites to inhibit pituitary LH secretion was markedly augmented by TCDD. The increased potency of testosterone was accompanied by a reduced efficiency of gonadotropin-releasing hormone to stimulate LH secretion and by a diminished number of pituitary gonadotropin-releasing hormone receptors.

Hence, the anterior lobe of the pituitary gland has turned out be a target for TCDD toxicity, exhibiting both exaggerated and suppressed responses to its regulatory hormones after TCDD exposure. Therefore, it is of special interest that hypophysectomy has been shown to aggravate the acute toxicity of TCDD by both increasing the mortality rate and curtailing the mean time to death (Gorski et al., 1988a). The exceptional susceptibility of hypophysectomized rats to TCDD was partially normalized by corticosterone or T_4 supplementation, but it appeared to involve one or more unknown auxiliary factors residing in the pituitary. It was not associated with alterations in hepatic Ah receptors (Carlstedt-Duke et al., 1979).

b. POSTERIOR PITUITARY HORMONES. In a recent study, plasma concentrations of oxytocin were utilized as a biochemical index to assess the severity of nausea produced by TCDD in rats (Pohjanvirta et al., 1994b). TCDD did not influence the basal levels of this hormone in either H/W rats treated with a high, but sublethal, dose (1000 μg/kg) or L-E rats exposed to a lethal dose (50 μg/kg). However, by 8 days after exposure, TCDD-treated L-E rats became hyperresponsive in terms of plasma oxytocin to a model nausea-provoking agent, lithium chloride. Thus, the outcome represents an intriguing analogy to the impact of TCDD on the regulation of LH secretion.

2. Adrenal gland. a. CORTICOSTERONE. Vos et al. (1973) reported that in TCDD-exposed guinea pigs serum levels of cortisol and corticosterone remained unaffected. The time of sampling was, unfortunately, not reported. Lin et al. (1991b) observed a moderate dose-dependent upward trend in plasma corticosterone for C57BL/$6^{b/b}$ mice (but not for C57BL/$6^{d/d}$ mice) that were killed a few hours after the lights were turned on 7 days postdosing. With regard to rats, the data reported in the literature vary considerably. Balk and Piper (1984) recorded a decrease in corticosterone versus ad libitum-fed controls 14 and 21 days after a single oral administration of 25 μg/kg to male S-D rats; the rats were killed 4 h before lights were turned off. Neal et al. (1979) monitored plasma fluorescence due to 11-hydroxycorticosteroids in young male S-D rats given 50 μg/kg TCDD and found up to a 40% depression during the first 4 days, followed by a three-fold increase at days 7 and 14; the times of killing were not stated. Peterson et al. (1984a; Moore et al., 1985) reported that, when measured at the diurnal maximum (late in the light phase), there was no change or only a slight decrease in plasma corticosterone, regardless of the TCDD dose, 7 days after treatment, but there was a significant reduction by 12 days after a high nonlethal dose (15 μg/kg). In contrast, Gorski and his colleagues (1988c) found elevated levels of plasma corticosterone from day 8 on following exposure to either a nonlethal (25 μg/kg) or lethal (125 μg/kg) dose of TCDD in male S-D rats killed during the dark hours of the day. Although rats pair fed to those given the lower dose did not differ from their ad libitum-fed counterparts, the pair-fed controls for the lethal dose exhibited changes that were highly similar to those seen in the TCDD-treated rats. Finally, in the most recent study, Bestervelt et al. (1993a) recorded increased plasma corticosterone values in male S-D rats 1 and 5 days after administration of 50 μg/kg TCDD; the rats were killed 3 h after lights were turned on. The increase vanished by day 7, and from day 10 on it had turned into a decrease.

The discrepancies in results may, at least partly, be attributable to different times of sampling during the diurnal cycle. It has been shown that TCDD interferes with the normal light/dark rhythm of corticosterone, causing the curve to flatten out (DiBartolomeis et al., 1987; Jones et al., 1987b). During the early light hours, corticosterone concentration may increase, but late in the light phase the opposite is true. Only the latter effect showed specificity in the sense that it did not occur in pair-fed animals (DiBartolomeis et al., 1987). In view of the findings by Gorski et al. (1988c), it is possible that there is another specific period during the dark phase. Our recent experiments showed that a lethal dose (50 μg/kg) of TCDD increased plasma corticosterone in male L-E rats 6 to 7 days after exposure, regardless of whether

the rats were killed midway through the dark period or during the early light hours (R. Pohjanvirta and J. Tuomisto, unpublished data).

The way TCDD disturbs glucocorticoid metabolism has been elucidated in some detail. Balk and Piper (1984) discovered an unknown steroid metabolite in the plasma of rats treated with TCDD 14 days earlier and identified it as 11-β-hydroxyprogesterone. The accumulation of this compound suggested inhibited activity of 21-hydroxylase in the biosynthesis chain of corticosterone by TCDD. In subsequent work done in the same laboratory, the hypothesis was confirmed. A dose of 50 μg/kg TCDD administered to male S-D rats suppressed adrenal 21-hydroxylase activity by 30 to 40% 7 and 14 days after exposure. Similar decreases were detected in adrenal microsomal cytochrome P-450 (Mebus and Piper, 1986). Both of these findings were replicated by DiBartolomeis et al. (1987). Nevertheless, the latter group concluded that the depressions were physiologically insignificant; instead, they implicated cholesterol side chain cleavage as the step critically inhibited by TCDD. Inhibition of the enzyme responsible for this reaction, cytochrome P-450_{scc}, was associated with increased mitochondrial cholesterol (DiBartolomeis et al., 1986a). In an in vitro study with bovine adrenal cortical cells, however, they recorded reduced mitochondrial accumulation of cholesterol by TCDD-exposed cells in response to adrenocorticotropin stimulation (DiBartolomeis et al., 1986b). Thus, it appears that TCDD is able to interfere with several stages of glucocorticoid biosynthesis, but a major effect is the transfer of cholesterol into and out of mitochondria. This particular mechanism, which seems to show species (and tissue) specificity, may be operative in the actions of TCDD on steroids in general (see section II.D.5.b).

TCDD does not compete with glucocorticoids in binding to their specific intracellular receptors (Neal et al., 1979). It does, however, diminish the binding capacity of hepatic glucocorticoid receptors (usually without affecting binding affinity) in the rat (Nelson et al., 1989; Sunahara et al., 1989) and mouse (Nelson et al., 1989; Stohs et al., 1990a; Lin et al., 1991b). This effect seems to be tissue and cell dependent; in addition to the liver, TCDD has been reported to bring about a decrease in the skeletal muscle (Max and Silbergeld, 1987) and the thymus (Csaba et al., 1991) of the rat as well as in the skin of the mouse (Stohs et al., 1990a), whereas in a human squamous cell carcinoma cell line (Greenlee et al., 1987) and (at a low dose) in the embryonic mouse palate (Abbott et al., 1992a), there was conversely an upregulation of glucocorticoid receptors.

Whether or not the physiological responses of animals to glucocorticoids are altered by TCDD is a matter of dispute. Rozman and associates have argued that the suppressed activities of certain liver enzymes (e.g., PEPCK) by TCDD might result from an interaction of TCDD with the glucocorticoid receptor system (Stahl et al., 1993a). On the other hand, pretreatment with 50 μg/kg TCDD 4 to 12 days before testing did not significantly inhibit the basal levels or induction by dexamethasone of tyrosine aminotransferase (a glucocorticoid receptor-regulated enzyme activity; Vanderbilt et al., 1987) in the liver of S-D rats compared with pair-fed controls (Neal et al., 1979). Lin et al. (1991b) used ad libitum-fed controls and found a slight depression in the basal activity of this enzyme in C57BL/$6^{b/b}$ and C57BL/$6^{d/d}$ mice treated with 100 or 300 μg/kg TCDD 7 days earlier. Because there was a decrease in activity over time in pair-fed rats, the disparate nutritional status of the controls in the two studies probably accounts for the dissimilar results. More difficult to reconcile with the results of the earlier studies is, however, the recent observation of significantly induced activity of hepatic tyrosine aminotransferase in S-D rats 8 days after exposure to 30 to 60 μg/kg TCDD, whether compared with ad libitum-fed or pair-fed controls (Weber et al., 1994).

Bilateral adrenalectomy drastically increased mortality of male S-D rats and shortened mean time to death after TCDD exposure (Gorski et al., 1988d). Because adrenal demedullation did not bring about the same effect, the crucial modulator(s) appeared to reside in the cortex. With corticosterone replacement therapy the mortality was similar to that for intact rats, but a further increase in corticosterone dose provided no additional benefit. An especially interesting finding was the fact that thyroidectomy did not influence the lethality of TCDD in adrenalectomized, corticosterone-supplemented rats (see section II.D.4). Other researchers have shown that adrenalectomy sensitizes rats to hepatic monooxygenase induction as well as glucocorticoid receptor downregulation by TCDD (Nelson et al., 1989), without modulating hepatic Ah receptor levels (Carlstedt-Duke et al., 1979). As for intact animals, dexamethasone attenuated TCDD-induced loss of body weight in male C57BL/6 mice (Taylor et al., 1992) but exacerbated it in male L-E rats (Pohjanvirta et al., 1988b), which may reflect their differential responses to endotoxin (see section II.G.2).

b. ADRENALINE. The study of Gorski et al. (1988d) of adrenal demedullated rats, which was cited above, renders a key role for adrenal medullary hormones in the acute lethality of TCDD unlikely. However, male outbred Long-Evans rats treated with a lethal dose of TCDD (1000 μg/kg) exhibited a progressive tendency with time (up to 8 days) toward elevated adrenaline concentrations in their adrenal glands compared with either ad libitum-fed or pair-fed controls. Concurrently, adrenal levels of noradrenaline remained unaltered (Unkila et al., 1993d). In H/W rats equipped with permanent indwelling intravenous catheters and exposed to the same dose (nonlethal to this strain) 2 days previously, plasma adrenaline concentrations did not differ from those of ad libitum-fed controls either at the basal state or after stimulation

with 400 mg/kg of 2-deoxyglucose (R. Pohjanvirta and J. Tuomisto, unpublished data).

3. Pancreas. a. INSULIN. Serum insulin concentration typically decreases after a lethal or a high sublethal dose of TCDD in rats, but the change does not occur until day 4. Thereafter, serum insulin stays at a low level for about 2 weeks (Gorski and Rozman, 1987; Gorski et al., 1988b; Pohjanvirta et al., 1989a). This depression was shown to be accompanied by a less pronounced decrease in pancreatic concentration of insulin on day 7 (Potter et al., 1983) and by unaltered insulin binding to hepatic plasma membranes on days 2 and 10 (Matsumura et al., 1984). Similar downward shifts were revealed to take place in guinea pigs but with a more rapid evolvement (Brewster, 1985). Already at 2 days postexposure, there was a moderate (35%) reduction in serum insulin along with a much greater decrease (85%) in pancreatic insulin compared with pair-fed animals. Furthermore, insulin binding to adipocyte membranes proved to be increased 3.4-fold. Recent studies of isolated pancreatic membranes from TCDD-treated and pair-fed guinea pigs showed TCDD to promote protein-tyrosine kinase activities (Ebner et al., 1993).

A different course of events was recorded in another TCDD-susceptible species, the rabbit. A dose of 50 μg/kg decreased serum insulin concentrations compared with pair-fed controls for the first 8 hours; the change reached statistical significance as soon as 15 min after TCDD administration. This initial depression was followed by an elevation at 2 days, which leveled off by day 10 (Ebner et al., 1988). Because serum glucose was not affected by TCDD in guinea pigs (Brewster, 1985) and was affected only marginally in rabbits until day 10 (Ebner et al., 1988), TCDD might be capable of exerting a direct impact on pancreatic production of insulin. An alternative explanation is that the lowered insulin concentration represents an attempt by the body to retain serum glucose within a safe range in the face of hampered gluconeogenesis. At sublethal doses in rats, the serum insulin profile was also different from pair-fed animals, but the pair-fed controls for lethally intoxicated rats showed a similar (although slightly weaker) suppression in insulin levels (Gorski et al., 1988b).

Gorski and Rozman (1987) discovered that S-D rats exposed to a lethal dose (125 μg/kg) of TCDD gradually became hypersensitive to the hypoglycemic effect of insulin. The insulin-induced (20 units/kg) mortality rate increased progressively, attaining a level of 100% by day 8, whereas all pair-fed control rats tolerated the insulin challenge. This was later replicated in H/W rats (Pohjanvirta et al., 1990c). It turned out to be a remarkably persistent effect, because a few H/W rats displayed it even after recovery from the acute toxicity of TCDD (3 months after dosing with 1000 μg/kg) (Pohjanvirta et al., 1990b). It is well known that insulin elicits feeding as an emergency response to inhibited cerebral utilization of glucose (for a review, see Ritter, 1986). However, the response was totally lacking (Pohjanvirta et al., 1990c) or markedly dampened (Pohjanvirta et al., 1990b) in TCDD-treated H/W rats (see also section II.E.1.a), thus providing at least a partial explanation for the exceptional lethality of insulin to TCDD-exposed rats. This inhibition of a vital response can be seen as indirect evidence for a profound downregulation of the control systems for energy metabolism.

b. OTHER HORMONES. A high nonlethal dose of TCDD (25 μg/kg) to S-D rats tended to lower plasma glucagon, whereas a lethal dose (125 μg/kg) significantly promoted it from day 16 on (Gorski et al., 1988b). The increase may be causally related to the drastic 90% reduction in glucagon binding to hepatic plasma membranes observed in S-D rats treated with 115 μg/kg TCDD 10 days earlier. A lower dose of 25 μg/kg did not cause any change (Matsumura et al., 1984). TCDD did not influence serum or pancreatic concentrations of somatostatin as assessed 7 days after a dose of 45 μg/kg to S-D rats (Potter et al., 1983). Likewise, pancreatic β-endorphin levels were unaffected by 50 μg/kg TCDD in both L-E and H/W rats during the first 10 days postexposure (Pohjanvirta et al., 1993b).

4. Thyroid gland. TCDD reduces serum T_4 concentrations rapidly in rats (within the first 24 h; Jones et al., 1987b). Both the magnitude and duration of the change are moderately dose related, with lethal doses causing an irreversible 50 to 70% decrease compared with the ad libitum-fed controls (Potter et al., 1986b; Gorski and Rozman, 1987; Pohjanvirta et al., 1989a). Pair feeding decreases T_4 levels as well, but not nearly to the extent that TCDD does (Potter et al., 1986b; Gorski et al., 1988b). Serum total and free T_4 values have usually shown parallel alterations in response to TCDD (Potter et al., 1983, 1986b; Gorski and Rozman, 1987; Gorski et al., 1988b).

Extremely variable results have been published regarding levels of serum T_3 in rats after TCDD exposure. Several studies have found concentrations indistinguishable from those of controls at doses ranging from nonlethal to lethal and a follow-up extending up to 32 days (Potter et al., 1983; Rozman and Greim, 1986b; Gorski and Rozman, 1987; Jones et al., 1987b). By contrast, dose-dependently increased T_3 values in TCDD-treated (25 to 100 μg/kg) versus pair-fed or ad libitum-fed control rats 7 days postexposure were reported by Potter et al. (1986b), whereas decreased levels (versus ad libitum-fed controls) 10 to 14 days after exposure to 100 μg/kg TCDD were observed by Rozman et al. (1985a) and by Pazdernik and Rozman (1985). Gorski et al. (1988b) and Pohjanvirta et al. (1989a) recorded alterations (shifts in either direction in various strains) on day 16 only. Changes in serum free T_3 concentrations also appeared to be negligible (Gorski et al., 1988b). Serum concentrations of the biologically inactive reverse T_3 have been reported to

decline initially in rats, without clear dose response (days 2 through 8), but to return thereafter to the control level (Gorski and Rozman, 1987).

Conspicuously dissimilar changes in serum thyroid hormone concentrations have been recorded in other species. Although TCDD-treated (2 μg/kg) guinea pigs displayed decreased T_4 and T_3 values shortly before death, these declines were equal or more prominent in their pair-fed controls (Huang Lu et al., 1986). However, McKinney et al. (1985b) found that TCDD (0.5 to 2.0 μg/kg) increased serum T_4 concentrations in guinea pigs at 9 days. In both C57BL/$6^{b/b}$ and C57BL/$6^{d/d}$ mice, doses of TCDD of approximately the LD_{50} value elevated T_4 and reciprocally decreased T_3 levels at 35 days, whereas lower doses were ineffective (Birnbaum et al., 1990). In hamsters, a dose of TCDD as low as 100 μg/kg caused a sustained two- to three-fold increase in T_4 concentration during the whole observation period (7.5 weeks). T_3 levels were also high, but the change reached significance only during the first week. Reverse T_3 followed the same pattern as T_4 (Henry and Gasiewicz, 1987).

One of the main mechanisms by which TCDD brings about its effects on serum thyroid hormones in rats appears to be accelerated clearance of T_4 (McKinney et al., 1985b) through selectively enhanced biliary excretion (Bastomsky, 1977). The proportional excretion of T_4 glucuronide was also increased considerably, which suggests stimulated activity of the corresponding UDPGT. This idea was reinforced by enzyme induction studies in which the catalytic activity of hepatic UDPGT toward *p*-nitrophenol was measured (Lucier et al., 1973, 1975; Hook et al., 1975; Marselos et al., 1978; Rozman et al., 1985a; Pohjanvirta et al., 1989a, 1990a; Goon and Klaassen, 1992). Because T_4 has not proved to be a selective substrate for a special subtype of UDPGT, but rather is metabolized by the same forms of this enzyme that are inducible by TCDD (Saito et al., 1991; Barter and Klaassen, 1992), the assays with *p*-nitrophenol are probably qualitatively representative measures of T_4 glucuronidation rate in relation to control activity. In one study (Henry and Gasiewicz, 1987), T_4 was actually used as the substrate, and TCDD (10 μg/kg 7 days prior to the measurement) was found to bring about a 70% augmentation in UDPGT activity per total liver. However, because this is a fairly modest increase and because a similar induction, in relative terms, also occurred in hamsters (despite their opposite response in terms of serum T_4), alterations in UDPGT activity may not totally account for the TCDD-induced decline in rat serum T_4. There is some recent evidence that hydroxylated metabolites of TCDD might compete with T_4 for binding to transthyretin and by this means contribute to the change (Lans et al., 1993).

A contributing factor to the consistent decrease in serum T_4 concurrently with a variable response in T_3 in TCDD-exposed rats could also be an enhanced activity of the enzyme responsible for the peripheral conversion of T_4 to T_3, 5′-deiodinase. In the few studies conducted so far to investigate this point, only hepatic 5′-deiodinase was determined. Contrary to expectation, TCDD appeared to inhibit this enzyme activity by 50 to 60% (Lans et al., 1991; Eltom et al., 1992).

In addition to altering thyroid hormone levels, TCDD may modulate the concentrations of thyroid hormone receptors. An increase in the mRNA for c-*erb-A* oncogene product was recorded after TCDD exposure in the livers of C57BL/6 mice but not in those of DBA/2 mice (Bombick et al., 1988), implying elevated concentrations of nuclear T_3 receptors by TCDD in the former strain.

To what extent the changes in serum thyroid hormone levels by TCDD portray shifts in their functional status in the body is still unknown. Based on similarities in molecular reactivity between TCDD and thyroid hormones (McKinney et al., 1985a), TCDD has been proposed to act in the body as a potent and persistent partial agonist for thyroxine (McKinney et al., 1985b). In several studies designed to verify this issue by assessing a variety of end points, TCDD-treated rats have, by and large, proved to be functionally euthyroid (Potter et al., 1986b; Kelling et al., 1987b; Roth et al., 1988). Nevertheless, thyroid hormones do modulate TCDD toxicity in rats. Thyroidectomy markedly protracted the manifestation of TCDD lethality without affecting mortality rate (Rozman et al., 1984, 1985b). This could be due to a decreased basal metabolic rate in thyroidectomized rats, which retards the expenditure of body energy stores, because acceleration of metabolism by cold exposure curtailed survival time after TCDD administration (Rozman and Greim, 1986b). Thyroidectomy also interacted with TCDD immunotoxicity. It prevented the TCDD-elicited decline in spleen anti-sheep red blood cell plaque-forming cell response but only partially reversed thymic atrophy (Pazdernik and Rozman, 1985). Concomitantly, in thyroidectomized rats, the inducibility of the characteristic hepatic monooxygenases by TCDD remained unaffected (Rozman et al., 1985a, 1987a; Henry and Gasiewicz, 1986). Supplementation of the diet with T_3, on the other hand, in S-D rats exacerbated in an additive manner TCDD-induced retardation of growth and renal accumulation of retinol and retinyl palmitate (Powers, 1988). Thyroid hormones may not be involved in TCDD toxicity the same way in mice as in rats, because daily treatment of TCDD-exposed mice with T_3 slightly prolonged survival time dose dependently (Neal et al., 1979).

5. Gonads. a. OVARIAL HORMONES. Although altered steroid hydroxylation reactions were observed in hepatic microsomes from pregnant rats treated with TCDD, in these animals the concentrations of serum estradiol remained at the control level (Shiverick and Muther, 1983). Serum estradiol was not changed by TCDD in virgin CD-1 mice at doses that elicited significant monooxygen-

ase induction (DeVito et al., 1992a,b). The steroid-specific form of UDPGT was not inducible by TCDD in guinea pigs or in various strains of mice (Umbreit et al., 1989a).

In spite of these negative findings with regard to the major circulating estrogen, TCDD has been shown to diminish hepatic and/or uterine estrogen receptor densities (without affecting binding affinity) in rats, mice, and guinea pigs (Romkes et al., 1987; Romkes and Safe, 1988; Hruska and Olson, 1989; Lin et al., 1991b; DeVito et al., 1992a,b). Hamsters, in contrast, displayed unchanged hepatic and increased uterine binding capacities (Hruska and Olson, 1989). The estrogen receptor response was also tissue specific, because in CD-1 mice ovariectomy prevented it in the uterus but not in the liver (DeVito et al., 1992a). Time-course experiments in CD-1 mice revealed that the depression was fully developed by 1 to 2 days after exposure, with recovery occurring by 14 to 21 days (DeVito et al., 1992a).

Both cytosolic and nuclear receptors appeared to be downregulated by TCDD (Romkes and Safe, 1988; DeVito et al., 1992a). Although progesterone exerted similar effects, there was a difference between TCDD and progesterone in the ability of various protein or RNA synthesis inhibitors to interfere with the phenomenon. Whereas the decrease caused by progesterone was inhibited by inhibitors of both types, TCDD-induced reduction was counteracted only by the RNA polymerase inhibitor actinomycin D (Romkes and Safe, 1988). Ah receptors (see section II.F) seem to be involved in the mediation of the estrogen receptor response (Romkes et al., 1987; Lin et al., 1991b), but it may require other regulators as well (DeVito et al., 1992b). The suppressive effect on estrogen receptors is probably a major factor underlying the potent antiestrogenic activity TCDD has exhibited in several experimental settings (Gallo et al., 1986; Umbreit et al., 1988; Astroff and Safe, 1990; Astroff et al., 1990, 1991; Johnson et al., 1992a). Coadministration of estradiol with TCDD to female CD-1 mice had little influence on the acute lethality of TCDD, although it increased the severity of TCDD-provoked ascites. By contrast, tamoxifen, a competitive inhibitor and a mixed agonist of the mouse estrogen receptor, exacerbated TCDD toxicity in terms of mortality and duration of survival time (Umbreit et al., 1989b). Subsequent studies disclosed that the potentiation was due to protracted hepatic retention of TCDD and aggravated liver toxicity (MacKenzie et al., 1992).

There is a lack of data concerning serum progesterone responses to TCDD treatment in rodents. Long-term exposure to TCDD was shown to reduce serum progesterone concentrations in five of seven rhesus monkeys, but these findings cannot be generalized directly to rodents, because four of the five monkeys exhibited a concurrent decrease in serum estradiol concentration (Barsotti et al., 1979). Nevertheless, TCDD decreased concentrations of cytosolic and nuclear progesterone receptors dose dependently in the rat uterus and liver (Romkes and Safe, 1988).

b. TESTICULAR HORMONES. TCDD was shown to reduce plasma concentrations of testosterone and dihydrotestosterone in sexually mature male S-D rats by up to 90 and 75%, respectively (Moore et al., 1985). The downward trends emerged by 2 days, reached their troughs on day 7, and persisted for at least 12 days. As inferred from pair-fed control data, only about half of the changes could be accounted for by hypophagia and body weight loss. A slightly different time course for serum testosterone in S-D rats was observed by Piper and coworkers (Mebus et al., 1987). In their study, the decrease was dose-dependently progressive during the whole 14-day follow-up period, eventually amounting to 85% at 50 μg/kg.

The mechanism by which TCDD affects plasma testosterone, as well as the history of its gradual elucidation, to some degree resembles the situation for adrenal steroids and TCDD (see section II.D.2). Keys et al. (1985) showed altered catalytic activities of hepatic testosterone-metabolizing hydroxylases in TCDD-treated immature rats. The activity of 7α-hydroxylase was induced, whereas 6β-, 16α-, 16β-, and 2α-hydroxylase activities were inhibited, along with decreased androstenedione formation. TCDD did not appear to induce hepatic glucuronidation of testosterone (Lucier et al., 1975). Moore and Peterson (1988) assessed the rate of testosterone catabolism at the whole animal level by monitoring the disappearance of radioactively labeled testosterone from plasma and its excretion into bile and by analyzing plasma androgens in castrated rats with testosterone implants. Their data indicated that the proximate cause of the androgenic deficiency must be in the production or secretion of testicular testosterone. In support of the former alternative, Kleeman et al. (1990) discovered that reduced testosterone secretion by testicles from TCDD-treated rats, compared with ad libitum-fed controls, in response to human chorionic gonadotropin ex vivo was paralleled by a similarly decreased content of intratesticular testosterone. Impeded biosynthesis of testosterone was also suggested by the findings that TCDD markedly decreased testicular heme synthesis and microsomal (but not mitochondrial) cytochrome P-450 at the same time as it depressed the cytochrome P-450-associated activities of 17-hydroxylase and 17,20-lyase (Tofilon and Piper, 1982; Mebus et al., 1987).

Further studies by Peterson and colleagues (Kleeman et al., 1990; Moore et al., 1991) step-by-step narrowed the primary biochemical lesion in testosterone biosynthesis, finding that it occurs at an earlier stage in the reaction chain than that proposed by Piper's group. Although they confirmed that TCDD inhibits cytochrome P-450$_{scc}$ (side chain cleavage) activity, the effect was not considered severe enough to inhibit testicular

steroidogenesis in vivo. Instead, the primary impact of TCDD appeared to be inhibition of LH-stimulated mobilization of cholesterol to cytochrome P-450$_{scc}$, subsequent to cyclic AMP formation. It is interesting that TCDD also impairs adrenal steroidogenesis by inhibiting cholesterol movement to cytochrome P-450$_{scc}$ in primary cultures of bovine adrenal cortical cells in vitro, but it does so by inhibiting cytochrome P-450$_{scc}$ activity and increasing mitochondrial cholesterol concentration in the rat in vivo (see section II.D.2).

In the past few years, evidence has emerged to show that the rate-limiting step in testicular and adrenal production of steroids, i.e., the transport of cholesterol from the outer to the inner mitochondrial membrane, is mediated by specific receptor molecules, the mitochondrial (or "peripheral") benzodiazepine-binding sites (for reviews, see Gavish et al., 1992; Krueger and Papadopoulos, 1992). In view of the data mentioned above, the critical target of TCDD in the testis might well be these receptors in Leydig cells. In a pilot experiment, we examined the effect of a lethal dose of TCDD (50 μg/kg) on testicular mitochondrial benzodiazepine receptors in L-E rats on day 7 postexposure (R. Pohjanvirta and J. Tuomisto, unpublished data). As expected, TCDD reduced plasma testosterone by almost 60%. This decline was accompanied by a 20% decrease in the maximal binding capacity of mitochondrial benzodiazepine receptors without any alteration in their binding affinity. Although preliminary, these results definitely indicate that, to establish the role of this new class of receptors in TCDD toxicity, further studies are warranted.

Because testosterone and other androgens are anabolic, it is conceivable that the androgenic deficiency brought about by TCDD could be involved in the pathogenesis of the wasting syndrome and TCDD lethality. However, the experimental data argue against it having an important role. Beatty et al. (1978) obtained 20-day LD_{50} values of 60 versus 40 μg/kg for intact versus castrated male rats and 25 versus 45 μg/kg TCDD for intact versus testosterone-treated female S-D rats, respectively. Androgens that originate in the testis thus appear to play only a minor, if any, modulatory part in the lethal action of TCDD.

6. Pineal gland. TCDD has been shown to exert a pronounced impact on circulating melatonin, the chief hormone produced by the pineal gland. In L-E rats treated with 50 μg/kg TCDD 6 days earlier, serum melatonin concentrations were 50% or less of the control values at all times during the daily lighting cycle (Pohjanvirta et al., 1989b). The change did not appear to be correlated with susceptibility to TCDD lethality, because a similar decrease was observed in H/W rats exposed to the same dose of TCDD (Linden et al., 1991). The alteration became manifest in H/W rats within 24 h after TCDD administration and persisted for at least 28 days.

Somewhat surprisingly, pineal histopathological findings were unremarkable, indicating that the decrease in serum melatonin concentration did not result from direct damage by TCDD to the secreting gland (Linden et al., 1991). Furthermore, pineal melatonin content was not lowered by TCDD (if anything, it was slightly higher than in controls), and there was only a modest inhibition in the activity of the rate-limiting enzyme of pineal melatonin synthesis, N-acetyl transferase (R. Pohjanvirta and J. Tuomisto, unpublished data). This finding pointed to the possibility of accelerated peripheral clearance of melatonin in response to TCDD exposure. However, the excretion of melatonin and its principal metabolite, 6-hydroxymelatonin sulphate, in urine were similarly and dose-dependently diminished in TCDD-treated L-E rats (Pohjanvirta et al., 1992). Additional studies revealed that the elimination of exogenous melatonin from the plasma was indeed hastened, but yet the disappearance of [^{3}H]melatonin-derived radioactivity was slowed by TCDD (50 μg/kg, 6 days earlier), suggesting that TCDD may induce an alternative metabolic pathway for melatonin, which results in metabolites that are less readily excreted than 6-hydroxymelatonin sulphate (R. Pohjanvirta and J. Tuomisto, unpublished data).

The decline in plasma melatonin is not likely to be intimately involved in the lethal action of TCDD due to the sensitivity of H/W rats to the effect. However, such a drastic change in the circulating levels of the most important day/night signaling substance in the body (for reviews, see Amstrong, 1989; Reiter, 1991) may well mediate, or contribute to, certain biological effects of TCDD, e.g., the shifts in corticosterone (section II.D.2.a) or feeding rhythms (section II.E.1.a).

E. Neurobehavioral and Neurochemical Effects

Despite the clear anorexia caused by TCDD in most animal species, behavioral changes and the role of the central nervous system in TCDD intoxication have failed to arouse interest until recently. Data concerning the direct effects of TCDD on the central nervous system are still contradictory. TCDD does not penetrate into the brain very well, and the concentrations are far lower than in the liver or adipose tissue (Abraham et al., 1988; Pohjanvirta et al., 1990d; Stahl and Rozman, 1990; Unkila et al., 1993a). On the other hand, they may be higher in the hypothalamus than in other parts of the brain (Pohjanvirta et al., 1990d). Sensitive biochemical effects, such as enzyme induction, are seen even in the brain (Unkila et al., 1993a). There is also preliminary evidence that TCDD may cause cellular injury in the neurones, as indicated by an increase in intracellular calcium (Hanneman et al., 1993).

1. Neurobehavioral aberrations. a. DISORDERS IN THE REGULATION OF FEED INTAKE AND BODY WEIGHT. Drastic hypophagia is a consistent clinical sign in most animal species exposed to TCDD. It is also by far the most important reason for the wasting syndrome characteris-

tic of the acute toxicity of TCDD. In comparison with other anorexigens or weight-reducing compounds, TCDD ranks among the most potent. In view of these facts, it is astonishing how little attention the mechanisms by which TCDD suppresses feeding have attracted. During the last two decades of intensive TCDD research, there have actually been only two research groups focusing on this issue.

The essential features that distinguish TCDD hypophagia from most other anorexias induced by chemical compounds in rats are its gradual emergence—progressive over days (Pohjanvirta et al., 1987; Pohjanvirta and Tuomisto, 1987; Unkila et al., 1993b) or even weeks (Seefeld et al., 1984a) after exposure—and its persistence following a single treatment. Both feed intake and body weight curves appear to lie permanently below the control level in response to a high, but sublethal, dose of TCDD (Seefeld et al., 1984b; Pohjanvirta and Tuomisto, 1990a,b). If corrected for the reduced metabolic mass of TCDD-exposed rats, however, daily feed intake comes close to the control value after the initial decrease (Seefeld et al., 1984b; Pohjanvirta et al., 1987; Pohjanvirta and Tuomisto, 1987, 1990a). Total aphagia is not a regular finding but may occur at high doses for variable periods (Pohjanvirta et al., 1987, 1993a).

There is a wealth of data implying that TCDD has a specific effect on the regulation of feed intake and/or body weight. By a variety of behavioral, biochemical, and pharmacological approaches, Pohjanvirta et al. (1994b) demonstrated that general malaise or nausea cannot explain TCDD hypophagia. Peterson and coworkers conducted an inventive set of behavioral experiments and arrived at the conclusion that TCDD may lower the putative set point for body weight in rats. They showed that rats treated with a sublethal dose defended their diminished body weight against exogenous manipulations, such as feeding a high-energy diet or restricting feed intake, in a way that was identical with control rats. The same was true for adjustments in feed consumption and body weight to changes in the caloric density or palatability of the diet (Seefeld et al., 1984b). If rats were feed restricted prior to exposure to TCDD, they displayed relative hyperphagia and body weight gain immediately after TCDD treatment, until their body weight converged with the unrestricted TCDD group (Seefeld et al., 1984a). In further support of this line of reasoning, TCDD-treated H/W rats were found to consistently react the same way as controls to repeated 24-h feed deprivations (Pohjanvirta and Tuomisto, 1990b); TCDD-dosed L-E and H/W rats reduced the intake of all three macronutrients proportionately when these were offered simultaneously (Pohjanvirta and Tuomisto, 1990a), and H/W rats, made obese by a prior ventromedial hypothalamic lesion, initially exhibited a strikingly more severe anorexia and body weight loss after TCDD than did sham-operated rats but finally reached and maintained the same level of body weight (Tuomisto et al., 1993c).

If allowed to eat ad libitum, pair-fed control rats catch up with the body weight level of unrestricted rats even after 7 weeks of pair feeding (Seefeld et al., 1984b). A mechanistic explanation for the conspicuous lack of this phenomenon in TCDD-treated rats was given by Pohjanvirta and Tuomisto (1990a), who discovered that, after recovering from the early phases of TCDD toxicity, H/W rats exhibited a peculiar discrimination in their avidity for consuming sweet liquids. They drank as much or more saccharin, but less sucrose solutions, than did control rats. At the same time, there was no deviation in their preferences for various concentrations of saccharin, thus proving that TCDD did not affect the ability of the rats to perceive tastes. Because the essential difference between saccharin and sucrose lies in their nutritive values and because TCDD-treated rats also diminished their intake of all three macronutrients proportionately, it was concluded that TCDD treatment resulted in an aversion to feed energy by rendering rats hypersensitive to postingestive satiety signals. This hypothesis was later substantiated by preloading experiments (Pohjanvirta et al., 1991). When TCDD-dosed rats, fasted for 24 h, were allowed to drink either a 20% sucrose or a 0.25% saccharin solution before access to feed, those animals that had had sucrose ate only about 50% of the amount consumed by the saccharin group. Although the preloads were quantitatively similar in control rats, there was no such divergence in subsequent feeding. When infused directly into the stomach, the sucrose solution also produced a longer lasting suppression of feed intake in TCDD-treated rats than in control rats, suggesting a mediator role for gastrointestinal factors in the response.

The exaggerated satiety response was not clearly manifest until body weight had restabilized to a new, lower level (Pohjanvirta and Tuomisto, 1990a). This pointed to a depressed drive to eat during the actual phase of losing weight. The view was strengthened by studies with insulin and the nonmetabolizable derivative of glucose, 2-deoxy-D-glucose. Both of these compounds lead to severely hampered utilization of cellular glucose (glucoprivation), which normally triggers a compensatory feeding response initiated in the hindbrain, but modulated by central and peripheral factors (for review, see Ritter, 1986). However, the eating response was totally lacking in TCDD-treated H/W rats at the time when hypophagia was at its most severe, rendering the animals exceptionally susceptible to insulin hypoglycemia (Pohjanvirta et al., 1990c). After restabilization of body weight, the rats were still refractory to 2-deoxy-D-glucose but less so to insulin. They also displayed an abnormal reduction in feed consumption when challenged with an inhibitor of fatty acid β-oxidation, sodium 2-mercaptoacetate (Pohjanvirta and Tuomisto, 1990b).

Hence, accumulating evidence seems to show that the

early anorexia and decline in body weight of TCDD-treated rats result from depressed motivation (hunger) to eat. This, in turn, may be due to a shift in a regulated set point, to an uncoupling of metabolic processes from the control systems for feed intake, or to effective damping of the mechanisms that initiate eating. Later, survivor rats (apparently recovering from the early insult to a sufficient degree or developing compensatory ways to overcome it) become hypersensitive to satiety signals, which prevents them from catch-up growth. How these phenomena are mediated is still unknown. The responses of TCDD-treated H/W rats to two putative physiological mediators of satiety, cholecystokinin and bombesin, were not markedly altered (Pohjanvirta et al., 1991). Likewise, treatment of S-D rats with the neurotoxin 5,7-dihydroxytryptamine, which depleted brain 5-HT, did not influence TCDD-induced hypophagia (Stahl et al., 1991). Transfusion of blood from control, satiated S-D rats reduced feed intake in recipient rats receiving scheduled feedings by approximately 40%, whereas blood from TCDD-exposed (8 days after a lethal dose) rats was ineffective, which argues against a humoral suppressor of feeding at that stage (Rozman et al., 1991). Moreover, complete subdiaphragmatic vagotomy before TCDD exposure or repeated treatment with various chemical compounds known to modulate aminergic neurotransmission and feed intake had no or negligible influence on the hypophagia and wasting syndrome in L-E rats (Pohjanvirta et al., 1988b; Tuomisto et al., 1994).

On the other hand, the ability of the nonselective opioid antagonist, naloxone, to inhibit feeding was slightly attenuated in H/W rats treated with TCDD 3 months earlier (Pohjanvirta and Tuomisto, 1990b), which may be of importance because opioid peptides are integral for the glucoprivic feeding response (see above). In response to chemically induced nausea, TCDD was shown to potentiate secretion of oxytocin (Pohjanvirta et al., 1994b). Physiological satiety is, under certain conditions, also associated with an increase in plasma oxytocin concentrations (Verbalis et al., 1986). Moreover, oxytocin appears to be an inhibitory transmitter for feeding regulatory centers in the brainstem (Wellman et al., 1993). Therefore, oxytocin is a candidate mediator of TCDD anorexia and should be further explored. In mice, an endogenous mediator involved in the early hypophagia may be TNF (see section II.G.2), because a specific antibody to this cytokine significantly attenuated TCDD-induced weight loss during the first 11 days after exposure (feed consumption was not measured) (Taylor et al., 1992). The relevance of this finding to other species is as yet unknown.

Although no studies of feeding microstructure after TCDD exposure have been conducted so far, it has become clear that TCDD alters the feeding patterns of rats in at least two ways. First, the normal circadian rhythm is distorted such that TCDD-dosed rats tend to eat more of their total daily intake during the light hours. TCDD seems to selectively suppress nocturnal feeding, while having a notably lesser inhibitory (or even a modestly fortifying) effect on daytime feeding (Christian et al., 1986a; Pohjanvirta et al., 1988b; Pohjanvirta and Tuomisto, 1990a,b). Because the time course of this shift in feeding rhythm paralleled the emergence of the enhanced satiety response (Pohjanvirta and Tuomisto, 1990a), it may represent an attempt of the rats to circumvent the postingestional satiety block by increasing the number of eating bouts. Second, TCDD dose-dependently increases feed spillage. In S-D rats the increase was progressive with time for at least 2 weeks after exposure (Seefeld et al., 1984a). It is tempting to speculate that this behavior aberration is also a reflection of the gradually appearing overresponsiveness to satiety factors.

Strikingly little information is available concerning TCDD-elicited changes in feeding behavior in species other than the rat. Hamsters were highly refractory to the early suppressive effect of TCDD (Henry and Gasiewicz, 1987). TCDD anorexia emerged more gradually in guinea pigs and, especially, in C57BL/6 mice (Kelling et al., 1985; Huang Lu et al., 1986) compared with rats.

Changes in water intake after TCDD exposure usually follow the patterns of feed consumption but are less marked (Seefeld and Peterson, 1984; Kelling et al., 1985; Pohjanvirta et al., 1987, 1988b; Pohjanvirta and Tuomisto, 1987, 1990a). A tight coupling between feed and water intake in the rat is well established (Fitzsimons and Le Magnen, 1969). This is the plausible reason for the reduction in water consumption in TCDD-treated rats, because TCDD did not interfere with the drinking responses of H/W rats to an osmotic challenge (Pohjanvirta and Tuomisto, 1990b). However, a moderate rebound in water intake after the initial decrease, accompanied by a decrease in urine specific gravity, has been described for TCDD-exposed H/W rats (Pohjanvirta et al., 1987).

b. CHANGES IN OTHER FORMS OF BEHAVIOR. As a drastic contrast to its profound effects on feeding behavior, TCDD largely appears to leave most other forms of behavior untouched (for alterations in motor activity, see section II.B.4). In spite of the expected impact on body weight, a high nonlethal dose (1000 μg/kg) did not influence the performance of H/W rats in an open field, in an elevated plus-maze test for anxiety, in a passive avoidance test for learning, in a rotating rod test for motor coordination and balance, or in a hot plate test for nociception. The only positive finding was a slight transient impairment 16 h postexposure in an elevated horizontal bridge test of motor coordination (Sirkka et al., 1992). In mice, TCDD has been reported to potentiate the analgesic response to 2-deoxy-D-glucose but not to morphine (Fujimoto et al., 1986).

Nevertheless, there appears to be another specific area of behavior apart from feeding that is highly vulnerable

to TCDD in rats, at least during limited periods of ontogenesis: masculine sexual behavior. Mably et al. (1992) found strong evidence that in utero and lactational TCDD exposure impairs sexual differentiation in the central nervous system, resulting in demasculinization and feminization of male rats. Doses as low as ≥0.064 or ≥0.16 μg/kg given to dams on gestation day 15 caused dose-related prolongations in latencies to mount, intromission, and ejaculation in male offspring at maturity. Although the involvement of physical impairments in these effects cannot be excluded, the probable primacy of behavioral alterations was implied by the finding that, after castration, estrogen priming, and progesterone treatment, rats exposed to TCDD perinatally displayed dose-dependent increases in lordosis quotient and lordosis intensity in response to being mounted by another male. Some of these findings were recently replicated in another strain of rat (Gray et al., 1993). In favor of the hypothesis of disrupted sexual differentiation in the central nervous system, Schantz et al. (1991) found reversed gender-related performance in the open field and radial maze tests for rats exposed perinatally to TCDD.

Some subtle, but significant, behavioral alterations were observed in the offspring of rhesus monkeys chronically fed a diet containing low levels (5 or 25 pg/g) of TCDD. TCDD-exposed mother-infant dyads spent more time than controls in close social contact in the absence of any obvious physical effects of the exposure on the progeny (Schantz et al., 1986). In one of the two cognitive tests used (discrimination-reversal learning), monkeys exposed perinatally to TCDD exhibited retarded learning of shape, but not color, reversals (Schantz and Bowman, 1989). For performance in spatial reversals, a curvilinear regression with the TCDD concentration in body fat was obtained; at low concentrations, there was clear facilitation of learning, which turned into impairment at high doses (Bowman et al., 1990). In social peer groups, TCDD-exposed offspring initiated more rough-tumble play, retreated less during play bouts, and were less often displaced from preferred positions in the playroom as compared with their control counterparts (Schantz et al., 1992).

2. Effects of a direct application of 2,3,7,8-tetrachlorodibenzo-p-dioxin into the central nervous system. A slow infusion of TCDD over 7 days into brain ventricles with Alzet minipumps caused a typical reduction in feed intake in both L-E and H/W rats (Pohjanvirta et al., 1989c). The response was significantly more pronounced than after subcutaneous infusion. However, these findings could not be replicated in S-D rats when TCDD was given as a single bolus injection in corn oil (Stahl and Rozman, 1990). The studies are somewhat difficult to compare because of the different solvents (dimethyl sulfoxide and corn oil, respectively) and different modes of administration (the same intracerebroventricular and subcutaneous doses as infusion versus a bolus of 8 μg/kg intracerebroventriculaly and 72 μg/kg intravenously, respectively). There are problems with the bioavailability of TCDD with Alzet pumps, because TCDD tends to adsorb to pump membranes, and oil is not an ideal vehicle in central nervous system studies; therefore, the discrepancy needs clarification. Because TCDD is practically insoluble in the aqueous phase and does not dissolve very well in lipids, dimethyl sulfoxide could potentiate its effects by promoting its kinetics.

3. Neurochemistry. a. CATECHOLAMINES. Neurochemical studies with TCDD are also quite recent, and as yet the rat is the only species that has been used. In the earliest study it was found that TCDD caused an almost two-fold increase in turnover of hypothalamic dopamine and a consequent decrease in prolactin secretion (Russell et al., 1988). These findings have not, however, been corroborated. Dopamine and levels of dopamine metabolite in ten brain areas were found to be unchanged or marginally decreased (Tuomisto et al., 1990). In another study, a slight increase of dopamine concentration in the hypothalamus was seen at one late time, but there were no changes in dopamine or its metabolite concentrations in the striatum (Rozman et al., 1991). No significant changes were found in single hypothalamic nuclei and median eminence (Unkila et al., 1993b,d).

Noradrenaline concentrations (Russell et al., 1988, Tuomisto et al., 1990, Rozman et al., 1991) or turnover rate (Unkila et al., 1993b) have also been found to be unchanged. Thus, there is no good evidence that catecholaminergic systems are involved in the anorexia caused by TCDD, although there is considerable evidence that many other chemicals (notably, many drugs) reduce food intake by acting on or through these systems (for reviews, see Morley and Levine, 1985; Sugrue 1987; Samanin and Garattini, 1993).

b. HISTAMINE. Histamine is another hypothalamic neurotransmitter that has been implicated in the regulation of food intake (Lecklin and Tuomisto, 1990; Sakata, 1991). A slight increase was found in histamine levels in the whole hypothalamus at 28 h (Tuomisto et al., 1990) and in the median eminence at 25 h after TCDD (Tuomisto et al., 1991). The significance of these findings is still unclear.

c. SEROTONERGIC NEUROTRANSMISSION. The role of 5-HT in TCDD anorexia is more complicated. A trend toward decreased 5-hydroxyindoleacetic acid (the major metabolite of 5-HT) levels was seen at early times after TCDD administration, suggesting a decreased 5-HT turnover (Tuomisto et al., 1990). However, at later times (3 to 16 days), consistent increases in brain tryptophan and 5-hydroxyindoleacetic acid have been observed (Tuomisto et al., 1990; Rozman et al., 1991; Stahl et al., 1991; Weber et al., 1992c; Unkila et al., 1993b,c, 1994a,b).

Tryptophan is the precursor amino acid of 5-HT, and the converting enzyme, tryptophan hydroxylase, is nor-

mally not saturated (Ashcroft et al., 1965). Therefore, it is not surprising that brain 5-HT turnover also turned out to be stimulated by TCDD, as indicated by increased levels of 5-hydroxyindoleacetic acid (Tuomisto et al., 1990; Rozman et al., 1991; Unkila et al., 1993b,c, 1994a,b) and by 5-hydroxytryptophan accumulation after administration of the decarboxylase inhibitor NSD-1015 (Unkila et al., 1994a). However, because depletion of 5-HT by the selective neurotoxic analogue, 5,7-dihydroxytryptamine, did not alter the feed intake or starvation syndrome after TCDD, it was concluded that activation of serotonergic neuronal systems may not be the cause of reduced feed intake and lethality (Stahl et al., 1991; Weber et al., 1992c).

The increase in tryptophan has been attributed to inhibition of tryptophan pyrrolase (tryptophan 2,3-dioxygenase), which is the key enzyme of an alternative biochemical pathway for tryptophan metabolism, and the inhibition was suggested to lead to an increased level of plasma tryptophan (Stahl et al., 1993a; Weber et al., 1992c, 1994). This increase was approximately double in TCDD-treated S-D rats, whereas in pair-fed controls there was only a slight decrease (Rozman et al., 1991). This would then be reflected in the hypothalamus and striatum as almost a doubling of tryptophan and up to a 50% increase in 5-HT and 5-hydroxyindoleacetic acid. This mechanism, however, is not in line with other results.

Recent studies have indicated an interesting difference between the sensitive L-E rat and the resistant H/W rat in terms of their tryptophan metabolism. Both brain tryptophan and 5-hydroxyindoleacetic acid levels were elevated in the sensitive strain after TCDD but not in the resistant one (Unkila et al., 1993b, 1994a,b), except for a slight elevation when a huge dose of 9600 μg/kg was administered (Unkila et al., 1993c, 1994b). At variance with the results in S-D rats, however, only a modest and variable increase was seen in total plasma tryptophan.

The inhibition of tryptophan pyrrolase was also moderate and variable (Unkila et al., 1993c, 1994a,b). On the other hand, the brain alterations seemed to follow the pattern of free tryptophan in the plasma, which increased as much as three- to four-fold in the sensitive strain (Unkila et al., 1993c, 1994a,b). Tryptophan is, in fact, the only amino acid known to bind reversibly to plasma albumin (Kragh-Hansen, 1981). The explanation for the decreased binding is not clear, but one possibility is that elevated free fatty acids (Curzon et al., 1973) or bilirubin (Kragh-Hansen, 1981) might compete with tryptophan for protein binding and thereby increase the amount of free amino acid that is immediately available for transport to the brain. Another possibility is the presence of a factor that can change the affinity of tryptophan for albumin (e.g., allosterically).

Changes in tryptophan metabolism, like many other changes after TCDD administration, suggest that there is a basic regulatory derailment that is able, secondarily, to cause several metabolic changes, including an inhibition of tryptophan pyrrolase and an increase in free fatty acids. Such changes often seem to correlate with lethality, but not with 7-ethoxyresorutin O-de-ethylase induction; they are also caused by other clorinated dibenzo-*p*-dioxins given at a lethal or near-lethal dose (Weber et al., 1992a,b, 1994). Changes in brain serotonergic neurotransmission may be secondary to such factors and not a causative factor for anorexia. This is also suggested by the latency; anorexia, after a high dose, occurs in hours, whereas altered tryptophan metabolism in the brain is typically a late phenomenon, becoming detectable after 3 to 4 days and increasing up to at least 16 days (Tuomisto et al., 1990, Rozman et al., 1991).

d. NEUROPEPTIDES. There is much less information available concerning peptide neurotransmitters. Hypothalamic levels of β-endorphin after TCDD were reported to be first increased (the first day after TCDD) and then decreased, and the B_{max} of mu opioid binding increased 3 days after TCDD administration (Bestervelt et al., 1991). In another study, however, no changes in β-endorphin-like immunoreactivity could be seen on days 1, 4, or 10 after TCDD (Pohjanvirta et al., 1993b). Interestingly, in the TCDD-sensitive L-E strain, plasma β-endorphin-like immunoreactivity was decreased by TCDD at all times, whereas there was an increase in pair-fed controls on day 4. In the resistant H/W strain, no significant changes were noticed (Pohjanvirta et al., 1993b).

e. CHOLINERGIC NEUROTRANSMISSION. There is no information regarding the cholinergic system except for an early note that serum chlolinesterase is inhibited after a large dose of TCDD is given (Buu-Hoï et al., 1972a). The coplanar polychlorinated biphenyl compound, 3,3′,4,4′-tetrachlorobiphenyl, has been described as causing developmental behavioral and cholinergic muscarinic receptor changes in the mouse brain after administration to the newborn (Eriksson, 1988; Eriksson et al., 1991).

f. SIGNAL TRANSDUCTION. The effects of TCDD on second messengers and signal transduction mechanisms in the central nervous system are virtually unknown. Inositol levels were found to be decreased in most areas of the brain in both the sensitive L-E and the resistant H/W rat after TCDD (Pohjanvirta et al., 1994a). This may be due to inhibition of synthesis caused by hypoglycemia and substrate deficit. No consistent changes in inositol phosphates were found.

In the liver of S-D rats, cyclic AMP levels were increased 2 to 8 days after a single lethal dose of TCDD (Stahl et al., 1993a). This was also the case in the white adipose tissue of guinea pigs treated with 1 μg/kg TCDD 2 days earlier (Brewster, 1985). No studies have so far examined the influence of TCDD on brain cyclic AMP

concentrations, but that information would be highly desirable, because inhibition versus stimulation of cyclic AMP in the brain has been proposed as the final common pathway to feeding or satiety (Chance and Fischer, 1993).

In conclusion, there is an almost complete ignorance as to which neurone systems convey the regulation of TCDD anorexia in the centers of the brain that regulate feeding. However, crucial participation of catecholaminergic systems seems to be ruled out. Serotonergic and histaminergic systems may be partially involved but certainly do not play a prominent role. Changes in tryptophan levels in rats indicate that the doses of TCDD used have been close to lethal doses. Elucidation of the underlying mechanism of these changes might help us to interpret the metabolic disarray obviously involved in the acute toxicity of TCDD. Amino acid and peptide neuronal systems are the obvious areas that should be investigated further. The effects produced by TCDD seem to be highly selective, and the compound may actually prove to be a useful tool for studying the physiology of feeding regulation in the central nervous system and the long-term control of body weight.

4. Hypothalamic lesions. Lesioning various hypothalamic nuclei has been an important part of the research methodology used to elucidate the regulation of feed intake in animals. As yet, little work has been done with TCDD in this area. It is interesting that lesioning of ventromedial hypothalamic nuclei, which normally causes increased feed intake and promotes weight gain, does not alleviate the anorexia caused by TCDD. On the contrary, after a nonlethal dose of TCDD, animals made obese by a ventromedial hypothalamic lesion stopped eating completely, until their weight was at the same level as that of TCDD-treated, sham-operated controls (Tuomisto et al., 1993c). This favors the "body weight set point" hypothesis (Peterson et al., 1984b; Seefeld et al., 1984a), according to which TCDD regulates the set point at a subnormal level. Other regulatory functions, however, operate normally and attempt to direct the body weight toward the new preferred weight.

F. Aromatic Hydrocarbon Receptors, Enzyme Induction, and Acute Toxicity

The effects of TCDD mediated by Ah receptors have been thoroughly reviewed; the most conspicuous and unambiguous of these effects is the induction of cytochrome P-450-dependent enzymes (cf. recent reviews by Durrin et al., 1987; Whitlock, 1987, 1989, 1990, 1993; Safe, 1988; Nebert and Jones, 1989; Whitlock et al., 1989; Denison, 1991; Landers and Bunce, 1991; Swanson and Bradfield, 1993; Okey et al., 1994). It is also quite likely that many forms of toxicity are mediated by Ah receptors, e.g., those involving EGF receptors.

Involvement of Ah receptors in acute lethality, however, is far from clear, and the prevailing hypothesis has also been challenged on several grounds. In fact, a drug or chemical with only a single mechanism of action would probably be the first of this kind in the history of pharmacology and toxicology, and, therefore, one should be quite prepared to find other mechanisms. The best argument for a single mechanism is the potency of TCDD; thus, any other mechanisms that the compound undoubtedly has may be relevant only at higher doses never achieved. One problem is that by far the most extensive work on Ah receptors has been done using the liver (as well as thymus and some epithelial tissues such as skin). Little is known about other tissues that may be relevant for short-term toxicity and its manifestations, notably wasting syndrome and disturbances in the regulation of food intake.

1. Phylogeny and ontogeny of aromatic hydrocarbon receptors. There is relatively little information concerning the stage of phylogenetic development at which Ah receptors first appeared. This information would be quite important in that it could provide hints about the possible physiological role and physiological ligand of this receptor, if any. Ah receptors have been demonstrated in the rainbow trout (Heilmann et al., 1988) and in a rainbow trout hepatoma cell line (Lorenzen and Okey, 1990). Among marine animals, labeling of cytosolic proteins with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin was observed in seven teleost and elasmobranch fish and in beluga whales (Hahn et al., 1992). No labeled proteins were found in cytosol from two species of agnathan fish or in any of the nine invertebrate marine animals investigated. The presence of specifically labeled polypeptides corresponded to P-4501A inducibility and sensitivity to toxicity. Thus, the Ah receptor system seems to have evolved early in vertebrate evolution, or even earlier, because Ah receptors were also found in three of four strains of *Drosophila melanogaster* (Bigelow et al. 1985), although there was no relationship to induction of aryl hydrocarbon hydroxylase.

Biochemical and physicochemical characteristics of Ah receptors appear to be reasonably similar among species (Gasiewicz and Rucci, 1984; Denison and Wilkinson, 1985; Denison et al., 1986b) even though the sensitivity of these species to TCDD varies greatly. Apparent molecular weights of the receptors seem to vary even within one species (Poland and Glover, 1990). The mouse Ah receptor was found to be resistant to salt-mediated subunit dissociation, differing from other species (Denison and Vella, 1990). When the dioxin-responsive enhancer (see below) oligonucleotide was used for binding cytosolic Ah receptors from various species, no apparent species-dependent, nucleotide-specific differences were found (Bank et al., 1992). This implies that the dioxin-responsive enhancer and Ah receptor are highly conserved, although in several species of fish no receptor-oligonucleotide complexes were found (Bank et al., 1992).

In the rat, liver Ah receptor concentrations were low shortly prior to birth. They then increased rapidly and

peaked at 3 weeks, after which there was some decline with age (Carlstedt-Duke et al., 1979; Gasiewicz et al., 1984). A similar pattern, but with an earlier peak (at 8 days), was recorded in the lung. Thymic receptor concentrations were more constant (Gasiewicz et al., 1984). In the chick embryo, the liver concentrations were high during the first half of the hatching period with a subsequent decrease, whereas enzyme induction remained high (Denison et al., 1986a). This was interpreted to indicate that only a small fraction of the Ah receptor population is required for maximal aryl hydrocarbon hydroxylase induction.

2. Cytochrome P-450 induction. The sequence of events leading to induction of cytochrome enzymes has been resolved in some detail, and only the main points will be dealt with here. It should be emphasized that an induction of the enzymes of xenobiotic metabolism, as such, is not a toxic end point. Occasionally, it can lead to toxicity as a side effect (e.g., CYP1A1 and CYP1A2 through the increase of reactive intermediates; cf. Nebert, 1989), but the phylogenetically essential characteristic of metabolism and induction of metabolism is detoxification of chemicals. In some cases, this also means a protection against carcinogens (Cohen et al., 1979; DiGiovanni et al., 1980).

TCDD binds to a cytosolic protein called Ah receptor or dioxin receptor (Poland et al., 1976), which was recently cloned (Burbach et al., 1992; Ema et al., 1992; Dolwick et al., 1993). Actually, two forms of Ah receptors have been found in rat cytosol (Denison, 1992). An apparent translocation of this TCDD-receptor complex from the cytosolic compartment to the nucleus takes place. However, there is no final proof that the unbound receptor is located in the cytosolic compartment and not the nucleus (Whitlock, 1987, 1990; Landers and Bunce, 1991; Swanson and Bradfield, 1993). Recent immunohistochemical results imply that at least in some cells from untreated mice (unliganded?) Ah receptors may be nuclear (Abbott et al., 1994).

The Ah receptor was earlier thought to belong to the group of steroid/thyroid family, and there are indeed functional similarities between these receptor systems (Whitelaw et al., 1993a), although there is hardly any cross-reactivity (Whitlock, 1987, 1990). However, the receptors are not structurally related (Burbach et al., 1992; Ema et al., 1992), and the Ah receptor seems to belong to a group of transcription factors with an NH_2-terminal basic helix-loop-helix motif essential for DNA binding (Burbach et al., 1992; Ema et al., 1992; Swanson and Bradfield, 1993). The murine b-1 type receptor cloned is a protein of 805 amino acids. TCDD binding seemed to require only a fragment of amino acids 230 to 421 (Whitelaw et al., 1993a).

TCDD binding converts the receptor to its functional, DNA-binding form (fig. 2). The inactive form of Ah receptor is assumed to exist as a stable heteromer with

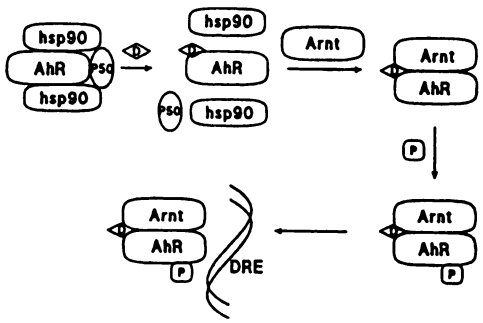

FIG. 2. Diagram of the proposed sequence of activation of the Ah receptor to DNA-binding form. D, dioxin; AhR, Ah receptor protein; hsp90, heat shock protein 90; P50, 50 kDa protein; Arnt, Ah receptor nuclear translocator protein; P, phosphate; DRE, dioxin-responsive element.

heat shock protein hsp90 (Denis et al., 1988; Perdew, 1988) and possibly other proteins (Perdew, 1992). The hsp90 has been suggested to hold the receptor in a conformation able to bind the ligand reversibly and to prevent DNA binding of the unliganded receptor (Pongratz et al., 1992; Henry and Gasiewicz, 1993). It is released before the receptor binds to DNA, and after its release, TCDD becomes very tightly bound (Henry and Gasiewicz, 1993). Another dimerization, probably with Ah receptor nuclear translocator factor (cf. Hoffman et al., 1991; Johnson et al., 1993), is needed for binding (Poellinger et al., 1992; Reyes et al., 1992; Probst et al., 1993; Whitelaw et al., 1993b). Protein kinase-dependent phosphorylation has been proposed (Carrier et al., 1992; Okino et al., 1992; Berghard et al., 1993, Kurl et al., 1993b) but also questioned (Schafer et al., 1993). The Ah receptor nuclear translocator protein and Ah receptor have similarities in their amino acid sequences (Burbach et al., 1992; Ema et al., 1992). They may dimerize via the basic helix-loop-helix domains present in both monomers to generate the functional, DNA-binding heteromeric transcription factor (Hankinson, 1993; Swanson and Bradfield, 1993).

The Ah receptor then binds to a region of DNA flanking the 5′-end of the CYP1A1 gene (Jones et al., 1985). These domains are located upstream from the transcription start site of the CYP1A1 gene and are termed dioxin-responsive transcriptional enhancers (dioxin or xenobiotic response elements). The enhancers are relatively inaccessible to DNA-binding proteins in the absence of activation (Wu and Whitlock, 1993). The liganded receptor is assumed to bind within the major DNA groove and make contact with the core recognition sequence (Denison et al., 1988; Hapgood et al., 1989; Neuhold et al., 1989; Shen and Whitlock, 1989; Saatcioglu et al., 1990; Yao and Denison, 1992). The enhancer has been proposed to contain at least six binding sites (Lusska et al., 1993; Wu and Whitlock, 1993).

By a mechanism that is not yet understood, an increasing rate of transcription of the CYP1A1 gene follows (Gonzalez et al., 1984; Israel and Whitlock, 1984;

Whitlock et al., 1989; Morgan and Whitlock, 1992). In vitro the initiation of the transcription takes place minutes after exposure to TCDD and even in the presence of protein synthesis inhibitors (Israel and Whitlock, 1984), which suggests that other newly synthesized proteins are not needed. In fact, protein synthesis inhibitors cause "superinduction," a greater induction by TCDD in the presence of inhibitors than by TCDD alone (Gonzalez and Nebert, 1985; Israel et al., 1985). This has been explained by the existence of labile inhibitory proteins that modulate the action of TCDD (Takimoto et al., 1991; Lusska et al., 1992).

3. Enzymes induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. It is assumed that the Ah receptor-enhancer system regulates a number of genes, although CYP1A1 has been studied most thoroughly. Thus, in addition to mono-oxygenases dependent on P-450IA1, a number of other enzymes are also induced (table 6). The mechanisms of induction are not necessarily the same for all enzymes and, in some instances, can be secondary. For a discussion of their association with the Ah receptor, see Bigelow and Nebert (1986) and Nebert (1989). In some cases, regulation by two genes is required, one linked with the *Ah* locus and the other one not linked (Kumaki et al., 1977).

4. Occurrence of aromatic hydrocarbon receptors in various tissues. By far the most work has been done with liver Ah receptors. Ah receptors and related phenomena, such as enzyme induction, have also been found in many other tissues (table 7). Quantitatively, the highest levels have been found in the liver, lung, intestine, and thymus (Mason and Okey, 1982; Gasiewicz, 1983). In several tissues of various species the receptors seem to be qualitatively rather similar (Henry et al., 1989). It may be anticipated that cloning the receptor will lead to rapid progress in the mapping, and perhaps typing, of Ah receptors in various organs by molecular biological techniques.

TABLE 6
Some enzymes inducible by TCDD

Enzyme	Reference
Acetanilide 4-hydroxylase	Negishi and Nebert, 1979
Acyl-coenzyme A:retinol acyltransferase	Jurek et al., 1990
Aldehyde dehydrogenase	Deitrich et al., 1977
δ-Aminolevulinic acid synthetase	Poland and Glover, 1973a,b
Aryl hydrocarbon hydroxylase	Poland and Glover, 1973a, 1974
Benzyloxyresorufin *O*-dealkylase	Beebe et al., 1990
DT-diaphorase [NAD(P)H: quinone reductase; NAD(P)H:menadione oxidoreductase]	Beatty and Neal, 1976 Kumaki et al., 1977; Gasiewicz et al., 1986b
17β-Estradiol-2-hydroxylase	Graham et al., 1988
7-Ethoxycoumarin *O*-deethylase	Gasiewicz et al., 1986b
7-Ethoxyresorufin *O*-deethylase	Phillipson et al., 1984
γ-Glutamyltranspeptidase	Gupta et al., 1981
Glutathione *S*-transferase	Baars et al., 1978; Manis and Apap, 1979; Gregus et al., 1989
Malic enzyme	Kelling et al., 1987b; Roth et al., 1988
Ornithine decarboxylase	Nebert et al., 1980
Phospholipase A_2	Bresnik et al., 1981
Protein kinases, tyrosine kinase	Bombick et al., 1985, 1987 Kramer et al., 1987
RNA polymerase	Kurl et al., 1982
Testosterone 7α-hydroxylase	Keys et al., 1985
Transglutaminase	Puhvel et al., 1984
UDP glucuronosyl transferase	Lucier et al., 1973, 1975; Marselos et al., 1978; Thunberg et al., 1984

5. Structure-activity relationship information of aromatic hydrocarbon receptors. In addition to TCDD, all dibenzo-*p*-dioxins with chlorines in the 2-, 3-, 7-, and 8-positions have an affinity for the Ah receptor, although the affinity decreases with increasing chlorination. Congeners of dibenzofuran also behave in the same way, and some non-*o*-polychlorinated biphenyl compounds are active as well. This information has been used for two purposes. First, because of the lack of toxicity data (especially data concerning long-term toxicity, such as carcinogenicity), Ah receptor-binding affinity has served as a surrogate for toxicity, along with other in vitro information regarding dioxin-like compounds. Second, these data have been utilized to deduce the structure of the dioxin-binding site of the Ah receptor. This area has been reviewed recently (Safe, 1986; Safe et al., 1989), and only some points are addressed here.

By calculating a toxic equivalency factor for each compound, the toxicity of their mixtures can also be calculated. The toxic equivalency factor concept relies on three assumptions. First, it assumes that the mechanism of action is the same for all compounds. Second, it assumes that the interactions of different compounds in a mixture are additive (implying that there are no partial antagonists). Third, it assumes that pharmacokinetic and other secondary factors do not cause too much deviation after a continuous exposure. All of these assumptions can be questioned, and the concept can only be taken as an administrative approximation rather than scientific fact. The less potent polychlorinated biphenyl compounds, in particular, may also have non-dioxin-like actions that determine their actual risk to humans (Ahlborg et al., 1992). It is also likely that metabolism and faster kinetics will reduce the long-term risk of exposure to certain dibenzofurans as compared with dibenzodioxins.

Recently, the toxic equivalency factor concept was put to the test in a short-term rat toxicity study (Stahl et al., 1992b). Four dioxin congeners, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin, 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin, 1,2,3,4,6,7,8-heptachloro-dibenzo-*p*-dioxin, and a mixture of the four in the presumed equipotent ratio were given to S-D rats. It was found that lethality (Stahl et al., 1992b), as well as several biochemical parameters associated with acute toxicity (Weber et al., 1992a), followed the toxic equiva-

TABLE 7
Occurrence of Ah receptors in various tissues (some variation among species and strains exists)

Tissue	Reference
Brain (cerebrum, cerebellum)	Carlstedt-Duke, 1979; Gasiewicz and Rucci, 1984
Heart	Gasiewicz and Rucci, 1984
Intestine	Mason and Okey, 1982; Gasiewicz and Rucci, 1984
Kidney	Carlstedt-Duke, 1979; Mason and Okey, 1982; Gasiewicz and Rucci, 1984; Kurl et al., 1985; Henry et al., 1989
Leukocytes	Gillner et al., 1989; Waithe et al., 1991
Liver	Poland et al., 1976; Carlstedt-Duke, 1979; Mason and Okey, 1982; Gasiewicz and Neal, 1982
Lung	Carlstedt-Duke, 1979; Mason and Okey, 1982; Gasiewicz and Rucci, 1984; Henry et al., 1989
Palatal shelves, embryonic	Pratt et al., 1984a
Placenta	Manchester et al., 1987
Prostate	Mason and Okey, 1982; Söderkvist et al., 1986
Skin epithelial cells	Hudson et al., 1983; Roberts et al., 1985
Spleen	Gasiewicz and Rucci, 1984; Roberts et al., 1989
Testis	Carlstedt-Duke, 1979; Gasiewicz and Rucci, 1984; Henry et al., 1989
Thymus	Carlstedt-Duke, 1979; Lund et al., 1982; Mason and Okey, 1982; Gasiewicz and Rucci, 1984; Henry et al., 1989
Tonsils	Lorenzen and Okey, 1991
Uterus	Furuhashi et al., 1986

lency factor assumption quite well (table 8). The potency was decreased to about one-fifth by every additional chlorine in this series. The study results suggest that the toxic equivalency factor concept may serve satisfactorily to predict acute toxicity, at least among the most potent dioxins. This did not hold for the resistant H/W rat strain, however, because the hexachlorodibenzo-*p*-dioxin was more potent than TCDD (Pohjanvirta et al., 1993a). It may be that in this exceptionally resistant strain some other mechanism of toxicity will surface when the TCDD-like effect is not lethal.

6. Segregation of toxicity with aryl hydrocarbon hydroxylase inducibility. One of the basic arguments for Ah receptor-mediated altered gene expression being involved in toxicity is segregation of toxicity with aryl hydrocarbon hydroxylase inducibility in cross-breeding studies between the inbred mouse strains C57BL/6 and DBA/2. The responsive phenotype segregates as a dominant trait, which is under the control of a single autosomal gene (Nebert et al., 1972; Thomas et al., 1972). The alleles governing the responsive and nonresponsive phenotypes of inducibility are designated Ah^b and Ah^d, respectively. There is a wealth of data showing differences between these phenotypes in carcinogenesis, bone marrow toxicity, hepatotoxicity, and other forms of toxicity caused by a number of chemicals (Nebert, 1989). The differences are often due to metabolism of the toxic compound, such as benzo[*a*]pyrene or aflatoxin, and could, in principle, be in either direction, depending on the metabolic routes. Enzyme induction caused by potent ligands such as TCDD could then change the toxicity of compounds metabolized by these enzymes.

TABLE 8
*Single-dose oral LD_{50} values for S-D rats along with corresponding toxic equivalency factor (TEF) and international (I-TEF) values of five chlorinated dibenzo-p-dioxins (CDD)**

CDD isomer	LD_{50} (μg/kg)	TEF	I-TEF
2,3,7,8-tetra-CDD	43	1	1
1,2,3,7,8-penta-CDD	206	0.2	0.5
1,2,3,4,7,8-hexa-CDD	887	0.05	0.1
1,2,3,4,6,7,8-hepta-CDD	6,325	0.007	0.01
Octa-CDD (estimated)	(30,000)	(0.0014)	0.001

* From Stahl et al., 1992b (with permission granted by Springer-Verlag GmbH and Co.).

The toxicity of TCDD itself, however, also segregates with the dominant responsive trait in such a way that $Ah^{b/b}$ is about ten-fold more sensitive than the $Ah^{d/d}$ phenotype. This holds for lethality and a variety of other toxic end points (see section V.B). The difference between the Ah-responsive phenotype and the nonresponsive phenotype is usually about ten-fold, agreeing with the biochemical data on Ah receptor-related phenomena.

What makes the case even more convincing is the segregation of toxicity in cross-breeding of congenic strains of C57BL/6J mice which are only assumed to differ at the *Ah* locus such that one strain is Ah-responsive ($Ah^{b/b}$) and the other Ah nonresponsive ($Ah^{d/d}$) (Birnbaum, 1986). The LD_{50} for the nonresponsive phenotype was approximately 20-fold as high as that for the responsive strain (Birnbaum et al., 1990). Several other indicators of acute toxicity exhibited a qualitative similarity in the strains, but the $Ah^{d/d}$ mice required eight to 24 times higher doses of TCDD.

Including other species, however, renders the picture much more confusing. The Ah receptors are quite similar in various species (Gasiewicz and Rucci, 1984; Denison et al., 1986b; see also section II.F.1). In addition, enzyme induction (indicating the active response range of the Ah receptor) occurs at doses within 1 order of magnitude in various species with widely different susceptibilities to the acute lethality (Gasiewicz et al., 1986b; Henry and

Gasiewicz, 1987). Finally, within another species, the rat, there are no differences in either receptor binding of TCDD or in doses causing enzyme induction, whereas lethality is different by 3 orders of magnitude (Pohjanvirta et al., 1988a, 1993a; Unkila et al., 1994b; see section V.C). This must mean that either there is no correlation between acute lethality and Ah receptors in species other than the mouse, or there must be a remarkable portion of spare receptors not needed for enzyme induction (Denison et al., 1986a; Rucci and Gasiewicz, 1988) but necessary for toxic responses, or there must be other determinants in addition to the Ah receptor-initiated gene transcription that are crucial for lethality. It is, of course, also possible that there are other Ah receptor-like receptors (cf. Poland and Glover, 1987, 1990) that have subtle differences from the receptors mediating CYP1A1 transcription or that are transformed differently after the ligand binding. These receptors could be in target tissues other than the liver.

A different possibility is that, in more resistant species, there might be antagonistic or physiological modifying mechanisms that would decrease TCDD toxicity (cf. Umbreit and Gallo, 1988). It is of interest that hamsters, as hibernators, must have very powerful regulatory mechanisms for different stages of metabolic activity and, thus, could have better correcting mechanisms against metabolic derailment. It is more difficult to explain in this way differences among various (sub)strains within a single species, e.g., between H/W and L-E rat strains (Pohjanvirta et al., 1988a) or among various Long-Evans rat substrains (Pohjanvirta and Tuomisto, 1990c).

One of the obvious problems in the interpretation is our ignorance as to what genes are essential to acute lethality; the "lethality genes" are conceivably not those coding xenobiotic metabolizing enzymes. A possibility that has raised curiosity for a long time is the possible existence of an endogenous hormone-like ligand, the actions of which TCDD would disturb or mimic. However, such a ligand has not been found so far, and if it exists, it should probably participate in the regulation of very basic vegetative functions, such as energy metabolism and nutrition.

G. Immune Suppression

1. General characterization. The immune system, especially its specific (acquired) limb, has turned out to be highly sensitive to TCDD in laboratory animals. For example, treatment with TCDD (1 μg/kg once a week for 4 weeks), which did not produce clinical or pathological changes, decreased resistance to *Salmonella* infection in mice (Thigpen et al., 1975), and a single subcutaneous dose of 0.01 μg/kg reduced the relative number of lymphocytes with the surface marker for T-helper cells in peripheral blood of marmoset monkeys (Neubert et al., 1990b). Because there are recent comprehensive reviews concerning this section of TCDD toxicity (Holsapple et al., 1991a,b; Vos et al., 1991), only the main points will be briefly described here. Thymic atrophy by TCDD has already been discussed (see section II.C.1.a).

In immunotoxicological studies of TCDD, the mouse has been the preferred test species, and the following description mainly applies to it. However, no major qualitative interspecies differences in responses have been discovered so far, including in vitro studies with human lymphocytes (Mehta et al., 1992). A notable exception to this rule was recently disclosed in the antibody plaque-forming cell response to sheep red blood cells, which was dose-relatedly suppressed by TCDD in mice but either unaffected or enhanced in rats (Smialowicz et al., 1994).

Both B- and T-lymphocytes are functionally compromised by TCDD. Assessments of their relative sensitivities to TCDD have yielded variable results (Vos et al., 1973; Faith and Moore, 1977; Faith and Luster, 1979; Vecchi et al., 1980; Chastain and Pazdernik, 1985; Dooley and Holsapple, 1988; Dooley et al., 1990; Lundberg et al., 1991). Essential determinants in this respect may be the species and age of the animal, as well as the duration of exposure (Luster et al., 1989).

TCDD impairs stimulated antibody production (Vecchi et al., 1980, 1983a,b), with a variable effect on basal antibody production (Sharma and Gehring, 1979; Moran et al., 1986; Kramer et al., 1987), probably by interfering with the differentiation of B-cells into plasma cells (Tucker et al., 1986; Luster et al., 1988). In addition to the impact on the differentiation process, TCDD may also hamper B-cell proliferation (Mehta et al., 1992; Morris et al., 1993; Karras and Holsapple, 1994). In vitro the suppressed antibody response is accompanied by protein phosphorylation (Clark et al., 1991a), and both effects have been shown to be dose-dependently reversible by human γ-interferon (Snyder et al., 1993).

Regarding T-cells, TCDD seems to selectively interfere with their antigen-specific activation (Lundberg et al., 1992; Neumann et al., 1993). TCDD also inhibits the generation or activity of cytotoxic T-lymphocytes (Clark et al., 1984; Kerkvliet et al., 1990a; Holladay et al., 1991). The inhibition may be mediated by induced suppressor T-cell activity (Clark et al., 1981; Nagarkatti et al., 1984), although other studies have failed to substantiate this (Luster et al., 1980; Dooley et al., 1990). In addition, in mice TCDD reduces the number of progenitor stem cells (Luster et al., 1980, 1985; Fine et al., 1989, 1990a,b), decreases the cytolytic activity of polymorphonuclear cells (Ackermann et al., 1989), and depresses complement activity (White et al., 1986).

On the other hand, a few essential components of host defense systems against microbial agents are relatively refractory to TCDD. For example, numerous investigators have reported unaffected capability for normal function (although not necessarily the number) of macrophages, T-helper cells, and natural killer cells after

TCDD exposure (Faith and Moore, 1977; Vos et al., 1978; Faith and Luster, 1979; Luster et al., 1979; Mantovani et al., 1980; Dooley and Holsapple, 1988; Dooley et al., 1990; House et al., 1990; Kerkvliet and Oughton, 1993). In some cases, however, indications of suppressed T-helper function (Kerkvliet et al., 1990b; Tomar and Kerkvliet, 1991) and either augmented (Funseth and Ilbäck, 1992) or depressed (Selgrade et al., 1992) natural killer cell activity have been observed.

There appear to be certain peculiarities in the dose-response relationships for immunological impacts of TCDD. At low doses, the effect may be reversed. Neubert et al. (1992) showed that, whereas a weekly dose of 1.5 ng/kg TCDD administered to marmoset monkeys reduced the percentage and absolute number of "helper-inducer" T-cells in the blood, a still lower dose of 0.3 ng/kg per week induced an increase in this lymphocyte subpopulation. Another interesting feature is that the degree of *Ah* dependence (see section II.F.6) of the immune suppression may differ between acute and subacute exposures. A 14-day treatment with TCDD was notably more effective in causing thymus atrophy than the same cumulative dose given as a single injection in DBA/2 mice, an *Ah* low-responder strain, but not in B6C3F1 mice, an *Ah* high-responder strain (Morris et al., 1992). Furthermore, suppression of humoral immunity was aggravated approximately ten-fold by the subacute regimen in DBA/2 mice without the accompanying hepatomegaly that was observed in B6C3F1 mice under comparable conditions. In line with these findings, 14-day treatment with a congener of TCDD devoid of affinity for the Ah receptor, 2,7-dichlorodibenzo-*p*-dioxin, produced suppression of the antibody responses to both a T-dependent and a T-independent antigen in B6C3F1 mice but did not induce hepatic microsomal enzyme activities or cause hepatomegaly (Holsapple et al., 1986b).

2. Endotoxin hypersensitivity and tumor necrosis factor. TCDD exposure has been reported to increase the sensitivity of mice to endotoxin, as assessed by 48-h mortality (Vos et al., 1978; Rosenthal et al., 1989). This hypersensitive state was not manifest on day 1 but occurred by day 5 after TCDD exposure. Although serum endotoxin levels were not changed by TCDD, the clearance of injected endotoxin tended to be slowed. This, together with a protective influence of uridine, suggested impaired hepatic detoxification of endotoxin as the reason for the hypersensitivity (Rosenthal et al., 1989). In contrast to these findings, TCDD did not affect the susceptibility of L-E or H/W rats to endotoxin lethality, although it should be noted that mortality was recorded at 24 h (Pohjanvirta et al., 1990b). However, endotoxin appeared to exert an additive effect with TCDD on lipid peroxide concentration in the liver of H/W rats. The differential outcome in these two species of rodents suggests that TCDD-induced endotoxin hypersensitivity might be a phenomenon unique to mice. This hypothesis is supported by both distinct pathology [severe ascites or subcutaneous edema is a characteristic feature in mice but not in rats (McConnell et al., 1978b; Kelling et al., 1985)] and an opposite effect of dexamethasone on TCDD toxicity [aggravation in rats (Pohjanvirta et al., 1988b), amelioration in mice (Taylor et al., 1992)].

Endotoxin itself is not highly toxic to most mammalian tissues; nor does it exert most of its effects on the host's metabolism directly. However, it stimulates the production of a molecule, cachectin or TNF, which is the primary mediator of endotoxin shock (for reviews, see Beutler and Cerami, 1987, 1988). Therefore, it is not surprising that TCDD turned out to promote TNF secretion in a dose-related manner in endotoxin-exposed mice (Clark et al., 1991b). Peritoneal macrophages from NMRI mice treated with 25–75 μg/kg TCDD 14 days earlier also showed dose-dependently induced secretion of TNF (Massa et al., 1992). Similarly to dexamethasone, which is known to potently inhibit TNF biosynthesis (Beutler and Cerami, 1987), anti-TNF antibody diminished TCDD lethality in C57BL/6J mice (Taylor et al., 1992). However, dexamethasone was clearly a more effective alleviator, and in replicate experiments, the difference in TCDD exposure survival rates between anti-TNF antibody and the control substance (hamster immunoglobulin G) groups did not reach statistical significance. Both dexamethasone and the anti-TNF antibody tended to mitigate TCDD-induced alterations in serum clinical chemistry values. Dexamethasone also counteracted TCDD-elicited liver damage but concomitantly enhanced its microsomal monooxygenase induction; the anti-TNF antibody was not examined for these end points (Taylor et al., 1992). Both dexamethasone and anti-TNF antibody significantly antagonized TCDD-stimulated lipid peroxidation in mice (Alsharif et al., 1994a), which is in agreement with findings from other studies that have demonstrated the ability of TNF to promote oxidative stress (Janssen et al., 1993). Thus, there is accumulating evidence for an important role for TNF, and possibly other mediators of inflammation as well, in the acute toxicity of TCDD in mice. To what degree these findings can be extended to other species remains to be determined.

3. Changes in other inflammatory mediators and in responsiveness to them. Theobald et al. (1983) demonstrated that TCDD dose-dependently increased the potency, but not the maximum efficacy, of carrageenan and dextran in producing paw edema in rats. The effect was apparent within a few hours after TCDD exposure and lasted for at least 29 days. At 5 days, the ED_{50} of TCDD in enhancing the carrageenan response was 6 μg/kg. TCDD also amplified carrageenan-induced pleural edema but did not affect the number or type of leukocytes in the pleural exudate. Granuloma formation in response to subcutaneous cotton pellets was not influenced by TCDD, which implied a selective impact on edema for-

mation. Similar potentiation was obtained with other 3-methylcholanthrene-type enzyme inducers as well but not with phenobarbital-type inducers.

A possible mechanism for the enhanced irritant-induced edema formation could be stimulated synthesis of vasoactive prostaglandins by TCDD. However, although there is a report of promoted cardiac prostaglandin release by TCDD in chick embryos at certain low doses (Quilley and Rifkind, 1986), prostaglandin synthetase activity in rabbit liver or kidney medulla microsomes was not altered by TCDD (Kohli and Goldstein, 1981); nor was arachidonic acid turnover altered in XB/3T3 cells (Knutson and Poland, 1984b). A subsequent study with anti-inflammatory agents by Katz et al. (1984) reinforced the view that prostaglandins were not critically involved in TCDD-enhanced edema formation. On the other hand, TCDD turned out to increase the edemagenic potency of bradykinin and histamine (but not 5-HT or prostaglandin E2), which suggests that TCDD augments the effect of these two mediator substances on vascular endothelium and by this means produces the amplified edema response.

TCDD was shown to increase the amounts of mRNAs for interleukin-1β and plasminogen activator inhibitor-2 in a human keratinocyte cell line (Sutter et al., 1991). However, in vivo exposure of B6C3F1 mice to 10 μg/kg TCDD 7 to 10 days earlier did not result in any detectable effect on interleukin-1 production by peritoneal macrophages (House et al., 1990). The sera from these mice also contained unaltered titers of interferon.

H. Biochemical Alterations in Cell Membranes

1: 2,3,7,8-Tetrachlorodibenzo-p-dioxin and membrane fluidity. TCDD causes a number of changes in liver cell membranes (Jones and Butler, 1974; Jones, 1975), which are reflected in increased permeability (Davidson and Fujimoto, 1987) and alterations in various transport and enzymatic functions, including Na^+, K^+-ATPase, and Mg^{2+}-ATPase (Jones, 1975; Peterson et al., 1979; Brewster, 1985). Some changes are physicochemical effects in membranes, such as decreased hepatic microsomal, mitochondrial, and plasma membrane fluidity. These effects seem to be connected with oxidative damage (Alsharif et al., 1990; Stohs et al., 1990b), which has been suggested to contribute to an influx of calcium and related effects that may lead to cell dysfunction and death (Stohs et al., 1990b). The problem is knowing whether the effects are primary or secondary (see also section II.I.1); in addition, they may be associated with acute lethality but not necessarily as an etiological factor.

2. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and integral proteins of cell membranes. As shown by electrophoresis and concanavalin A binding, the levels of a number of proteins are reduced in liver plasma membranes after TCDD treatment (Brewster et al., 1982). Hepatic plasma membranes from TCDD-treated animals also had a lowered capacity to bind exogenous low-density lipoprotein (Bombick et al., 1984). This was interpreted as a decrease in low-density lipoprotein receptor activity, and in a subsequent study, decreases in several membrane receptors were found, including EGF, insulin (Madhukar et al., 1984), glucagon (Matsumura et al., 1984), and insulin-like growth factor receptors (Liu et al., 1992).

TCDD administered in vivo causes a profound increase in protein kinase activities in the hepatic plasma membrane of rats and guinea pigs. Kinases activated in the process include protein kinase C (Bombick et al., 1985) and several tyrosine kinases (Bombick and Matsumura, 1987). Induction of protein kinase C by TCDD has also been demonstrated in the rat testis (Al-Bayati et al., 1988b) and thymus (Bombick et al., 1988) in vivo, as well as in primary cultures of rat hepatocytes (Wölfle et al., 1993b) and thymocytes (DePetrillo and Kurl, 1993) in vitro. Protein kinase C was also activated in rainbow trout (Newsted and Giesy, 1993). In addition, TCDD has been reported to stimulate the expression of the c-*ras* protooncogene product in hepatic plasma membranes (Tullis et al., 1992). As for the adipose tissue of the guinea pig, however, the findings are conflicting. Whereas an early in vivo study recorded either unchanged or slightly depressed protein kinase activities after TCDD administration (Brewster, 1985), a more recent work using explant tissue culture revealed induction of protein phosphorylation, probably by way of augmented protein tyrosine kinase and protein kinase C activities (Enan and Matsumura, 1993). Such changes suggest that various signal transduction phenomena are involved in the effects of TCDD. Only those phenomena related to EGF receptors have been studied in depth.

There has been some discussion of what comes first, and c-*ras* expression has been suggested to occur so early that it would not be a result of EGF receptor downregulation but rather a cause of it (Tullis et al., 1992). In vitro protein kinase C activation in rat thymocytes and in murine Hepa-1 hepatoma cells has also been seen in minutes pointing to a primary role (Puga et al., 1992; DePetrillo and Kurl, 1993). On the other hand, prolonged exposure (3 to 48 h) to TCDD was needed to activate protein kinase C in primary cultures of rat hepatocytes (Wölfle et al., 1993b). The activation of EGF receptor through the phosphorylation of tyrosine residues and the inactivation of activity through phosphorylation of serine and threonine residues is one mechanism by which the EGF receptor activity in cell growth and differentiation might be controlled (cf. Newsted and Giesy, 1993, and below).

3. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and epidermal growth factor receptor. The EGF receptor is a transmembrane glycoprotein that has ligand-dependent tyrosine kinase activity (for recent reviews, cf. Velu, 1990; Carpenter and Wahl, 1991). Different polypeptide growth factors are able to bind and activate the receptor. Two

of these predominate, EGF and TGF_α. EGF seems to function principally via an endocrine mode, whereas TGF_α acts locally via a paracrine, autocrine, or juxtacrine mode (Velu, 1990).

The tyrosine kinase of the EGF receptor seems to represent its effector function. This results in phosphorylation of tyrosine residues from various cellular substrates (Carpenter and Wahl, 1991), as well as in receptor autophosphorylation, which may, in fact, be intermolecular cross-phosphorylation within an oligomeric receptor complex created by ligand binding (Schlessinger, 1988; Schlessinger and Ullrich, 1992). EGF and TGF_α are mitogenic but possess many other activities as well (Carpenter and Wahl, 1991).

a. DOWNREGULATION OF EPIDERMAL GROWTH FACTOR RECEPTOR AFTER 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN. TCDD has been demonstrated to decrease the binding capacity of EGF receptors in the liver (Madhukar et al., 1984) and in hepatoma cells (Kärenlampi et al., 1983). By using a 50% decrease in EGF binding as an indicator, Madhukar et al. (1984) found that the guinea pig was the most sensitive species, with the S-D rat and Golden Syrian hamster about 14 and 32 times less sensitive, respectively. In three mouse strains that tolerate TCDD differently (C57BL/6J, CBA/J, and AKR/J), the decrease in EGF binding was more severe in the two sensitive strains (Madhukar et al., 1984). According to results from congenic strains of mice that are assumed to differ only at the *Ah* locus, this effect has been suggested to be dependent on the *Ah* locus (Lin et al., 1991a).

The mechanism by which TCDD alters EGF receptor binding is not known. As measured by immunohistochemical detection, by EGF stimulation of EGF receptor autophosphorylation, or by Western analysis, the hepatic EGF receptor is downregulated (Sewall et al., 1993, submitted), but TCDD was not found to decrease EGF receptor mRNA in the mouse liver (Lin et al., 1991a).

In keratinocytes EGF receptor downregulation may result from an overexpression of TGF_α and the consequent enhanced internalization and phosphorylation of the EGF receptor (Choi et al., 1991). The proposed mechanism of TGF_α increase is a posttranscriptional stabilization of TGF_α mRNA (Gaido et al., 1992).

Indications of EGF receptor activation, such as enhanced activities of protein kinases (Bombick et al., 1985; Bombick and Matsumura, 1987; Madhukar et al., 1988; Ma et al., 1992) and EGF receptor phosphorylation (Madhukar et al., 1984; Bombick et al., 1985; Newsted and Giesy, 1993; Sewall et al., 1993), have also been seen in the liver after TCDD administration. TCDD has not been shown to increase circulating EGF (Madhukar et al., 1988), however, which would explain enhanced EGF receptor activity. TCDD itself does not bind to the EGF receptor, although the evidence for this is circumstantial (Madhukar et al., 1988). Contrary to results in keratinocytes (Gaido et al., 1992), in mouse liver the amount of TGF_α mRNA did not change after TCDD (Lin et al., 1991a). It is of interest that, whereas the immunoreactivity to EGF receptor antibody at the plasma membrane decreased, the cytoplasmic immunoreactivity tended to increase (Sewall et al., 1993).

All of this implies that the apparent downregulation by TCDD is due to increased stimulation, internalization, and degradation of the receptor, but the exact mechanism that leads to this end point is not clear. In contrast to the increased phosphorylation seen after acute exposure to TCDD, phosphorylation was decreased in correlation with decreased receptor binding of EGF after prolonged exposure (Sewall et al., 1993). This is understandable if we assume that receptor synthesis does not keep pace with the stimulation-linked degradation.

Downregulation of the liver EGF receptor has been suggested to be more sensitive to TCDD than is 7-ethoxyresorutin O-deethylase induction. The ED_{50} values were calculated to be approximately 0.3 and 2.5 μg/kg in the rat (Ma et al., 1992) and 0.17 and 0.79 μg/kg in the trout (Newsted and Giesy, 1993). These values imply that the EGF receptor would be downregulated at a five- to nine-fold lower dose than that needed for 7-ethoxyresorutin O-deethylase induction. In Ah-responsive $Ah^{b/b}$ mice and nonresponsive $Ah^{d/d}$ mice, the ED_{50} values were 0.7 and 7 μg/kg, respectively (Lin et al., 1991a). The respective ED_{50} values for induction of 7-ethoxyresorutin O-deethylase activity were 1.6 and 16 μg/kg. After prolonged exposure in rats, the ED_{50} of the decrease in receptor capacity was close to the ED_{50} of CYP1A1 induction (Sewall et al., 1993). On the whole, it seems that in many cases doses causing EGF receptor downregulation and CYP1A1 induction are not too far from each other.

EGF receptor downregulation in the mouse liver has also been demonstrated after administration of some polychlorinated dibenzofuren compounds (Ryan et al., 1989).

b. EPIDERMAL GROWTH FACTOR RECEPTOR AS A MEDIATOR OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN TOXICITY. A proposal was put forth quite early that some of the toxic and morphological manifestations of TCDD are due to the effects of TCDD on the EGF receptor. In rats the decrease in body weight after a single dose of TCDD correlated with the decrease in EGF binding, and among differently sensitive inbred mouse strains, EGF binding was reduced in the sensitive strains (Madhukar et al., 1984). Several parameters in neonatal development, including eye opening and tooth eruption, were affected similarly by TCDD and EGF in the mouse (Madhukar et al., 1984, 1988), although not in the mink (Aulerich et al., 1988). Thus, at least initially and in certain species, the effect of TCDD resembles EGF receptor stimulation rather than downregulation. This becomes understandable if the cause of downregulation is actually increased

binding of the ligand, followed by mobilization of the receptor from the cell membrane, response, internalization, and degradation.

i. Acute lethality. Relatively little work has been done to elucidate the role of the EGF receptor in the acute lethality of TCDD. In an early study (Madhukar et al., 1984), the rat and the hamster were found to be more resistant than the guinea pig to EGF receptor downregulation, but the difference was nowhere close to the differences in their LD_{50} values. In congenic $Ah^{b/b}$ and $Ah^{d/d}$ mice, the ED_{50} of TCDD on the downregulation of the EGF receptor differed by a factor of 10, i.e., the same as that for the LD_{50} values, which suggested an association between Ah receptors and the EGF receptor downregulation, as well as with lethality (Lin et al., 1991a). Yet in most species, the effects on the EGF receptor are seen at doses that cause minimal systemic toxicity (Madhukar et al., 1984; Lin et al., 1991a). In rats, inhibition of protein kinase activity by quercetin has been reported to antagonize the lethal action of TCDD to some extent (Bombick and Matsumura, 1987).

There seems to be both a qualitative and a quantitative difference between H/W and L-E rat strains. Decreases in EGF-binding capacity and EGF receptor protein of liver membranes are time and dose dependent in the L-E rat, whereas doses of 50 and 500 μg/kg caused a similar reduction in the H/W rat, and there was no further decrease from day 4 to day 10 (Tuomisto et al., 1993a). This may mean that there is a ceiling for the downregulation in the H/W strain. Whether or not this has to do with the resistance of H/W rats is not known.

ii. Skin. Skin afflictions and EGF receptors as a mode for TCDD effects have been studied in some detail but will be discussed here very briefly because the lesions are not acute. The reader is referred to reviews of this particular topic (Greenlee et al., 1987). Skin epithelial cells are of special interest because, in general, very few of the various biochemical responses in cell cultures can be associated with TCDD toxicity (Poland and Knutson, 1982). Several epidermal cell lines or normal epidermal cells are an exception (Greenlee et al., 1987).

Keratinocytes are highly responsive to TCDD (cf. Poland and Knutson, 1982, Knutson and Poland, 1984b). At low concentrations, EGF receptors are downregulated in vitro in hours, and protein kinase activity and keratinization are increased (Kawamoto et al., 1989). In this case the downregulation is temporary, possibly because the cells differentiate to a less responsive level. As described above, the mechanism of downregulation is thought to be posttranscriptional induction of TGF_{α} (Gaido et al., 1992). The alterations in programing of cell growth and differentiation that follow may be essential for the pathogenesis of, for example, chloracne. However, one should note that glucocorticoid receptors, but not EGF receptors, were downregulated by TCDD in the skin of hairless mice (Stohs et al., 1990a).

iii. Developmental toxicity. Since the EGF receptor plays an important role in controlling growth and differentiation, its possible role in the teratogenicity and embryo/fetotoxicity of TCDD-like compounds is of particular interest. One of the best-studied sites is the palatal epithelium. EGF (Abbott and Birnbaum, 1990a) and EGF receptors (Abbott et al., 1988) are expressed in the developing palate and, along with other growth factors, may have an important role in regulating the proliferation and differentiation of epithelial and mesenchymal palatal cells (Abbott et al., 1992b; Brunet et al., 1993).

Cleft palate is induced in embryonic mice by TCDD (Courtney and Moore, 1971; Neubert et al., 1973), and this is due to a failure of palatal shelves to fuse in the midline even though they come into contact (Pratt et al., 1984a,b). The failure appears to be a consequence of altered differentiation and proliferation of medial epithelial cells, i.e., programed cell death and fusion of the opposing shelves does not occur (Abbott and Birnbaum, 1989a,b, 1990a). The continued proliferation is accompanied by continued expression and increase of EGF receptors (Abbott and Birnbaum, 1989a,b). Both EGF and TGF_{α} (as well as TGF_{β}) decreased (Abbott and Birnbaum, 1990a). It may be that several growth factors acting in concert are needed for normal palatogenesis, and cleft palate induced by TCDD is associated with disruption of this pattern (Abbott and Birnbaum, 1990a). Interestingly, Ah receptors were downregulated after TCDD throughout the palate (Abbott et al., 1994).

The alterations induced by TCDD were also observed in vitro in human embryonic palatal shelves, but they were less sensitive than mouse palatal shelves, suggesting that higher exposures would be needed in humans than in mice to induce this teratogenic end point (Abbott and Birnbaum, 1991).

Ureteral epithelium is another site of enhanced proliferation that is associated with increases in the levels of EGF receptors (Abbott et al., 1987; Abbott and Birnbaum, 1990b). The sensitive period for hydronephrosis is longer than that for cleft palate (Couture et al., 1990b), and hydronephrosis can be caused even by lactational exposure (Couture-Haws et al., 1991a,b). TCDD is also more potent in inducing hydronephrosis than it is in causing cleft palate in mice (Couture et al., 1990b).

Rats are more resistant to the teratogenicity/developmental toxicity of polychlorinated dibenzo-*p*-dioxins/polychorinated dibenzofurans than mice are, and the fetuses are only affected at doses that are also toxic to the dam (Schwetz et al., 1973; Couture et al., 1989, 1990a; Olson et al., 1990). It is of interest that developmental toxicity differs in the resistant H/W rat and the susceptible L-E rat in that, with little overlap, H/W offspring are susceptible to hydronephrosis and L-E offspring are susceptible to cleft palate (Huuskonen et al., 1994). EGF

receptors have not yet been studied in the offspring, but liver receptors were downregulated dose dependently in the L-E strain; in H/W the downregulation plateaued to a maximum at a dose level that is only a fraction of the LD_{50} for that strain (Tuomisto et al., 1993a).

In general, sensitivity differences in developmental toxicity among species and strains do not follow the sensitivity differences in acute lethality (Couture et al., 1990a; Olson and McCarrigle, 1992). Indirect effects due to a complex interplay of regulatory factors such as EGF and TGFs would be a natural track to follow in search of reasons for such differences.

iv. Carcinogenicity. Since EGF is a potent mitogen with tumor-promoting activity (Stoscheck and King, 1986; Aaronson, 1991; Carpenter and Wahl, 1991), some effort has been devoted to explaining the animal carcinogenicity, and especially hepatocarcinogenicity, of TCDD by changes in EGF regulation (Lev-Ran et al., 1986; Harris et al., 1987; Goldstein et al., 1990; Clark et al., 1991c; North et al., 1992; Schrenk et al., 1992; Sewall et al., 1993). These aspects are not dealt with in the present review of short-term toxicity of TCDD.

I. Other Short-Term Effects

1. Lipid peroxidation. A role for lipid peroxidation (and, more generally, oxidative stress) in the acute toxicity of TCDD was first proposed by Albro et al. (1978), who found a dose-related increase in the amount of lipofuscin in the hearts of TCDD-treated rats. The idea has since been further advanced, especially by Stohs (1990), who has recently reviewed this field in depth. Therefore, here we will limit the description to the salient features (for details of TCDD-induced cell membrane alterations, see section II.H).

Results of numerous studies have verified the fact that TCDD exposure results in enhanced lipid peroxidation in rodents (Stohs et al., 1983; Al-Bayati et al., 1987a; Hermansky et al., 1987; Shara and Stohs, 1987; Goel et al., 1988; Mohammadpour et al., 1988), although not all indices of lipid peroxidation are necessarily affected identically (Robertson et al., 1985; Shara et al., 1992). The promotion is most marked in the liver but has also been reported in extrahepatic tissues, such as heart, testes, thymus, brain, adrenal glands, and kidneys (Al-Bayati et al., 1987a, 1988b; Hermansky et al., 1988; Mohammadpour et al., 1988; Bestervelt et al., 1994), as well as in serum and urine (Bagchi et al., 1993). In the rat liver, lipid peroxidation (measured as the concentration of substances that react with thiobarbituric acid) may already be augmented 24 h after TCDD treatment but reaches its peak (three to five times the control value at doses of 50 to 120 μg/kg) 6 to 9 days postexposure (Al-Bayati et al., 1987a; Goel et al., 1988). Female rats appear to be more responsive than male rats (Al-Bayati et al., 1987a).

The effect of TCDD on lipid peroxidation may be more quantitative than qualitative, because, in both control and TCDD-treated rats, hepatic microsomal lipid peroxidation seems to stem primarily from hydrogen peroxide, although superoxide, hydroxyl radical, and singlet oxygen may also be involved (Al-Turk 1986; Stohs et al., 1986; Al-Bayati and Stohs, 1991). The chief sources of reactive oxygen species in the case of TCDD are probably microsomes (Stohs et al., 1983; Hassan et al., 1985a,c; Bagchi and Stohs, 1993), mitochondria (Stohs et al., 1990b,c; Bagchi and Stohs, 1993), and macrophages, as well as other phagocytes (Alsharif and Stohs, 1992; Alsharif et al., 1994b; Bagchi and Stohs, 1993). TCDD-induced lipid peroxidation has been found to be iron dependent (Albro et al., 1986; Al-Bayati and Stohs, 1987; Al-Turk et al., 1988), and changes in the subcellular distribution of iron (Al-Bayati et al., 1987a,b, 1988b; Wahba et al., 1988a, 1989b, 1990a), as well as of some other cations, such as copper (Al-Bayati et al., 1988b; Wahba et al., 1988a; Elsenhans et al., 1991), magnesium (Wahba et al., 1988a), and calcium (Al-Bayati et al., 1988a; Stohs et al., 1990b,c; Wahba et al., 1989b), coincide with, and probably contribute to, the effect.

In addition to producing reactive oxygen species, TCDD also promotes lipid peroxidation by inhibiting the activity of the most important defense enzyme against hydrogen peroxide and radicals derived from it, glutathione peroxidase (Hassan et al., 1985a,b; Shara and Stohs, 1987; Al-Bayati et al., 1988b; Al-Turk et al., 1988; Pohjanvirta et al., 1990b). The activity of superoxide dismutase may also be slightly depressed, at least in the heart (Hermansky et al., 1988) and testes (Al-Bayati et al., 1988b). TCDD-induced microsomal lipid peroxidation has recently been shown to be reversed in vitro by inhibitors of phospholipase A_2 (Al-Bayati and Stohs, 1991), which releases arachidonic acid from phospholipids. Arachidonic acid, in turn, serves as a precursor for lipid peroxidation (Iida et al., 1982). Thus, these findings have implicated phospholipase A_2 activity as a key mediator of microsomal lipid peroxidation by TCDD.

The actual relationship between lipid peroxidation and the acute lethality of TCDD is not yet clear. In an early work, Hassan et al. (1983) discovered that lethal doses of TCDD administered to rats and guinea pigs (120 and 3 μg/kg, respectively) stimulated hepatic microsomal lipid peroxidation (seven-fold in rats, but only 1.6-fold in guinea pigs), whereas no such effect was detected in hamsters to which a sublethal dose of TCDD (600 μg/kg) was administered. In inbred mouse strains, the responsiveness to TCDD-enhanced lipid peroxidation correlated with sensitivity to TCDD lethality (Mohammadpour et al., 1988). This was also the case in L-E and H/W rats (Pohjanvirta et al., 1990b). Although lipid peroxidation thus appeared to be associated with TCDD lethality, it was not certain which of the two phenomena was primary, because the drastic effect of TCDD on body and organ weights had not been taken into account in

the early studies. Indeed, lipid peroxidation proved to be similarly aggravated in L-E rats pair fed to their lethally intoxicated counterparts, with the impact emerging at a slightly earlier stage (Pohjanvirta et al., 1990b). Futhermore, when the resistant H/W rats were maintained with a restricted feed regimen in accord with the feed consumption pattern of L-E rats treated with a lethal dose of TCDD, they also displayed markedly enhanced hepatic lipid peroxidation (Pohjanvirta et al., 1990b). These findings were replicated and extended in S-D rats by Wahba et al. (1989b). In their study, pair feeding resulted in greater alterations in the hepatic mitochondrial and microsomal concentrations of iron and calcium than occurred after TCDD exposure, whereas lipid peroxidation was more effectively induced by TCDD. In all instances the changes occurred in the same direction in pair-fed and TCDD-dosed rats, except for microsomal iron, which increased in pair-fed rats but decreased in TCDD-dosed rats. An important additional finding in these two studies was that enhanced hepatic lipid peroxidation in feed-restricted animals coincided with the development of liver atrophy. This was also the case in TCDD-treated L-E rats (Pohjanvirta et al., 1990b). The experiments with feed-restricted rats further implied that the inhibition of glutathione peroxidase activity was a direct effect of TCDD, inasmuch as it could not be elicited by starvation (Wahba et al., 1989b; Pohjanvirta et al., 1990b).

Other approaches have also been exploited in an attempt to clarify the role of lipid peroxidation in TCDD toxicity. Albro et al. (1988) compared the effects of TCDD with those of carbon tetrachloride on the disposition of linoleic acid in the livers of Fischer 344 rats and arrived at the conclusion that the extent of TCDD-stimulated lipid peroxidation in rats in vivo appeared to be too modest to account for the toxicity of TCDD. Surgical thyroidectomy, which markedly modulates TCDD toxicity (see section II.D.4), slightly attenuated the effects of TCDD on hepatic lipid peroxidation and glutathione peroxidase (Hermansky et al., 1987). Maintaining rats on an iron-deficient diet totally prevented TCDD-enhanced hepatic lipid peroxidation but failed to influence the body weight loss caused by TCDD (Al-Turk et al., 1988). In line with that observation, a diet supplemented with the potent antioxidant, butylated hydroxyanisole, tended to increase the survival rate and time of TCDD-exposed adult male L-E rats without modifying the initial decline in body weight (Pohjanvirta et al., 1990b).

More pronounced protection by butylated hydroxyanisole against TCDD-promoted lethality, body weight loss, and hepatic lipid peroxidation was recorded in young female S-D rats (Stohs et al., 1984; Hassan et al., 1985a, 1987). However, although both vitamins A and E entirely abolished the hepatic microsomal lipid peroxidation caused by TCDD in these rats, they exhibited a negligible effect on TCDD lethality or weight loss (Stohs et al., 1984; Hassan et al., 1985b). The same lack of effect on change in body weight and mortality was true for vitamin C, which aggravated the hepatic microsomal lipid peroxidation produced by TCDD (Hassan et al., 1987). An optimal concentration of selenium in the diet (0.1 ppm) increased the survival rate of TCDD-exposed young female S-D rats without affecting initial loss of body weight or the magnitude of microsomal lipid peroxidation in the liver (Hassan et al., 1985c). The link between hepatic lipid peroxidation and overt toxicity of TCDD is, therefore, not tight, and it is feasible that the ameliorating effects of butylated hydroxyanisole were at least partially attributable to some of its other actions unrelated to the antioxidant function, such as induction of hepatic glucuronidation (Gregus and Klaassen, 1988). As to more specific toxic end points, butylated hydroxyanisole has been shown to afford moderate protection against the porphyria (Sweeney et al., 1984), but not against the impaired bile flow and biliary ouabain excretion (Choe and Yang, 1983), caused by TCDD.

In hepatocyte and macrophage nuclei, TCDD-promoted oxidative stress may lead to single-strand breaks in DNA (Wahba et al., 1988b; Alsharif et al., 1994b). The DNA effect exhibited a slight temporal lag compared with nuclear lipid peroxidation (Stohs et al., 1990c; Alsharif et al., 1994b). It was dose dependent and appeared to be unrelated to hypophagia and the associated changes in body and organ weights, because it was not seen in pair-fed S-D rats (Wahba et al., 1989a,b). A 10-day dosing regimen with the antioxidant, oltipraz, prevented the increase in hepatocyte nuclear single-strand breaks by TCDD, whereas clofibrate was less effective (Wahba et al., 1989a). Both microsomes and mitochondria, but not cytosol, from TCDD-treated rats were able to induce DNA single-strand breaks in hepatocyte nuclei from untreated rats in vitro, and the induction was reversible by deferoxamine. Addition of Fe^{2+} or Fe^{3+} ions to the nuclei of untreated rats brought about the same outcome, which was again reversible by deferoxamine (Wahba et al., 1989a). However, when deferoxamine was given in vivo to young female S-D rats during a period of 10 days, TCDD-induced nuclear lipid peroxidation tended to be mildly suppressed, whereas the number of DNA single-strand breaks in hepatocytes turned out to be almost doubled (Wahba et al., 1990b). Likewise, a combination of dexamethasone and anti-TNF antibody reduced both hepatic microsomal and mitochondrial lipid peroxidation by TCDD more effectively than either treatment alone, whereas there was no positive coeffect in the inhibition of DNA single-strand breaks (Alsharif et al., 1994a). Hence, factors other than lipid peroxidation may also participate in the production of DNA single-strand breaks by TCDD.

More studies are needed to settle questions concerning the contribution of oxidative stress to the short-term

toxicity of TCDD. At present, the weight of evidence favors the view that it may be involved as a primary factor in certain specific end points such as DNA single-strand breaks, but with regard to the wasting syndrome, it seems to be a consequence rather than the cause.

2. *Vitamin A.* A review of the interference of TCDD and related chemicals with vitamin A homeostasis appeared recently (Zile, 1992). We will summarize the key points here, confining our discussion to findings concerning TCDD.

A consistent effect of TCDD in laboratory animals is a reduction in the hepatic storage of vitamin A (Thunberg et al., 1980; Thunberg, 1984; Håkansson et al., 1991a). An almost equally common decrease in vitamin A levels occurs in the lung (Håkansson et al., 1991a). The dose-response curves for the liver (and apparently the lung) effect follow approximately those for acute lethality, except in the hamster, which seems to be more sensitive to vitamin A reduction than are other rodents (Hanberg et al., 1990; Håkansson et al., 1991a). In rats, TCDD characteristically increases renal vitamin A at high nonlethal doses, but this change vanishes at lethal doses (Powers, 1988; Hanberg et al., 1990; Pohjanvirta et al., 1990a; Håkansson et al., 1991a). Rats also tend to respond to TCDD by slightly or moderately increasing their serum concentrations of vitamin A (Thunberg et al., 1979; Håkansson et al., 1987, 1991a; Powers, 1988; Pohjanvirta et al., 1990a). The effects of TCDD on vitamin A levels in rats are thus conspicuously reminiscent of those seen in mild vitamin A deficiency (Morita and Nakano, 1982).

Renal and serum vitamin A alterations are uncommon in other species (Håkansson et al., 1991a). The changes in vitamin A status appear within a couple of days after TCDD exposure and tend to persist for at least several months (Thunberg et al., 1979; Håkansson and Hanberg, 1989; Pohjanvirta et al., 1990a; Håkansson et al., 1991a). They do not arise as a consequence of hypophagia (Bank et al., 1989; Håkansson et al., 1989). As to the chemical forms of vitamin A, both retinol and its esters, foremost retinyl palmitate, are similarly affected by TCDD in the rat (Bank et al., 1989; Brouwer et al., 1989), although the alterations in renal retinol concentrations may be smaller than those in retinyl esters (Bank et al., 1989; Jurek et al., 1990).

The pathogenesis of the TCDD-elicited disturbances in vitamin A homeostasis has been scrutinized in the rat. Similarly to vitamin A deficiency, the initial hepatic uptake of newly administered retinyl acetate appeared to remain intact in animals treated with TCDD (Håkansson and Ahlborg, 1985), but the transfer of vitamin A from parenchymal hepatocytes to stellate cells, the cell population that harbors >80% of the vitamin A in the liver, seemed to be impaired (Håkansson and Hanberg, 1989). However, it is not certain whether this defect in normal partitioning was a direct impact of TCDD or whether it merely resulted from the TCDD-generated vitamin A deficiency (Blomhoff et al., 1991). The excretion of radioactivity from [11,12-^{3}H]retinyl acetate or [15-^{3}H]retinol in bile, urine, and feces was greater in TCDD-exposed rats than in controls (Håkansson and Ahlborg, 1985; Håkansson et al., 1986, 1988). TCDD treatment also led to augmented mobilization of the hepatic and extrahepatic stores of vitamin A (Håkansson et al., 1988; Brouwer et al., 1989) and was accompanied by enhanced retinol esterification in the kidney because of induced activity of acyl-coenzyme A:retinol acyltransferase (Jurek et al., 1990). These effects, in tandem, probably account for the renal accumulation of vitamin A after TCDD exposure in rats.

In the liver, TCDD surprisingly turned out to be a noncompetitive inhibitor in vitro of the principal retinyl ester-degrading enzyme, retinyl palmitate hydrolase, but the inhibition was considered not to be of toxicological importance in vivo (Powers, 1988). On the other hand, TCDD appeared to accelerate retinoid catabolism in the liver by stimulating microsomal retinol oxidase and retinoyl UDPGT activities but had no influence on the oxidation of retinal to retinoic acid or on the microsomal oxidation of retinoic acid (Powers, 1988; Bank et al., 1989). The contribution of enhanced retinoid biotransformation to the depleting effect of TCDD on hepatic vitamin A stores may still not be a major one, because the changes observed in enzyme activities were not marked (Powers, 1988; Bank et al., 1989) and because TCDD reduced hepatic retinol storage to the same extent in heterozygous and homozygous Gunn rats, despite the fact that the latter did not exhibit UDPGT induction (analyzed toward *p*-nitrophenol) (Thunberg and Håkansson, 1983).

In the serum of S-D rats, a slightly disproportionate elevation between the retinol bound to retinol-binding protein and that bound to retinol-binding protein-transthyretin complex (50 and 25%, respectively) was recorded after TCDD treatment (Brouwer et al., 1989), possibly suggesting the inability of transthyretin synthesis to keep pace with retinol mobilization or indicating disturbed association of the two carrier proteins.

The role of altered vitamin A homeostasis in the diverse manifestations of TCDD toxicity is still poorly understood. As mentioned in the preceding section (section II.I.1.), vitamin A supplementation had little influence on the loss of body weight or mortality after TCDD exposure. The opposite challenge, considerably restricted dietary supply of vitamin A, moderately aggravated TCDD lethality in young S-D rats (Håkansson et al., 1991b). In mice, vitamin A depletion did not affect the decline in body weight or thymic atrophy but appeared to potentiate the cutaneous toxicity brought about by TCDD (Puhvel et al., 1991). Excess dietary vitamin A exerted a nearly additive suppressive effect with TCDD on serum T_4 in S-D rats, but neither that nor a vitamin

A-deficient diet interfered with the induction by TCDD of UDPGT or cytochrome P-450-dependent enzyme activities (Rozman et al., 1987a). The biologically active form of vitamin A, retinoic acid, turned out to have a synergistic effect with TCDD in producing cleft palate in mouse fetuses (Birnbaum et al., 1989).

3. Polyamines. The polyamines, spermidine and spermine, and the diamine (often regarded as a polyamine), putrescine, are organic cations that play an essential role in both normal and neoplastic growth of cells (for reviews, see Tabor and Tabor, 1984 and Pegg, 1988). The rate of their biosynthesis is regulated by the activity of ornithine decarboxylase. An early study in S-D rats by Potter et al. (1982) revealed that, at doses of 5 to 135 μg/kg during the first 72 h postexposure, TCDD totally or substantially inhibited the hepatic activity of ornithine decarboxylase induced by partial hepatectomy, dexamethasone, or aminophylline, without itself influencing the constitutive activity. Further support for the view that TCDD decreases tissue responsiveness to ornithine decarboxylase induction in general has come from studies with prolactin (cf. section II.D.1.a). As to putrescine and the polyamines proper, a low dose (5 μg/kg) of TCDD diminished the hepatic concentrations of putrescine and spermidine, but not spermine, measured 1 week after the treatment (Potter et al., 1982). These findings were later largely verified and extended by Farrell and Safe (1986), who confirmed that TCDD doses in the range of 1 to 100 μg/kg did not affect basal hepatic ornithine decarboxylase activity in Wistar rats within the first week after administration. By contrast, a high dose (250 μg/kg) produced a transient, approximately four-fold induction at 6 h, which could be eliminated with the irreversible and specific inhibitor of ornithine decarboxylase, difluoromethylornithine. An important observation was that multiple administrations of difluoromethylornithine did not modify TCDD-elicited body weight loss or thymic atrophy.

A somewhat different outcome was recorded in mice. Both 2 and 100 μg/kg TCDD promoted a similar, transient ornithine decarboxylase induction response in the livers of C57Bl/6 mice, whereas only the higher dose was effective in DBA/2 mice (Nebert et al., 1980; Raunio and Pelkonen, 1983). The activity peaked at 12 to 24 h and leveled off by 48 h. Again, difluoromethylornithine was capable of reversing the response almost entirely (Raunio and Pelkonen, 1983). A more recent report by Thomas et al. (1990) showed that 2 days after treatment with a dose of 30 μg/kg TCDD reduced the concentrations of both putrescine and the polyamines in the thymuses of CD1 mice. In the liver, the levels of spermine and spermidine declined, whereas in the spleen there was no change in polyamine or putrescine concentrations. Thus, factors in polyamine turnover other than ornithine decarboxylase activity are likely to be critically interfered with by TCDD.

Changes in the biosynthesis rate and concentrations of polyamines appeared to influence TCDD toxicity. Coadministration of difluoromethylornithine in drinking water aggravated TCDD lethality and the severity of TCDD-generated ascites, whereas putrescine (given the same way from day 7 on) decreased mortality from 33 to 0%. However, if putrescine treatment was initiated on the same day as TCDD was injected, an increase in mortality ensued (Thomas et al., 1990). Keeping in mind that TCDD did not elicit ascites and difluoromethylornithine did not modify its toxicity in rats, these findings further underscore the divergences in responses to TCDD between the two rodent species and call for caution in generalization and extrapolation across species.

III. Toxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Vitro

In view of its high toxicity to mammals in vivo, it is surprising how innocuous TCDD is to cultured mammalian cells. Concentrations as high as 0.1 to 1.0 μM did not produce detectable toxic effects in any of the various cell types examined (Beatty et al., 1975; Knutson and Poland, 1980; Yang et al., 1983a). Fetal and neonatal epithelial cells may be somewhat more susceptible to TCDD, because formation of enlarged cells was observed (Nau et al., 1981). Similarly, concentrations of TCDD that resulted in marked enzyme induction caused only mild, if any, histological alterations in a human hepatoblastoma and a mouse hepatoma cell line (Eisen et al., 1986; Schecter et al., 1987). TCDD did not appear to adversely affect intercellular communication among monolayer cells of C3H/10T1/2 murine fibroblasts (Boreiko et al., 1987, 1989) and inhibited it only transiently in Chinese hamster V79 cells (Lincoln et al., 1987; de Haan et al., 1993). A more sustained inhibition was found in murine Hepa-1c1c7 cells (de Haan et al., 1993).

However, some effects that bear a resemblance to those seen in vivo can be elicited by TCDD in cell or organ culture. One of these, enzyme induction, has largely constituted the basis for assignment of toxic equivalency factors (relative to TCDD) to various congeners of halogenated Ahs to be used in the toxicological evaluation of their complex mixtures (for review, see Safe et al., 1989). Enzyme induction has also been fruitfully exploited in investigating the mechanism of gene regulation by TCDD, using wild (inducible) and variant type (weakly or not inducible because of defective Ah receptor ligand binding or nuclear accumulation or because of exceptionally low levels of Ah receptors) mouse hepatoma cells (Legraverend et al., 1982; Hankinson, 1983; Whitlock et al., 1984; Hankinson et al., 1985; Whitlock, 1990). Furthermore, the same cell types have made it possible to study in vitro the role of Ah receptors as mediators of other impacts of TCDD, e.g., the decrease in estrogen receptor levels (Zacharewski et al., 1991) and protooncogene activation (see below).

In cultured human epidermal cells, TCDD may trigger proliferation, differentiation, or inhibition of differentiation, probably depending on the cell system, culturing conditions, and/or growth dynamics of the cells at the time of TCDD treatment (Greenlee et al., 1985b,c; Hudson et al., 1986; Hébert et al., 1990; Van Pelt et al., 1992; reviewed by Greenlee et al., 1987). Likewise, in a cell line derived from mouse teratoma, XB, TCDD brings about both proliferation and differentiation to stratified squamous epithelium (Knutson and Poland, 1984a). A dissimilar response occurs in 5L, a particular rat hepatoma-derived cell line, in which TCDD hinders cell proliferation and increases cell volume by blocking entry into the S-phase without affecting other phases of the cell cycle (Göttlicher and Wiebel, 1991; Wiebel et al., 1991). The inhibition of growth seemed to be dependent on Ah receptors (Göttlicher et al., 1990) and could partially be antagonized by dexamethasone and other glucocorticoids (Cikryt et al., 1992).

An interesting difference in the coeffect of estrogen and TCDD on the proliferation of certain human and rat cells has been shown to occur. In two human breast cancer cell lines, MCF-7 and T47D, whose growth requires or is enhanced by estrogen, TCDD acts as a growth inhibitory agent and antagonizes estrogen-promoted growth (Gierthy and Lincoln, 1988; Biegel and Safe, 1990; Fernandez and Safe, 1992). By contrast, in primary cultures of rat hepatocytes, ethinylestradiol additively or even synergistically augmented the comitogenic action of TCDD on EGF-stimulated DNA synthesis (Schrenk et al., 1992).

Incubation of either rat peritoneal macrophages, hepatic mitochondria, or microsomes with TCDD results in enhanced generation of the superoxide anion (Bagchi and Stohs, 1993). In rat primary hepatocytes, TCDD counteracts the induction of PEPCK activity by glucagon/dexamethasone treatment (Stahl et al., 1993b). TCDD is further able to modify, either qualitatively or quantitatively, the responses of various cell types to such effectors as hydrocortisone (Rice and Cline, 1984), dexamethasone (Wölfle et al., 1993a), adrenocorticotropin (DiBartolomeis et al., 1986b), insulin (Fernandez and Safe, 1992; Liu et al., 1992; Wölfle et al., 1993a), estrogen (Gierthy et al., 1987; Biegel and Safe, 1990; Narasimhan et al., 1991; Fernandez and Safe, 1992), EGF (Fernandez and Safe, 1992), and vitamin A (Rubin and Rice, 1988). The recently demonstrated ability of TCDD to increase intracellular calcium concentration rapidly in cultured rat hippocampal neural and astroglial cells (Hanneman et al., 1993) may have a bearing on some of the neurobehavioral effects of TCDD.

In vitro studies can be of considerable aid in attempts to delineate the mechanisms of TCDD carcinogenicity. In murine Hepa-1 hepatoma cells, 15 nM TCDD swiftly (within a couple of minutes) triggered the influx of calcium ions, which was followed by activation of protein kinase C, induction of the immediate-early protooncogenes c-*fos*, *jun*-B, c-*jun*, and *jun*-D and (between 2 and 4 h after initiation of the treatment) substantially increased activity of the transcription factor AP-1. It is noteworthy that these effects did not appear to be mediated by Ah receptors (Puga et al., 1992). Exposure to ≥0.1 nM TCDD for 2 weeks transformed immortalized, but not primary, human epidermal keratinocytes. When transplanted into nude mice, the transformed cells showed morphological alterations and induced squamous cell carcinomas (Yang et al., 1992). TCDD can also enhance the transformation of N-methyl-N′-nitro-N-nitrosoguanidine-initiated rat tracheal (Tanaka et al., 1989) and C3H/10T1/2 mouse embryo fibroblast cells, with the optimal concentrations being 0.3 and 0.04 nM, respectively (Abernethy et al., 1985; Abernethy and Boreiko, 1987).

It is also feasible to study the immunomodulatory effects of TCDD with cell culture. TCDD diminishes both the proliferation of (Luster et al., 1979; Sharma and Gehring, 1979; Pavlyak et al., 1989) and stimulated antibody production in cultured splenic lymphocytes (Luster et al., 1984, 1988; Holsapple et al., 1986a; Tucker et al., 1986), while augmenting their basal antibody secretion (Kramer et al., 1987). However, the responses of splenic B-lymphocytes to TCDD in vitro are crucially dependent on serum-derived growth factors in the culture medium, and some lots of serum can actually support lymphocyte activation (induced proliferation and immunoglobulin M secretion) by TCDD (Morris et al., 1991; Morris and Holsapple, 1991). B-lymphocytes may also exhibit stimulated protein tyrosine phosphorylation in response to TCDD treatment (Clark et al., 1991a). Coculture of thymocytes with TCDD-treated thymic epithelial cells leads to suppressed thymocyte maturation (Greenlee et al., 1985a; Cook et al., 1987), and after incubation with TCDD, immature rat thymocytes undergo a suicide process (apoptosis) (McConkey et al., 1988), which is preceded by, or associated with, transiently induced protein kinase C activity (DePetrillo and Kurl, 1993), as well as increased RNA synthesis, poly(A) polymerase activity, and protein synthesis (Kurl et al., 1993a). Moreover, exposure of bone marrow cells to TCDD results in inhibition of granulocyte colony formation (Luster et al., 1984, 1985; Ackermann et al., 1989).

In selected cases, valuable information concerning the action mechanisms of TCDD has been gained by organ cultural techniques. Embryonal or fetal thymus organ culture has turned out to be a useful model for studies of TCDD-induced thymic atrophy (Dencker et al., 1985; Nikolaidis et al., 1988; d'Argy et al., 1989). Research regarding TCDD teratogenicity has, in turn, benefited from the finding that embryonal palatal cultures, as well as ureteral organ cultures, display the same alterations in differentiation as are seen in response to TCDD in

vivo (Abbott et al., 1989; Abbott and Birnbaum, 1990b; Abbott and Buckalew, 1992), which has provided a scientific basis for the assessment of risk to humans (Abbott and Birnbaum, 1991).

IV. Toxicokinetics of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

The toxicity of every chemical agent in an organism is critically influenced by its toxicokinetic behavior. This aspect is also often the reason for differences in sensitivity among species and strains. Therefore, it is necessary to include here a brief discussion of the predominant features of TCDD toxicokinetics in laboratory animals. An exhaustive review of this issue was recently published (Van den Berg et al., 1994).

A. Absorption

TCDD is fairly well absorbed from the gastrointestinal tract in laboratory animals, if it is completely dissolved in the vehicle used. In S-D rats, 70 to 85% of orally administered TCDD was found to be absorbed (Piper et al., 1973; Allen et al., 1975; Rose et al., 1976). The absorption capacity of the jejunum proved to be independent of the age of the animal (Hébert and Birnbaum, 1988). The uptake of TCDD from the gut was similarly effective in hamsters (Olson et al., 1980a). By contrast, a much lower proportion (approximately 30%) of the administered dose appeared to be absorbed in ICR/Ha Swiss mice (Koshakji et al., 1984). However, because the vehicle used in this case was saline based, it is likely that TCDD was partly undissolved. Other studies in rats have clearly demonstrated the dependence of absorption efficiency on the state of TCDD in the vehicle (Poiger and Schlatter, 1980). In line with this reasoning, the acute intragastric LD_{50} value for TCDD in guinea pigs was shown to be more than four times higher when the compound was given in water than when it was administered in peanut oil, indirectly pointing to critical differences in absorption (Kumar et al., 1986).

The solvent also plays a decisive part in determining the dermal absorptivity of TCDD. As much as 77% of topically applied undiluted TCDD penetrated rat skin within 4 days (Roy et al., 1990). When dissolved in acetone, a maximum of 40% of the applied dose left the administration site within 3 days (Brewster et al., 1989). If adsorbed on soil, <10% of the dose usually penetrated (Shu et al., 1988; Roy et al., 1990). Absorption also depended on the age of the rats, being higher in weanling animals than in adults and decreasing with age in adulthood (Banks et al., 1990; Banks Anderson et al., 1993).

For the absorption of TCDD, intraperitoneal, intratracheal, and subcutaneous routes seem to be at least as effective as oral administration (Olson et al., 1980a; Lakshman et al., 1986; Abraham et al., 1988, 1989b; Nessel et al., 1992).

B. Distribution

TCDD is transported after absorption mainly in the blood and lymph associated with lipoproteins and chylomicrons (Marinovich et al., 1983; Lakshman et al., 1986). A portion may also be bound to transthyretin (McKinney et al., 1985a). Distribution to the tissues is rapid, with the principal sites of deposition in the rat, mouse, hamster, and guinea pig being the liver and adipose tissue (Piper et al., 1973; Allen et al., 1975; Olson et al., 1980a; Birnbaum, 1986; Olson, 1986; Gillner et al., 1987; Abraham et al., 1988; Pohjanvirta et al., 1990d; Weber et al., 1993) (see table 9). In rats and mice, increasing doses of TCDD tended to produce a shift in its proportional partitioning between the liver and adipose tissue in favor of the former (Abraham et al., 1988; Diliberto et al., 1993). A peculiarly high accumulation of TCDD has also been recorded in the olfactory mucosa of rats and mice (Appelgren et al., 1983; Gillner et al., 1987), although in a more recent study this could not be confirmed (Weber et al., 1993). In all other tissues, the levels are low (e.g., in the rat, usually <10 to 20% of the liver concentration) (Piper et al., 1973; Allen et al., 1975; Rose et al., 1976; Van Miller et al., 1976; Abraham et al., 1988; Pohjanvirta et al., 1990d). From findings in the rhesus monkey, it was previously believed that primates as a whole constitute an exception to this general pattern of distribution. In rhesus monkeys, the muscle and skin appear to harbor the greatest concentrations of TCDD (table 9). However, recent studies in marmosets have revealed a similar accumulation of TCDD in the liver to that in rodents (Abraham et al., 1989a; Neubert et al., 1990a) (table 9).

The role of adipose tissue as a tissue depot for TCDD is hardly unexpected, considering the high lipophilicity of TCDD. The probable reason for the selective accumulation of TCDD in the liver, however, has been elucidated only recently. Although hepatocyte cytosol contains several binding species for TCDD, such as Ah receptors (see section II.F) and lipoproteins of variable sizes (Lesca et al., 1987; Souès et al., 1989), the retention of TCDD appears to be related to tissue-specific enzyme induction (see section II.F) (Leung et al., 1988, 1990; Andersen and Greenlee, 1991). In the liver, TCDD (similar to 3-methylcholanthrene) induces both cytochromes CYP1A1 and CYP1A2, whereas in almost all extrahepatic tissues [nasal epithelium may be an exception (Gillner et al., 1987)] only the former cytochrome is induced (Goldstein and Linko, 1984; Tuteja et al., 1985; Degawa et al., 1987; Pasco et al., 1988; Sesardic et al., 1990). Induced cytochrome CYP1A2, in turn, seems to be a crucial binding species for TCDD in rodents (Voorman and Aust, 1987, 1989; Poland et al., 1989a,b; Kedderis et al., 1993). The hepatocellular binding of TCDD is so firm that only a very limited amount will be released back to the circulation (Tsuda et al., 1988). The tight noncovalent binding to CYP1A2 accounts for the uneven

TABLE 9
Distribution of radioactivity following administration of radiolabeled TCDD in various species and strains

Species/strain	Label	Dose (μg/kg TCDD)	Day (time post-exposure)	Liver	Adipose	Muscle	Skin	Reference
Guinea pig/Hartley	^{14}C	2 (ip)	11	2.2† (21.2)‡	3.0 NP	NP NP	NP (6.5)	Gasiewicz and Neal, 1979
Mouse/C57BL/6	^{3}H	10 (ip)	12	15.2 (24.0)	4.4 (5.9)	0.2 NP	NP NP	Gasiewicz et al., 1983a
Mouse/DBA/2	^{3}H	10 (ip)	12	2.6 (4.0)	2.4 (8.0)	0.2 NP	NP NP	
Mouse/B6D2	^{3}H	10 (ip)	12	15.8 (25.5)	3.7 (4.7)	0.2 NP	NP NP	
Rat/S-D	^{3}H	400 (ip)	7	4.54 (43.0)	3.46 NP	0.06 (4.6)	0.13 (4.4)	Van Miller et al., 1976
Rat/L-E	^{14}C	5 (ip)	8	2.23 (27.5)	1.63 NP	0.03 NP	0.28 NP	Pohjanvirta et al., 1990d
Rat/H/W	^{14}C	5 (ip)	8	2.00 (32.6)	0.96 NP	0.03 NP	0.17 NP	
Syrian hamster	^{3}H	650 (ip)	10	4.02 (16.7)	2.03 NP	NP NP	NP NP	Gasiewicz et al., 1983b
Rhesus monkey (adult)	^{3}H	400 (ip)	7	0.09 (10.4)	0.16 (16.2)	0.004 (8.62)	0.028 (13.1)	Van Miller et al., 1976
Rhesus monkey (infant)	^{3}H	400 (ip)	7	0.13 (4.51)	0.49 NP	0.096 (35.6)	0.24 (22.7)	
Marmoset monkey	^{14}C	5 (sc)	9	NP (20–30)	NP NP	NP NP	NP NP	Krowke, 1986

* Abbreviations: ip, intraperitoneal; sc, subcutaneous; NP, data not provided.
† Radioactivity as % dose/g tissue.
‡ Radioactivity as % dose/total tissue.

subcellular distribution of TCDD in hepatocytes with accumulation in the microsomal fraction (Vinopal and Casida, 1973; Allen et al., 1975; Gasiewicz and Neal, 1979). It also explains the difference in hepatic TCDD concentrations between C56BL/6 and DBA/2 strains of mice (C56BL/6 > DBA/2) observed in some studies (table 9): Doses of about 3 μg/kg result in maximal induction in the C56BL/6 strain, whereas a ten-fold higher dose is needed for the DBA/2 strain (Poland and Glover, 1975). If high enough doses, eliciting maximal induction in both strains, are used, the difference in the hepatic accumulation of TCDD largely disappears (Poland et al., 1976). Moreover, in vitro the hepatic uptake of TCDD is similar in both strains (Shen and Olson, 1987). There is some recent evidence to suggest that other, at present unknown, inducible hepatic factors apart from CYP1A2 contribute to the preferential accumulation of TCDD in the rodent liver (Kedderis et al., 1991).

The levels of TCDD in the blood remain strikingly low after a nonlethal dose (Pohjanvirta et al., 1990d; Weber et al., 1993). The mobilization of storage fat (and consequently TCDD) associated with the wasting syndrome accounts for the late increase in serum and tissue TCDD concentrations following lethal exposure (Unkila et al., 1993a; Weber et al., 1993). It is also surprising how little TCDD accumulates in the brain. In spite of its extensive blood supply and high lipid content, the brain attains concentrations of TCDD comparable to those in blood (Weber et al., 1993) or only slightly higher (Manara et al., 1982). The poor penetration of TCDD into the brain may result from its binding to lipoproteins in plasma.

C. Metabolism and Excretion

TCDD appears to be biotransformed at a fairly slow rate to numerous more polar metabolites in the mammalian liver. In the rat, at least eight metabolites have been found (Ramsey et al., 1982), most of which are excreted as glucuronides (Poiger and Schlatter, 1979). A major metabolic route has proved to be hydroxylation at a lateral position or periposition, although the cleavage of one of the ether bonds is also an important reaction, at least in the rat (Sawahata et al., 1982; Poiger and Buser, 1983, 1984). The available data suggest some differences in the metabolism of TCDD among species both in vivo (Gasiewicz et al., 1983b; Poiger and Buser, 1984) and in cultured hepatocytes (Wroblewski and Olson, 1985). However, no consistent correlation with sensitivity to TCDD toxicity has been established.

Although the in vitro binding of TCDD-derived radioactivity to microsomes has been suggested as an indication of reactive intermediate formation (Nelson et al., 1977; Guenthner et al., 1979) and a hypothesis has been advanced concerning the generation of quinones from TCDD in the body leading to oxidative stress (Ames and Kovacic, 1991), TCDD metabolism is generally regarded as a detoxification process (Beatty et al., 1978; Ramsey et al., 1982; Weber et al., 1982; Mason and Safe, 1986),

which may involve TCDD-inducible monooxygenase activities (Poiger and Schlatter, 1985; Wroblewski and Olson, 1985, 1988). Therefore, it is believed that TCDD toxicity is attributable to the unchanged parent compound.

TCDD may be able to induce its own metabolism in dogs (Poiger and Buser, 1984; Poiger and Schlatter, 1985), hamsters (Wroblewski and Olson, 1988) and, possibly, mice (Leung et al., 1990) and rats (Wroblewski and Olson, 1985, 1988), although other in vivo or ex vivo data obtained from mice and rats do not support this notion (Shen and Olson, 1987; Curtis et al., 1990; Kedderis et al., 1991, 1993; Kedderis and Birnbaum, 1992). Guinea pigs, at least, seem to be incapable of autoinduction (Wroblewski and Olson, 1985). Yet, it appears unlikely that the rate of biotransformation, which is overall sluggish in all species, plays a crucial role in determining species sensitivity to TCDD, because (*a*) in the uninduced state, hepatocytes from mice (irrespective of their Ah status) metabolize TCDD at least four times as fast as, for example, those from hamsters (Shen and Olson, 1987) and (*b*) TCDD metabolism occurs at a similar rate in hepatocytes from S-D rats and hamsters in both the uninduced and the induced states (Wroblewski and Olson, 1988).

The metabolites are readily excreted after they are formed, as indicated by the fact that all TCDD-derived radioactivity in the tissues of the rat, mouse, hamster, and ring-necked pheasant represented the parent compound (Rose et al., 1976; Olson et al., 1980a; Koshakji et al., 1984; Curtis et al., 1990; Pohjanvirta et al., 1990d; Nosek et al., 1992b; Weber et al., 1993). Nevertheless, in the guinea pig a portion (4 to 28%) of the total radioactivity stemmed from TCDD metabolites (Olson, 1986). On the other hand, little if any unmetabolized TCDD occurred in the bile or urine of rats, hamsters, mice, guinea pigs, and dogs (Olson et al., 1980a; Gasiewicz et al., 1983a; Neal et al., 1984; Poiger and Schlatter, 1985; Olson, 1986; Pohjanvirta et al., 1990d; Kedderis et al., 1991). In the feces, most of the TCDD excreted is as metabolites in hamsters (Gasiewicz et al., 1983b) but as intact parent compound in guinea pigs (Olson, 1986), implying luminal transfer across the intestinal wall in the latter species. For the mouse and rat, the reported findings are conflicting in this respect (Vinopal and Casida, 1973; Gasiewicz et al., 1983a; Koshakji et al., 1984; Pohjanvirta et al., 1990d; Weber et al., 1993). The high-performance liquid chromatography pattern of metabolites did not exhibit any consistent correlation with TCDD susceptibility in L-E and H/W rats (Pohjanvirta et al., 1990d).

The elimination of a sublethal dose of TCDD seems to follow first-order kinetics in the rat (Rose et al., 1976), hamster (Olson et al., 1980a), mouse (Koshakji et al., 1984), and guinea pig (Olson, 1986). However, S-D rats treated with a lethal dose of TCDD displayed a triphasic elimination pattern from the whole body (Weber et al., 1993). In all of these species, as well as in the rhesus monkey (Van Miller et al., 1976), fecal excretion is the predominant route of elimination, although in the hamster, in particular, urinary excretion is also substantial (approximately 40% of total elimination) (Olson et al., 1980a). The half-life of elimination from the whole body after single exposure is fairly similar in various strains of rat (16 to 31 days) (Allen et al., 1975; Piper et al., 1973; Pohjanvirta et al., 1990d; Rose et al., 1976; Weber et al., 1993) and in hamsters (11 to 15 days) (Olson et al., 1980a). Earlier studies found a half-life of about 30 days in guinea pigs (Gasiewicz and Neal, 1979; Nolan et al., 1979), whereas a more recent report concluded that the half-life was much longer (94 days) (Olson, 1986). The reason for the discrepancy is unclear as yet but might be related to the fact that, in the most recent study, the observation time was twice as long as in the earlier ones. In mice, the half-life of elimination depends on the strain, being 11 and 24 days for the C57BL/6 and DBA/2 strains, respectively (Gasiewicz et al., 1983a). This strain difference was attributed to a greater adipose tissue content and consequent sequestration of TCDD in the DBA/2 strain.

With regard to primates, there is some preliminary evidence from the rhesus monkey to suggest that the half-life may be remarkably longer (>1 year) than that found for small laboratory animals (Bowman et al., 1989), but again, the marmoset may resemble rodents more closely (Neubert et al., 1990a). Some interesting features of TCDD excretion in a species of bird (ring-necked pheasant) were recently disclosed by Nosek et al. (1992b). The elimination half-life turned out to depend decisively on age and reproductive status. For pheasant hatchlings, a whole-body elimination half-life of 13 days was obtained, whereas the corresponding figure for adult hen pheasants that were not producing eggs was as long as 378 days. When the hens started laying eggs, they translocated approximately 1% of their body burden to each egg for the first 15 eggs, thus demonstrating the importance of this route for elimination of TCDD. In mammals, secretion in milk may serve as a similarly effective means of elimination (Nau et al., 1986; Jones et al., 1987a; Bowman et al., 1989; Hagenmaier et al., 1990).

Repeated dosing with TCDD seems to accelerate its elimination, as implied by the elimination half-lives of 12 and 15 days for male and female rats, respectively, after daily exposure for 42 days (Fries and Marrow, 1975). Steady-state concentrations are approached within 13 weeks of repeated daily dosing in rats, with the rate constant being independent of TCDD dose over the range of 0.01 to 1.0 μg/kg/day (Rose et al., 1976). At a steady state, total retention of TCDD will be 10.5 to 29.0 times the daily dose in rats (Fries and Marrow, 1975; Rose et al., 1976).

Taken together, the high toxicity of TCDD and the wide interspecies or strain differences in sensitivity to TCDD clearly cannot be explained on kinetics grounds alone. However, certain kinetics features, such as the uninducibility of biotransformation coupled with slow elimination of TCDD in guinea pigs and the sequestration of TCDD in adipose tissue in DBA/2 mice (especially with repeated dosing), may act as contributory factors to these phenomena.

V. Main Animal Models for Studies of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Toxicity

A. Species Comparisons

The exceptionally wide variation among species and strains to the acute lethality of TCDD has been utilized in establishing animal models for studies of its mechanism(s) of action. One approach that has been used fairly often is to compare the effects of TCDD among guinea pigs, S-D, Wistar, or Fischer rats (representing animals with intermediate sensitivity), and hamsters. These species possess physicochemically similar hepatic Ah receptors (see sections II.F.1 and VI.A). Although the magnitude of microsomal enzyme induction varies across these and other species, e.g., being low in guinea pigs (Hook et al., 1975; Holcomb et al., 1988), the ED_{50} values are in a narrow range (Poland and Glover, 1974; Gasiewicz et al., 1986b; Henry and Gasiewicz, 1987). As to other biochemical and morphological effects, the responses show considerable qualitative diversity (see sections II.C. and II.D). The effective doses are also response and species dependent, but few studies have addressed the vital question of whether the effects bear similar ED_{50}/LD_{50} ratios across species. It seems that there is no general rule; the ED_{50} values for thymic atrophy and alterations in serum T_4 are approximately ten to 20 times as high in hamsters as in rats (Gasiewicz et al., 1986a; De Heer et al., 1993; Ernst et al., 1993), but mice (independent of *Ah* locus) are even more resistant than are hamsters to both impacts (Birnbaum et al., 1990). The specificity of the species variation to TCDD has been assessed by comparing sensitivities to another anorexigen producing a cachectic wasting syndrome, perfluorodecanoate. With this compound, hamsters and Fischer 344 rats proved to be slightly more susceptible than guinea pigs to wasting and lethality (Andersen et al., 1981; Olson et al., 1983), indicating that the species divergence in TCDD toxicity is indeed a specific phenomenon and does not merely reflect differences in the ability to cope with serious metabolic challenges.

However, the design of interspecies comparisons unavoidably suffers from major drawbacks and difficulties in data interpretation, emanating from the notably dissimilar physiology of these species, in particular, regulation of metabolism, food intake, and body weight. For example, guinea pigs are strict herbivores, whereas rats are omnivores. Hamsters, in turn, are hibernators with a system of feeding control that is in many respects unique, e.g., apparent lack of opioidergic regulation (Morley et al., 1983). For these reasons, comparisons within a given species would be preferable and would offer a physiologically less complicated starting point.

B. Mouse Strains

It was recognized in the late 1960s that inbred strains of mice can be classified according to their sensitivity toward monooxygenase induction by polycyclic Ahs, such as 3-methylcholanthrene (Nebert and Gelboin, 1969). Prototypes for responsive and nonresponsive strains soon evolved: C57BL/6 and DBA/2, respectively. Although DBA/2 mice were uninducible by 3-methylcholanthrene (Nebert and Gelboin, 1969), they responded to the more potent inducer TCDD but required about a tenfold higher dose than did C57BL/6 mice (Poland and Glover, 1975; Shen et al., 1989). In genetic crossings between these strains, sensitivity to microsomal enzyme induction was inherited as a simple, single-gene autosomal dominant trait (Gielen et al., 1972; Nebert et al., 1972; Thomas et al., 1972). The differential sensitivity turned out to arise from mutations in a genetic locus, designated *Ah*, which encodes a regulatory product, the specific receptor that mediates the induction (Poland et al., 1976; see section II.F.2). The Ah receptors of the DBA/2 strain proved to have a remarkably weak affinity for TCDD and their concentration was low (Okey et al., 1989).

Further studies of the two mouse strains and their congenic derivatives (obtained by genetic crossings) revealed that, in addition to microsomal enzyme induction, susceptibility to a variety of toxic effects of TCDD also segregated with the *Ah* locus. These included acute lethality, immune suppression, thymic atrophy, myelosuppression, teratogenicity, hepatic porphyria, endotoxin hypersensitivity, enhanced secretion of TNF, lipid peroxidation, and decreased binding of EGF (Courtney and Moore, 1971; Poland et al., 1979; Poland and Glover, 1980; Jones and Sweeney, 1980; Neal et al., 1982; Vecchi et al., 1983b; Nagarkatti et al., 1984; Chapman and Schiller, 1985; Luster et al., 1985; Mohammadpour et al., 1988; Rosenthal et al., 1989; Clark et al., 1991b; Lin et al., 1991b). TCDD appeared to bring about equal, or at least similar, alterations in body and organ weights, clinical chemistry values, and histopathological findings in congenic mice that differed only in the *Ah* locus, with approximately one order of magnitude difference in the effective doses (Birnbaum et al., 1990). An exception to this rule might be the liver, in which the degree of steatosis versus inflammation and necrosis has been reported to differ between TCDD-treated C57BL/6 and DBA/2 mice (Shen et al., 1991). The strain divergence seemed to be specific to TCDD and related compounds, because it did not extend to perfluorodecanoic acid,

which generates an analogous wasting syndrome (Brewster and Birnbaum, 1989; Harris et al., 1989).

C. Rat Strains

Although other mouse strains have also been used occasionally, the genetic difference between C57BL/6 and DBA/2 mice has constituted a cornerstone for mechanistic studies of TCDD toxicity. This, in turn, has strongly shaped current thinking and led to the view of a crucial determinant role for Ah receptor status in TCDD susceptibility. Inasmuch as no corresponding strain-based model was previously known in other species, generalization of the findings entailed elements of risk. In 1985, Walden and Schiller reported that Fischer rats were up to seven times more resistant to the acute lethality of TCDD than were S-D rats. This was one of the first clues that it could be possible to find a suitable pair of strains for a model in the rat as well. This proved to be the case a few years later, when it was observed that an outbred substrain of Wistar rats, H/W, showed outstanding resistance to TCDD lethality (Pohjanvirta et al., 1987, 1988a; Pohjanvirta and Tuomisto, 1987), whereas a Finnish inbred substrain of Long-Evans rats, L-E (for details of the historical background of these strains, see Pohjanvirta and Tuomisto, 1990c), turned out to be more susceptible than any other rat strain ever tested (Pohjanvirta et al., 1988a). With LD_{50} values of approximately 10 and ≥10,000 μg/kg for L-E and H/W rats, respectively (Pohjanvirta et al., 1988a, 1993a; Unkila et al., 1994b), these strains offered a powerful new means of exploring the mechanism of TCDD lethality.

Surprisingly, no appreciable differences were detected between L-E and H/W rats in hepatic Ah receptors or in the induction of enzyme activities known to be regulated by these receptors (Pohjanvirta et al., 1988a, 1989a, 1990a; Unkila et al., 1993a). Another major departure from the mouse model emerged in genetic studies. In the offspring of crosses between L-E and H/W rats, TCDD resistance was inherited as an autosomal dominant trait with two or possibly three genes contributing to the outcome (Pohjanvirta, 1990). A further discordance was observed in histopathology and clinical chemistry. Despite its resistance to TCDD lethality, the H/W strain displayed most of the characteristic toxic effects of TCDD exposure even at doses the same as, or only slightly higher than, those needed for L-E rats (Pohjanvirta et al., 1989a, 1990a, 1994a; Linden et al., 1991; Tuomisto et al., 1993a). Of the few exceptions, the most striking divergence between the two strains appeared in feeding behavior and changes in body weight (Tuomisto and Pohjanvirta, 1991) (see section II.E.1.a). This was accompanied by dissimilar responses in three regulators of feed intake: brain 5-HT turnover (Tuomisto et al., 1990; Unkila et al., 1993b,d), free tryptophan in plasma (Unkila et al., 1994a,b), and plasma β-endorphin (Pohjanvirta et al., 1993b). Other changes found only in L-E rats were enhanced lipid peroxidation, elevations in serum corticosterone and free fatty acids, and the typical liver lesion of TCDD accompanied by severe hepatic accumulation of fat (Pohjanvirta et al., 1989a, 1990b). Doses of TCDD nonlethal to both strains produced analogous alterations in vitamin A status, but doses lethal to L-E rats alone largely reversed the vitamin A changes in that strain (Pohjanvirta et al., 1990a). Similarly to the mouse model, the difference between the rat strains was specific for TCDD, as indicated by equal susceptibility of L-E and H/W rats to perfluorodecanoate-induced wasting and lethality (Unkila et al., 1992).

Hence, the new rat model has seriously challenged a number of previous notions based on findings in mice (the main features of both strain models and the species model are compared in table 10). This applies in particular to the Ah receptor hypothesis, which proposes a central role for these mediators of induction of cytochrome P-450-associated enzyme activities in all forms of TCDD toxicity. We will address this and other major hypotheses next in light of data gathered from the animal models. We will mainly confine the discussion to the gravest form of acute toxicity, lethality.

VI. Unifying Hypotheses for the Action Mechanism of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Their Validity in the Light of the Animal Models

A. Ah Receptor Hypothesis

During the past two decades of intensive TCDD research, numerous unifying hypotheses have been put forward to explain its acute toxicity. A selection of these hypotheses is listed in table 11. In the forefront is indisputably the Ah receptor hypothesis, according to which the Ah receptors determine the ensuing cell-, tissue-, organ-, individual-, strain-, and species-specific responses by regulating the activity of critical genes. The wide acceptance of this concept stems mainly from three sources: studies with the mouse model (see section V.B); structure-activity relationships in a given species revealing positive correlations among Ah receptor binding affinity, monooxygenase induction, and some manifestations of short-term toxicity including acute lethality (Safe et al., 1989; Kafafi et al., 1993); and the rapid and remarkable progress made in understanding the induction of CYP1A1 at a molecular level.

However, it should be acknowledged that there are also data that argue strongly against an all-dictating role for Ah receptors and the *Ah* locus in TCDD toxicity (for a more thorough discussion of the Ah receptor-dependent and -independent effects of TCDD, see Greim and Rozman, 1987, and Rozman, 1989). First, 3-methylcholathrene competes with TCDD for Ah receptor binding (Poland et al., 1976) and induces the same microsomal enzymes as does TCDD (Poland and Glover, 1974; Guengerich and Mason, 1979; Negishi and Nebert, 1979; De-

TABLE 10
Comparison of the animal models for TCDD toxicity studies

	Interspecies (guinea pig vs. rat vs. hamster)	Mouse (C57BL/6 vs. DBS/2)	Rat (L-E vs. H/W)
Genetic background	Distinct species	Inbred strains	Inbred (L-E) vs. outbred (H/W) strain
TCDD lethality	≈1000-fold difference	≈10-fold difference	≈1000-fold difference
Specificity; perfluorodecanoic acid lethality	Similar to all 3 species	Similar to both strains	Similar to both strains
Inheritance	Not applicable	Susceptibility dominant trait; 1 gene involved	Resistance dominant trait; 2 (3) genes involved
Ah receptors	"Normal" in all 3 species	Defective in DBA/2 mice	"Normal" in both strains
TCDD kinetics	≈7-fold difference in elimination half-life; proportional fecal excretion of metabolites varies	≈2-fold difference in elimination half-life	Similar in both strains
Monooxygenase induction	Effective doses similar; magnitude variable	≈10-fold difference in effective doses	Similar in both strains
Morphological and biochemical changes in general	Frequently dissimilar; effective doses response dependent	Mostly similar; ≈10- to 20-fold difference in effective doses	Mostly similar; minor differences in effective doses
Liver lesion	Qualitative and quantitative differences	Qualitative differences described	Qualitatively different
Total body fat content	≈4-fold difference	≈2-fold difference	Similar in both strains
Brain serotonin turnover	Not determined	Not determined	Accelerated in L-E rats alone
Feeding behavior	Differences in the magnitude and evolvement of suppression	Similar patterns (?) with ≈15-fold difference in effective doses	Initial suppression in both strains; abrupt recovery in H/W rats alone

gawa et al., 1987) with a 30,000-fold weaker potency on a molar basis (Poland and Glover, 1974) but does not produce the same signs of toxicity even with daily administration of 100 mg/kg for 20 days to rats (based on induction potencies, total dose equivalent to 80 μg/kg TCDD) (Neal et al., 1979). Second, the distribution, sedimentation coefficients, total specific binding capacities, and binding affinities for Ah receptors from guinea pigs, S-D rats, and hamsters are fairly similar and show little or no meaningful correlation with TCDD susceptibility (Gasiewicz, 1983; Gasiewicz and Rucci, 1984; Denison and Wilkinson, 1985; Denison et al., 1986b). In addition, the DNA-binding forms of the receptor are similar in these species (Henry et al., 1989), and there are no discernible differences in the ability of their Ah receptors to transform into a DNA-binding form and bind to their cognate recognition sites in the DNA (Bank et al., 1992). Despite the wide divergence in the LD_{50} values among species, their ED_{50} values for the established Ah receptor-mediated response, microsomal enzyme induction, are similar (Poland and Glover, 1974; Gasiewicz et al., 1986a,b; Henry and Gasiewicz, 1987). Third, the same facts have emerged from the rat model. In the face of a three-order of magnitude difference in

TABLE 11
Hypotheses of the primary mechanism of acute toxicity for TCDD

Hypothesis	Reference
Ah receptor-mediated pleiotropic responses	Poland and Knutson, 1982
Lowered body weight set point	Seefeld et al., 1984a
Thyroid hormone partial agonism	McKinney et al., 1985b
Deranged vitamin A status	Thunberg, 1984
Enhanced lipid peroxidation	Stohs et al., 1983
Brown adipose tissue damage	Rozman, 1984b,c
Inhibition of PEPCK activity	Weber et al., 1991b
Derailed estrogen homeostasis	Umbreit and Gallo, 1988
EGF receptor-mediated kinase activation	Brewster, 1985
Induced secretion of tumor necrosis factor	Taylor et al., 1992
Altered serum prolactin levels	Jones et al., 1987b
Dependence on total body fat content	Geyer et al., 1990, 1993

LD_{50} values, L-E and H/W rats proved to possess similar concentrations of functional hepatic Ah receptors and were equally sensitive to monooxygenase induction by TCDD (Pohjanvirta et al., 1988a). It can be argued that the liver may not be a valid surrogate for the critical, currently unknown, target organ(s), in which there might reside a disparity in Ah receptors. However, we recently examined Ah receptor function by measuring 7-ethoxyresorufin *O*-deethylase induction in another candidate target organ, the brain, and again detected a similar outcome in both strains (Unkila et al., 1993a).

The mouse model has clearly established an important role for Ah receptors in TCDD toxicity. However, the rat model, together with species comparisons, suggests that even more important regulatory steps (at least in terms of acute lethality) may exist. Inasmuch as the mouse model seems to be the only example of defective Ah receptors as the basis for a difference in TCDD susceptibility, it is uncertain whether Ah receptors in other species really play a permissive role for acute lethality to be manifested or whether they are necessary at all (this would require testing a variant of a susceptible species totally depleted of Ah receptors by, for example, gene technological methods). Evidence is also accumulating that some other end points of TCDD toxicity, such as disordered vitamin A status (Håkansson et al., 1991a), decreased binding capacity of hepatic glucocorticoid receptors (Lin et al., 1991b), and certain aspects of immune toxicity (Holsapple et al., 1991a), are independent of the *Ah* locus.

The rat model further points to the possibility that an interaction of two or three genes is decisively involved in acute lethality. Acute lethality might then be akin to two TCDD-induced toxic effects in mice, skin response and hepatotoxicity, both of which have been demonstrated to require cooperation of the *Ah* locus with other genes. In murine epidermis, sensitivity to enzyme induction is determined by the *Ah* locus, whereas sensitivity to histological tissue lesion (epidermal hyperplasia and hyperkeratosis along with squamous metaplasia of sebaceous glands) is determined by another genetic locus, designated *hr* (Knutson and Poland, 1982; Greenlee et al., 1990). The inheritances of sensitivities to hepatic porphyria and necrosis in inbred, TCDD-nonresponsive strains of mice, in turn, have turned out to be complex and to involve genes other than the *Ah* locus. Furthermore, the expression of the two traits may be determined independently (Greig et al., 1984).

If a scientific hypothesis or paradigm encounters findings that do not conform, there are only two possibilities. Either the hypothesis must be able to explain the deviating findings within the hypothesis or the hypothesis must be modified to accommodate them. Otherwise the hypothesis itself will lose credibility. Clearly, the explanation is not at hand as to accommodating the contradictory interspecies and interstrain data to comply with the Ah receptor hypothesis. The fashionable explanation is that Ah receptors are necessary but not sufficient. This may be true but it is equally possible that acute lethality arises by a mechanism independent of Ah receptors (see fig. 3).

B. Other Major Hypotheses

Most of the other hypotheses have already been dealt with in their relevant contexts. Therefore, we will make an attempt here to briefly assess the validity (in terms of acute lethality) of those hypotheses that have been examined using one or more of the animal models and give references to the sections where details can be found.

1. Body Weight Set Point. The decreased body weight set point hypothesis is based on a model for body weight regulation advocated by Keesey and Powley (1975, 1986). Although it does not provide a biochemical explanation for TCDD-induced alterations, it shows a way to interpret them. The production of a wasting syndrome, with its drastic changes in body weight, is one of the most consistent impacts of TCDD. Moreover, experimental data from diverse manipulations of body weight in TCDD-treated rats support the hypothesis (section II.E.1.a). It is also in agreement with the rat model, in which the most pronounced difference between the two strains occurs in feed intake and body weight (Tuomisto and Pohjanvirta, 1991). In addition to their toxicological

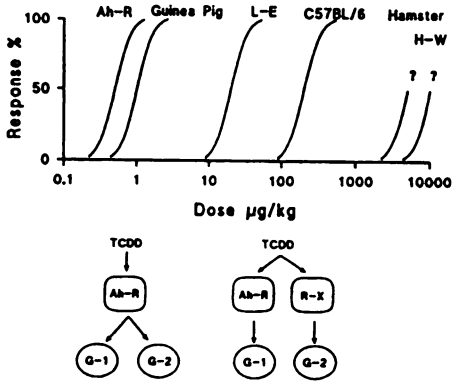

FIG. 3. A schematic graph illustrating some possibilities of explaining the variable lethality of TCDD to rodent species and strains. Enzyme induction related to Ah receptor binding occurs in different species at approximately 1 µg/kg. If the receptor is saturated in this range, it is not possible to explain lethalities in the range of 100 to >10,000 µg/kg by the Ah receptor, because 100% occupancy of the receptor (and hence the maximal effect) would take place well below LD_{50}. The spare receptor concept is a possibility (Rucci and Gasiewicz, 1988), but this would require a large excess of receptors, and enzyme induction would need ligand binding only in a minimal fraction. Varying sensitivities of genes (G-1 and G-2, bottom left graph) or other post-Ah differences will not help explanation, if the crucial receptor is the fully saturated Ah receptor. Another receptor (R-X; bottom right graph), with less affinity to TCDD in most species than that of the Ah receptor, remains an alternative explanation (G-2 responsive for lethality). If this is a variant of the Ah receptor (e.g., in key tissues other than the liver), genetic segregation data need not be contradictory to this possibility, but the model would not exclude the possibility of a "necessary but not sufficient" role of the Ah receptor either (both G-1 and G-2 are involved).

significance, these findings collectively suggest that TCDD might prove to be a valuable aid in physiological research on body weight control, because, in contrast to regulation at an elevated level, there is a paucity of regulation models at a decreased level of body weight (Le Magnen, 1984). However, further studies are needed in other species to verify the generality of the findings in rats. It should also be noted that, even if this mechanism is operative in TCDD-exposed animals, it probably cannot be the only one, because force-feeding does not prevent mortality (section II.B.7).

2. T_4 partial agonism. TCDD was postulated by McKinney and coworkers (1985b) to exert its toxicity by acting as a persistent and potent partial agonist for T_4. The hypothesis was subsequently extended by suggesting that Ah receptors might act as cytosolic acceptor sites for thyroid hormones and modulate their interactions with nuclear receptor sites (McKinney, 1989). However, the data from animal models are not in keeping with this hypothesis (section II.D.4): The degree of T_4 reduction in serum does not correlate with TCDD susceptibility between guinea pigs and rats; also, congenic strains of mice differing only at the *Ah* locus exhibit similar responsiveness to TCDD in terms of increased circulating T_4 levels. In line with this evidence, there was no divergence between L-E and H/W rats in TCDD-promoted changes in serum T_4, T_3, or thyroid-stimulating hormone concentrations until day 16 postexposure, when the lethally intoxicated L-E rats were already extremely debilitated. Nor did the thyroid architecture exhibit any difference (Pohjanvirta et al., 1989a).

3. Vitamin A status. Hypovitaminosis A shares a number of common clinical signs with the short-term toxicity of TCDD (summarized by Håkansson, 1988). The decrease in hepatic storage of vitamin A also appears to correlate with TCDD susceptibility in some species. Together these facts have led to a hypothesis proposing a key role for deranged vitamin A status in TCDD toxicity. However, the hamster is more sensitive to TCDD-induced reduction of hepatic vitamin A storage than it should be based on its resistance to TCDD lethality (section II.I.2). Furthermore, neither the mouse (Håkansson et al., 1991a) nor the rat model (Pohjanvirta et al., 1990a) gave evidence in support of this hypothesis.

4. Lipid peroxidation. Lipid peroxidation coupled with oxidative stress is an interesting hypothesis, because it is backed by evidence from all three animal models. Compatible with this hypothesis, TCDD enhanced hepatic lipid peroxidation in relation to its lethality in guinea pigs, rats, and hamsters (section II.I.1). The same result was obtained in inbred mouse strains and L-E and H/W rats. Nevertheless, a major obstacle to a straightforward interpretation of these findings arises from the fact that lipid peroxidation is a common unspecific sequel of tissue damage in general. Indeed, it was demonstrated that pair feeding as such results in lipid peroxidation of similar severity. It seems clear at present that TCDD can also cause oxidative stress and aggravate lipid peroxidation directly, but the question of its biological significance remains a matter of debate.

5. Brown adipose tissue. The exacerbation of TCDD toxicity by hexadecane led Rozman (1984b) to conclude, on kinetic grounds, that the critical target organ for TCDD might be the brown adipose tissue. Subsequent studies confirmed that TCDD does damage that tissue (section II.C.1.c). However, by far the most important established function of the brown adipose tissue in rodents is thermogenesis, and the thermogenic capacity turned out to be unaffected by TCDD (section II.B.4). The histopathological effect of TCDD on the tissue was also similar in L-E and H/W rats (Pohjanvirta et al., 1989a).

6. Phosphoenolpyruvate carboxykinase. Rozman and associates have strongly argued for the primacy of depressed gluconeogenesis by way of PEPCK inhibition (section II.B.5) as a mechanism explaining the acute lethality of TCDD. They pointed out that, in contrast, for example, to microsomal enzyme induction, which emerges at doses two to three orders of magnitude lower than those needed for acute lethality, PEPCK inhibition exhibits a similar dose response to that for acute lethality in S-D rats. These relationships appeared to hold in the mouse model as well (Smith and Rozman, 1993). In an American substrain of Long-Evans rats, however, PEPCK depression behaved like monooxygenase induction, lacking parallelism with acute lethality (Fan and Rozman, 1993). Likewise, recent studies with L-E and H/W rats uncovered a similar responsiveness in terms of PEPCK inhibition, although the total hepatic capacity for PEPCK activity was more severely affected in L-E rats (Tuomisto et al., submitted).

7. EGF receptors. Downregulation of hepatic EGF receptors is a highly sensitive biological response to TCDD (section II.H.3). It occurred at lower doses in guinea pigs than in rats or hamsters, but the difference was minor. The reduction of EGF binding in liver plasma membranes was also more substantial in C57BL/6 mice than in AKR/J (TCDD tolerant) mice (Madhukar et al., 1984). The rat model revealed that, in the early phases of TCDD intoxication, hepatic EGF binding decreased similarly in both L-E and H/W rats treated at a dose of 50 μg/kg. After day 4, there was a difference, with the change progressing in L-E rats alone (Tuomisto et al., 1993a). Although the findings in the three animal models are thus in accordance with this hypothesis, a word of caution is needed. The above-mentioned studies compared TCDD-treated animals with ad libitum-fed controls, and the effect of severe body weight loss on EGF receptor binding is not known. In view of the experience from, for example, lipid peroxidation studies, this aspect should definitely be scrutinized before any firm conclusions can be drawn about the nature of the relationships.

8. Estrogen homeostasis. Umbreit and Gallo (1988) ascribed a crucial role to estrogen receptor downregulation (section II.D.5.a) and to feedback countermechanisms attempting to reattain estrogen homeostasis in TCDD toxicity. They showed that, although the antiestrogenic, tamoxifen, aggravated TCDD toxicity, estrogen itself failed to interact with TCDD. Moreover, although there appeared to be an inverse correlation in untreated guinea pigs, rats, and hamsters between uterine estrogen receptor density and susceptibility to TCDD lethality, the decrease in density after TCDD treatment did not correlate with this susceptibility (Hruska and Olson, 1989). TCDD also suppressed estrogen-stimulated uterine imbibition at the same dose and to a similar extent in C57BL/6 and DBA/2 mice (Umbreit et al., 1988), and neither ovariectomy nor the potent antiestrogen, ICI 164,384, protected mice against TCDD-induced thymic atrophy and bone marrow alterations (Gasiewicz et al., 1993). This hypothesis has not been examined with the rat model.

9. Tumor necrosis factor. As discussed earlier (section II.G.2), TCDD sensitizes mice to endotoxin lethality and augments the secretion of TNF in response to endotoxin. Both of these effects were more pronounced in the congenic TCDD-responsive than in the -nonresponsive mice (Rosenthal et al., 1989; Clark et al., 1991b). Little is known about these phenomena in other species, although no evidence of endotoxin hypersensitivity (within 24 h after endotoxin) was observed in TCDD-treated L-E and H/W rats (Pohjanvirta et al., 1990b).

10. Prolactin. Jones and coworkers (1987b) implicated altered serum prolactin levels (section II.D.1.a) in TCDD toxicity. To date, no studies of the animal models have been carried out to put this hypothesis to the test. However, the failure of a subsequent study by another group (Moore et al., 1989) to substantiate the findings makes it unlikely that serum prolactin concentration is a general mediator of TCDD toxicity.

11. Body fat. Geyer et al. (1990, 1993) made the interesting discovery that there appeared to be a strong inverse correlation between susceptibility to TCDD lethality and total body fat content among mammalian species. They suggested that adipose tissue might act as a detoxication mechanism by which the compound is removed from sites of toxic action. Thus, this hypothesis seeks an explanation for interspecies differences and not directly for TCDD toxicity. At the strain level, the proposed relationship proved to hold in the mouse model (Gasiewicz et al., 1983a; Geyer et al., 1990, 1993). In contrast, L-E and H/W rats appeared to possess similar amounts of body fat (Pohjanvirta et al., 1990d), although this aspect has to be investigated in more detail. It is also noteworthy in this context that experimentally induced obesity with an accompanying accumulation of body fat, before TCDD exposure, did not confer protection against TCDD lethality in rats (Tuomisto et al., 1993c).

In summary, the three animal models have proved valuable as effective tools in the search for the ultimate mechanism(s) of TCDD toxicity at the whole animal level. If there is a single fundamental mode of action for TCDD across species, the models should give consistent, reproducible outcomes. An additional requirement is that the effect should not arise merely as a change secondary to anorexia and loss of body weight. The fact that none of the general hypotheses advanced so far fully meets all of these conditions may reflect, apart from the still mysterious pathogenesis, a scarcity of studies (e.g., body weight set point hypothesis), a complex, dynamic interaction of the mechanisms involved or different mechanisms in different species. The models are as yet unable to resolve whether or not the Ah receptors are a prerequisite for development of toxicity, but they suggest that factors other than these receptors determine the severity of the ensuing acute toxicity.

VII. Conclusions and Future Prospects

TCDD affects a wide variety of tissues and physiological functions by a number of mechanisms. One general mechanism stands out as especially salient and fundamental: potentiation or inhibition of normal responses to various stimuli (a few examples appear in table 12). A predominant clinical sign of the short-term toxicity of TCDD is a wasting syndrome, which may itself be fatal. However, prevention of this syndrome does not abolish TCDD lethality, which indicates that the cause of lethality must be more fundamental than mere lack of energy. Numerous hypotheses have been proposed as universal explanations for the acute lethality of TCDD,

TABLE 12
Augmented or depressed responses in TCDD-treated rats

Hypersensitivities	Hyposensitivities
Testosterone inhibition of luteinizing hormone secretion in the pituitary gland	Gonadotropin-releasing hormone stimulation of luteinizing hormone secretion in the pituitary gland
Plasma oxytocin increase by LiCl	Induction of testicular testosterone synthesis by luteinizing hormone
Analgesia by 2-deoxyglucose	Elicitation of feeding by insulin or 2-deoxyglucose
Satiety by postingestive signals	Satiety by naloxone
Hypoglycemia by insulin	Ornithine decarboxylase induction by prolactin
Edema by bradykinin and histamine	Promotion of thermogenesis in the brown adipose tissue by noradrenaline
Endotoxin lethality (mice)	Positive inotropic effect on the heart by isoproterenol

but they all suffer from either insufficient experimental data or lack of consistent support by the animal models.

Research concerning TCDD toxicity has largely been dominated by an Ah receptor-centered view. This has led to the current situation, in which attempts are being made to derive effects recorded at the whole animal level from in vitro findings at the molecular level. Could this partially account for the exceptional elusiveness of the basic toxicity mechanism? Would it really be in vain to follow the more conventional root-to-tip approach by focusing efforts on clarifying the pathogenetic steps first? For example, hypophagia is clearly by far the most important direct cause of TCDD-induced wasting. In spite of this, its underlying mechanisms have remained almost unexplored. Because the potency,of TCDD as an anorexigen ranks among the highest of all compounds, we envision that the elucidation of the mode of its anorexigenic action would substantially benefit not only toxicology but also physiological research concerning regulation of food intake and body weight.

Finally, it is important to note that susceptibility to the acute lethality of TCDD appears to lack correlation with susceptibility to some other forms of its toxicity. For instance, the laboratory animals most resistant to TCDD lethality, hamsters and H/W rats, are quite susceptible to TCDD fetotoxicity (Olson and McCarrigle, 1992; Huuskonen et al., 1994). This has significant bearing on assessment of human risk, because the evident resistance of humans to the acute lethality of TCDD and related compounds has been implicitly extended to cover other end points of their toxicity as well. Especially important in this regard would be data regarding the relative sensitivities of hamsters and H/W rats to the most relevant effects from the standpoint of human risk, such as immune toxicity and carcinogenicity.

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