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SUBCHRONIC TOXICITY OF SOME CHLORINATED DIBENZOFURANS IN RATS.

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ABSTRACT

Groups of rats were maintained for 13 weeks on diets containing different levels of 12378-PeCDF, 23478-PeCDF, 12348-PeCDF or 123678-HxCDF as well as a mixture of 23478-PeCDF, 12378-PeCDD, 123678-HxCDF and 2378-TCDD. Positive control groups received 2378-TCDD in the diet.

The parameters investigated included body weight development, food consumption, terminal organ weights, hematology and blood chemistry of terminal blood samples. Tissues were also examined microscopically. Liver and adipose tissues were analyzed for PCDF residues. The toxic potency of the isomers as compared to 2378-TCDD, was estimated and expressed by TCDD equivalence factors (TEFs): 12378-PeCDF, 0.01; 23478-PeCDF, 0.4; 12348-PeCDF, < 0.0003; 123678-HxCDF, 0.1. The toxic response of the animals towards the mixture was in accordance with the concept of additive toxicity.

INTRODUCTION

PCDFs and PCDDs are groups of chemicals which include very toxic and persistent members. The toxicity of the individual isomers is structure-dependent and the most effective compounds are halogenated at the 4 lateral positions; these are also retained most efficiently in animal tissue. To estimate the toxicity of a group of isomers as present for example in human milk the TCDD-equivalents concept is applied. The underlying toxicity equivalency factors are based mainly on acute toxicity or biochemical effects which for instance do not take into account the pharmacodynamics of these compounds and may not reflect the relevant situation of a chronic low level exposure. Subchronic or chronic toxicity data are available only on few PCDD isomers [1,2]. For some penta- and hexachloro-CDFs, which presumably have a high toxic potential and are present in relatively high concentrations in human food such data would be urgently needed. The main objectives of this study were therefore to provide additional data on some of these isomers. In addition we were also interested in the possible effects of an isomer which

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according to its structure, should be much less toxic (12348-PeCDF) and which is not present in animal tissue; it is probably formed during PCB-fires [14].

Apart from rare accidental episodes, humans are regularly exposed towards mixtures of PCDDs/PCDFs contained in animal fat which raises questions on toxic interferences of the individual isomers. In this study we have therefore administered a mixture of 4 isomers in proportions roughly equivalent to those present in cows milk [3]. By comparing the subchronic toxicity of the mixture with that of the individual isomers investigated simultaneously we expected to recognize possible synergistic or antagonistic interferences.

MATERIALS AND METHODS

Chemicals: 2378-TCDD (TCDD) was obtained from Givaudan S.A., Dübendorf, Switzerland. It was purified by several recrystallisations from hot anisole.

12348-, 12378-, and 23478-PeCDF and 123678-HxCDF were synthesized according to the method of Safe and Safe [4]. The products, after recrystallisation from methanol and subsequent HPLC-purification, contained no chlorinated impurities (detection limit 1.5 %).

12378-PeCDD was purchased from the Cambridge Isotope Laboratories (Woburn, MA, USA, specified purity: 99 %). Stock solutions of all these compounds were prepared, using xylene as solvent.

Preparation of the diets: Adequate volumes of the stock solutions were reduced to approx. 10 ml, diluted with 150 ml n-hexane and added to 200 g of pulverized rat diet (Nafag 890, Nafag, Gossau, Switzerland). The solvents were left to evaporate in a well-ventilated fume hood. Each of these premixes was mixed with 29.8 kg rat diet (same as above) in a ploughshare mixer. Food pellets were produced on a laboratory scale pelletizing device (Buehler AG., Uzwil, Switzerland). The resulting concentrations of the compounds in the diets, as calculated from the added amounts are summarized in Table 1. Due to technical reasons, the present study was carried out in two separate series (Table 1).

Table 1: Composition of the diets

Isomer	Dose groups	Conc. in the diet (µg/kg)
Experiment I:		
2378-TCDD	low/med/high	0.2/ 2/ 20
23478-PeCDF	low/med/high	2/ 20/ 200
Experiment II:		
2378-TCDD	ref	2
12348-PeCDF	low/high	600/ 6000
12378-PeCDF	low/med/high	2/ 20/ 200
123678-HxCDF	low/med/high	2/ 20/ 200
Mix: 2378-TCDD	low/high	0.2/ 2
Mix: 12378-PeCDD		1/ 10
Mix: 23478-PeCDF		2/ 20
Mix: 123678-HxCDF		1/ 10

Animals: For each dose group, 6 male and 6 female rats (Iva: SIV 50 (SD), source: Ivanovas, Kissleg, Germany), about 7 weeks old, were distributed to macrolon cages (using computer-aided randomization) with granulated soft wood bedding. The animals had free access to the diets and water. Body weights were recorded semiweekly and food consumption was measured weekly.

Necropsy: After 13 weeks all animals were killed by deep ether narcosis or i.p. administration of 25 mg of sodium pentobarbital. Liver, thymus, spleen, kidneys, heart, and testes were remo-

ved and weighed. In addition, lungs, uterus and ovaries, adrenals, mesenteric and submandibulary lymph nodes, and thyroid/parathyroid glands were removed, but not weighed. All these organs or parts of them were preserved in 4 % formalin. For light microscopic examination the tissues were embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Terminal blood samples were also analysed for hematology or blood chemistry parameters using routine methods. These results are presented elsewhere [12].

Residue analysis: Liver and adipose tissue were processed according to a method described by Smith et al. [13], with some slight modifications.

HRGC was carried out on a Fractovap 4160 gas chromatograph (Carlo Erba, It), equipped with a 20m glass capillary, coated with PS-255. A Finnigan 44510 mass spectrometer (EI mode) was used for quantification (MID). [^{37}Cl]-TCDD was used as internal standard for samples containing 2378-TCDD, 12348- and 12378-PeCDF, and 2378-TCDD for those containing 23478-PeCDF.

RESULTS

Spontaneous deaths: All female and one male rat, fed the high 23478-PeCDF diet died or were killed in the moribund state during the terminal period of the experiment. One male rat receiving the high TCDD also died during week 13, but this was not considered to be caused by TCDD, because typical symptoms were absent. No death occurred in the other groups.

Body weight gain and food consumption: Body weight development of the treated groups and the controls are shown in Fig. 1 a-f. Food intake was decreasing in a dose related manner in the rats fed 23478-PeCDF. In all other groups (except those fed 12348-PeCDF) feed consumption was decreased at the high dosage level.

Terminal body, liver and thymus weights: TCDD strongly affected total body, thymus and relative liver weights in all animals at the high dosage level (experiment I, Table 2). At the medium dosage level the females were more affected. The toxic lesions in the TCDD reference group (experiment II), which received the same diet than the TCDD med group, were in good accordance.

23478-PeCDF: Liver-to-body weight ratios were altered significantly at the medium and high dose levels in all rats except the females fed the high level diet (in these rats even the absolute liver weight was markedly lower than in all other animals, see Table 2). The weight of the thymus was significantly decreased in all animals except the males fed the low 23478-PeCDF diets. Kidney, spleen and heart weights were significant decreased at the high level (data not shown in Table 2). Generally, female rats seemed to be more affected than males.

12378-PeCDF: Significantly lower terminal body and thymus weights were recorded at the high dose level in both males and females and enlargement of the liver only in the males.

12348-PeCDF: Apart from a slightly reduced thymus weight in the males (low dose level), being of borderline statistical significance, no differences to the controls were observed.

123678-HxCDF: All animals at the high level diet had significant lower body, thymus and liver weights, and liver-to-body weight ratios were significantly increased. Reduction of thymus weights was significant also at the medium dosage levels.

Mixture: Feeding of the mix high diet markedly influenced total body, thymus and liver weights or liver-to-body weight ratio in the male and female rats. At the low level, a significant decrease of the thymus weight was observed in all animals.

Table 2: Terminal body, liver and thymus weights (mean ± SD)

Dose group	Total body (g)		Liver				Thymus (g)	
			g	g/100g	g	g/100g		
	♂	♀	♂		♀		♂	♀
Experiment I:								
Control	603 ±92	341 ±31	21.06 ±3.71	3.50 ±0.30	11.20 ±0.13	3.31 ±0.32	0.54 ±0.16	0.54 ±0.07
2378-TCDD low	541* ±43	315 ±32	20.12 ±2.76	3.71 ±0.33	10.33 ±1.13	3.28 ±0.21	0.54 ±0.16	0.51 ±0.13
med	547 ±46	283* ±23	22.56 ±2.17	4.12* ±0.23	10.37 ±0.77	3.67* ±0.18	0.32* ±0.05	0.31* ±0.03
high	401* ±49	236* ±21	18.77 ±4.69	4.62* ±0.74	12.20 ±2.96	5.13* ±0.87	0.14* ±0.05	0.06* ±0.07
23478-PeCDF low	565 ±52	314 ±14	21.09 ±1.89	3.75 ±0.34	10.85 ±0.82	3.46 ±0.24	0.54 ±0.11	0.41* ±0.08
med	491* ±38	278* ±26	21.56 ±2.17	4.40* ±0.30	10.99 ±0.77	3.97* ±0.32	0.25* ±0.06	0.24* ±0.04
high #	321* ±76	156* ± 8	17.90 ±5.64	5.48* ±0.87	5.22* ±0.46	3.37 ±0.41	0.06* ±0.04	0.05* ±0.04
Experiment II:								
Control	514 ±18	322 ±28	17.21 ±1.63	3.35 ±0.26	10.82 ±0.79	3.37 ±0.23	0.48 ±0.02	0.48 ±0.09
2378-TCDD ref	504 ±46	290* ±16	21.98* ±3.44	4.35* ±0.35	11.60 ±0.49	4.01* ±0.21	0.33 ±0.09	0.25* ±0.03
12348-PeCDF low	486 ±24	333 ±19	15.52 ±0.96	3.20 ±0.21	11.23 ±1.10	3.35 ±0.32	0.40* ±0.05	0.50 ±0.1
high	502 ±40	330 ±29	17.83 ±1.31	3.56 ±0.09	11.66 ±1.07	3.54 ±0.12	0.44 ±0.11	0.46 ±0.04
12378-PeCDF low	532 ±40	317 ±19	17.75 ±1.74	3.35 ±0.36	10.63 ±0.83	3.36 ±0.15	0.45 ±0.07	0.47 ±0.07
med	528 ±51	320 ±33	19.03 ±1.64	3.61 ±0.16	11.58 ±1.10	3.63 ±0.20	0.42 ±0.07	0.43 ±0.04
high	481* ±18	286* ±31	19.01* ±0.85	3.96* ±0.19	11.14 ±1.53	3.90 ±0.41	0.26* ±0.05	0.34* ±0.06
123678-HxCDF low	561 ±57	305 ±25	18.25 ±1.42	3.27 ±0.26	10.83 ±0.97	3.56 ±0.26	0.45 ±0.08	0.41 ±0.08
med	505 ±29	306 ±29	19.43 ±1.08	3.85* ±0.21	10.91 ±1.15	3.57 ±0.07	0.28* ±0.07	0.29* ±0.04
high	444*° ±22	257*° ±19	21.44* ±1.59	4.83* ±0.28	11.48 ±1.37	4.45* ±0.27	0.12*° ±0.02	0.10*° ±0.02
Mix low	498 ±37	307 ±14	16.83 ±1.93	3.37 ±0.21	11.19 ±0.51	3.65 ±0.28	0.34* ±0.05	0.33* ±0.04
Mix high	451*° ±44	258*° ±20	20.63* ±2.07	4.58* ±0.26	12.02 ±1.37	4.66* ±0.29	0.12*° ±0.03	0.11*° ±0.02

* significantly different from control (U-test, $p<0.05$); ° significantly different from 2378-TCDD ref group; # some data obtained before week 13 (females)

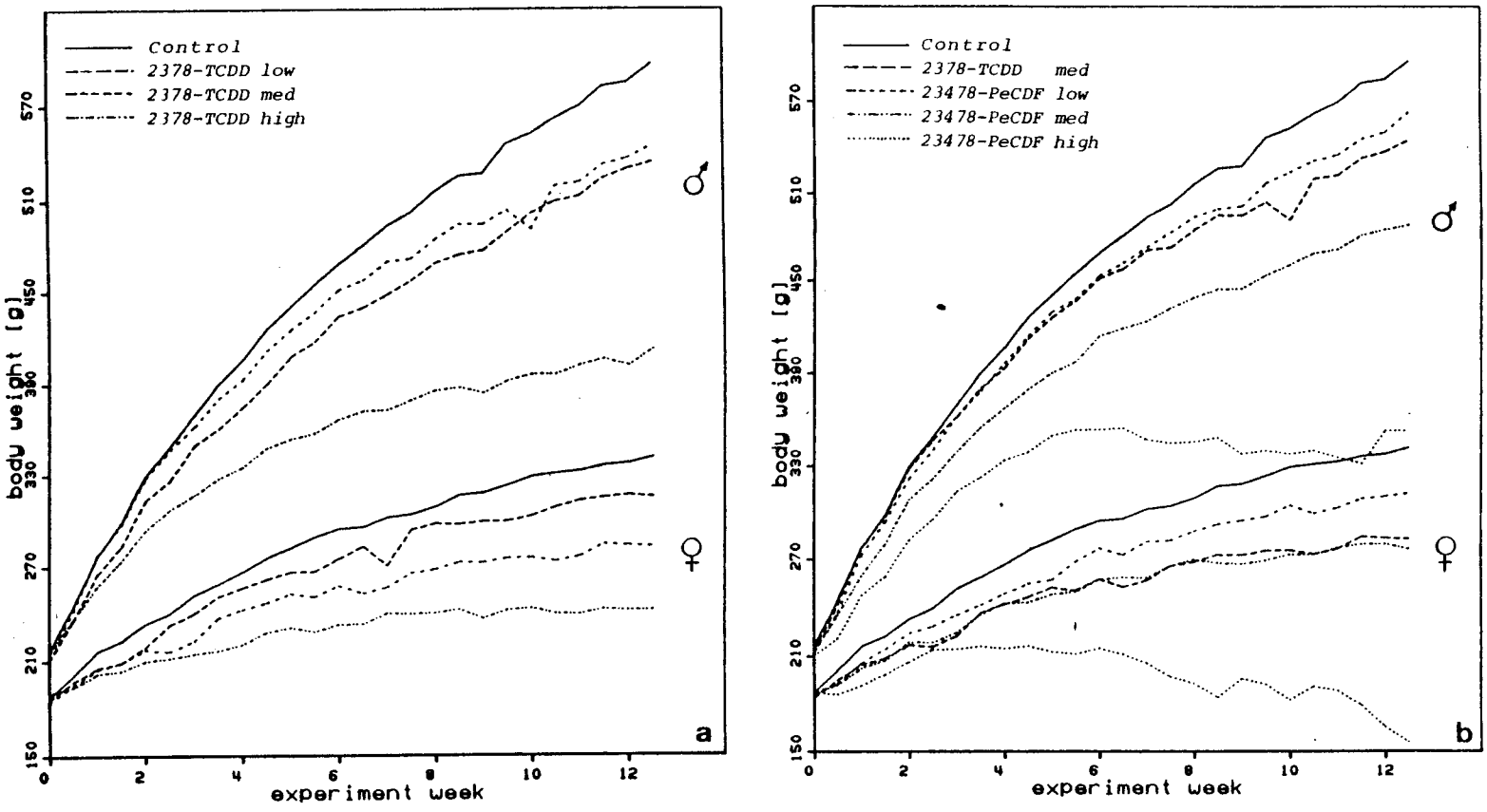

FIGURE 1: Mean bodyweights of male and female rats given (a) 2,3,7,8-TCDD and (b) 2,3,4,7,8-PeCDF in the diet for 13 weeks.

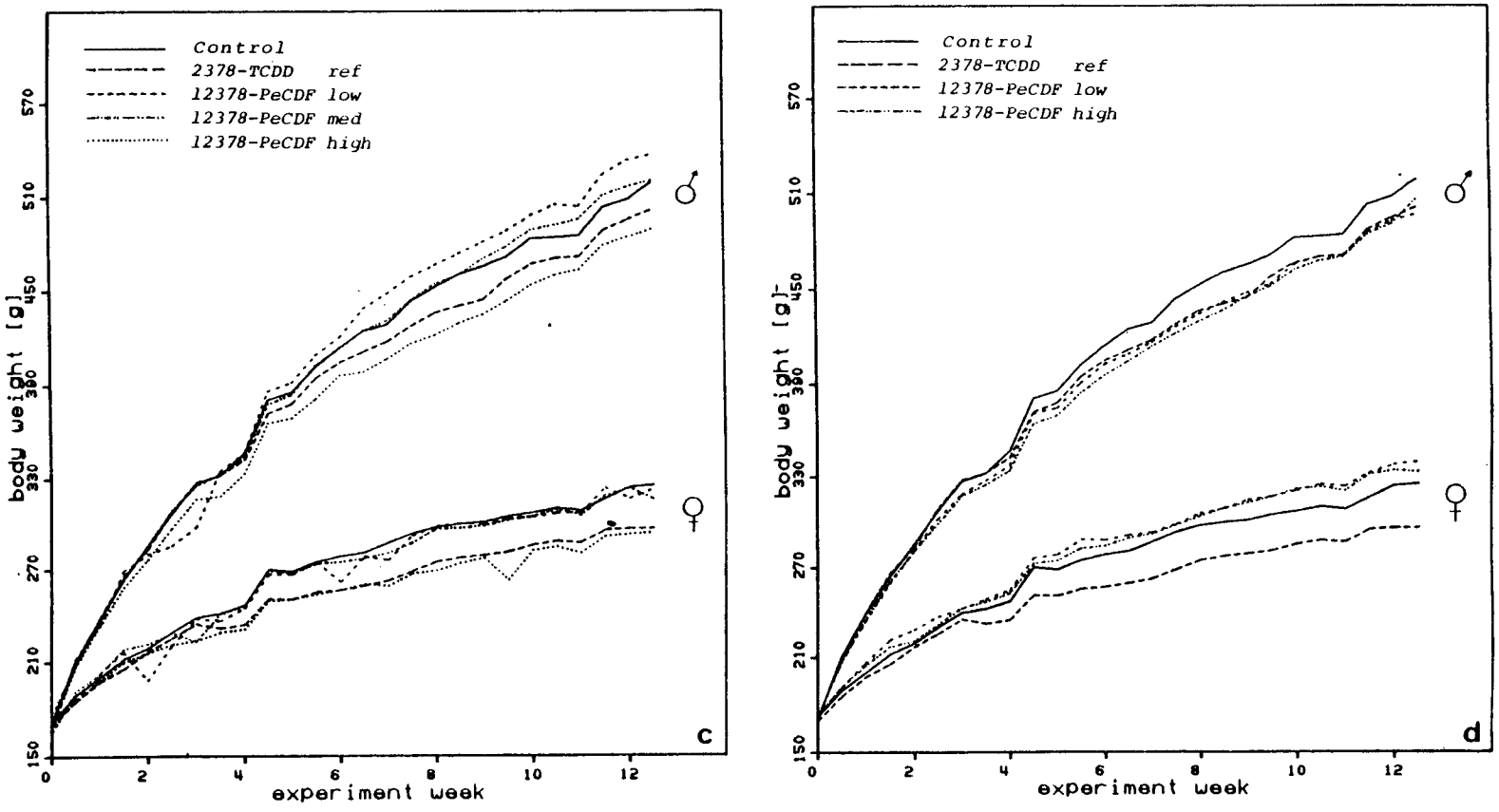

FIGURE 1 continued: (c) 1,2,3,7,8-PeCDF, (d) 1,2,3,4,8-PeCDF

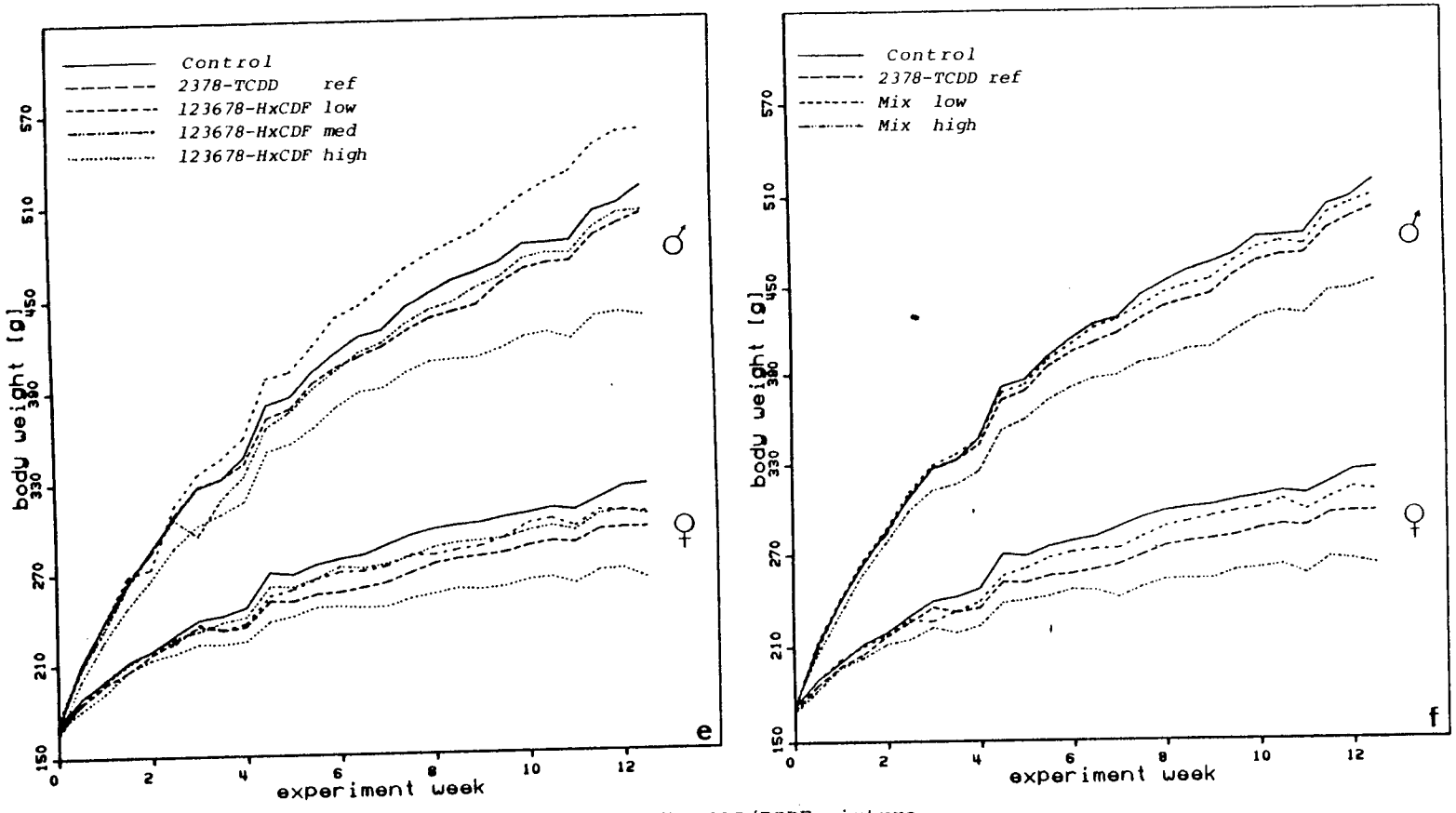

FIGURE 1 continued: (e) 1,2,3,6,7,8-HxCDF (f) PCDD/PCDF mixture

Gross and microscopic examination of tissues:

Experiment I (23478-PeCDF and TCDD): Gross pathological examination exhibited an intense reduction of the size of the thymus in rats of both sex maintained on the high level diets of 23478-PeCDF and TCDD. Upon microscopic examination a marked atrophy became apparent. Involutions were mainly in the cortical region where the number of thymocytes was considerably reduced. At the medium dose level a beginning atrophy of the cortex of the thymus was seen only with 23478-PeCDF and then only in the females. No microscopically discernible thymus lesions were seen at the medium TCDD and the low 23478-PeCDF/TCDD dose levels.

At the high dose levels atrophic alterations could occasionally also be observed in lymph nodes with reduction of lymphocytes mainly in the paracortical zone.

Gross examination of the livers of the rats given the high level diets showed accentuated lobular patterns (marmorization). Microscopically, severe toxic injury was seen in the liver. This finding was most pronounced in the female rats fed 23478-PeCDF. The nuclei of the liver cells showed minor to moderate atypia with variations in size and chromatin content. Considerable vacuolizations in - often polynucleated - hepatocytes progressed to pseudotubular transformation.

Focal necrosis of single or small groups of liver cells was frequent and often associated with activated macrophages containing lipogenic pigment. The regular lobular arrangement was disturbed from case to case, sometimes in connection with bridging fibrosis extending radially from the center to the periphery of the lobules. Ballooning and/or fatty change was present to a varying degree and distribution. Ectasis of the liver sinusoids reached the dimension of a prepeliosis, particularly in the subcapsular region of the liver and often with a zonal distribution from the portal area to the center of the lobule.

At the medium dose levels the above mentioned alterations of the liver were seen at a very early stage with both compounds. No essential toxic effects were observed at the low dose levels, except a slight disseminating fatty degeneration in the 23478-PeCDF treated animals. In the spleen the extramedullary haematopoesis in the red pulp was increased (high PeCDF and TCDD diets); the onset of these alterations was seen already with the medium PeCDF diet.

Experiment II (12378- and 12348-PeCDF, 123678-HxCDF and Mix): Gross pathologic examination showed a drastic reduction of the size of the thymus in rats of either sex maintained on the high 123678-HxCDF and the high mix diets. Upon microcopic examination, a marked atrophy was seen. The involutions affected pulp and cortex.

In the female groups fed the 12378-PeCDF high, 123678-HxCDF medium and mix high diets what appeared to be the start of thymus atrophy, mainly of the cortex was observed. A similar, but somewhat more distinct picture was seen in the TCDD group. No microscopically discernible thymus lesions were observed in other groups.

Examination of the livers revealed vacuolizations with increase in lipid content, necrosis of single cells and slight hyperplasia of Kupffer cells with the high level 123678-HxCDF and high mix diets. These alterations were less pronounced in the high 12378-PeCDF, the medium 123678-HxCDF, the low mix and the TCDD reference groups. No liver lesions could be detected in the other groups. No lesions were seen in other tissues which were attributable to the action of the administered compounds. No pathological changes whatsoever were found in the animals dosed 12348-PeCDF.

Tissue levels: In Table 3 only the data on the male animals are given, but tissue distribution in the females was similar. Tissue accumulation of the individual isomers differed markedly. Chlorine-substitution at the 1/9 and 4/6 positions seemed to be a substantial factor for liver retention. The isomers with a 4/6 substitution pattern also accumulated more strongly in the liver than in the adipose tissue. 12348-PeCDF, as expected, due to the presence of unsubstituted vicinal carbons was metabolized and excreted rapidly. With increasing dosage also liver retention increased somewhat. This could be a consequence of a saturation of the metabolizing enzyme systems and/or mobilization (and redistribution) of the isomers due to loss of body fat.

Table 3: PCDD/PCDF-Content of liver and adipose tissue (♂ rats):

Dose group	Dose/rat (µg)*	Conc. in liver (ng/g)•	Conc. in fat (ng/g)•	Liver retention (% of dose)
2378-TCDD low	0.46	0.44	0.51	1.9
med	4.8	9.4	2.6	4.4
high	40	136	19	6.5
23478-PeCDF low	4.9	66	3.4	28
med	43	599	6.3	30
high	365	7664	186	38
12378-PeCDF low	4.6	2.5	2	1
high	433	257	8.7	1.1
12348-PeCDF high	12888	0.3	26	0.000041
123678-HxCDF low	4.9	78	8	29
med	44	877	26	39
high	405	7727	69	41

*average intake calculated from food consumption. •Mean of 3 samples

DISCUSSION

Since detailed consideration of the toxicity of the vast majority of the PCDDs/PCDFs is limited by the lack of appropriate studies on most of the congeners, several health agencies as an interim approach have proposed the use of TCDD-equivalent factors (TEFs) for the risk assessment of PCDD/PCDF mixtures. For example, some TEFs issued by several authorities for applications for the isomers studied are listed in Table 4.
These TEFs are based on data from short term or in vitro studies, whose relationship to chronic toxicity is largely presumptive at this time. In view of the data given in this paper some of the TEFs should be reconsidered.

Summarizing all data on 23478-PeCDF it became evident that this isomer is only about 2 to 3 times less toxic than TCDD, therefore we propose a TEF of 0.4. 12378-PeCDF, at a diet level of 200 ppb caused atrophy of thymus and reduction in bodyweight comparable to that of the 2 ppb TCDD diet. Microscopic examination revealed less severe liver lesions and thymic atrophy than observed with 2 ppb TCDD. According to these data the TEFs presently used overestimate the toxicity of 12378-PeCDF; a TEF of 0.01 would be a more reliable figure. 123678-HxCDF, at the

same dose level elicited considerably greater toxicity than 12378-PeCDF, which especially became evident upon histopathological examination. Effects in the medium dose group (20 ppb) corresponded closely to those observed with 2 ppb TCDD. According to this a TEF of 0.1 for 123678-HxCDF seems to be suitable.

No toxic effects at all became apparent in rats fed the diet with 6 ppm 12348-PeCDF. As this dosage was 3000 times higher than that of TCDD the TEF of 12348-PeCDF must be below 0.0003.

Table 4: TCDD equivalency factors (TEFs)

Isomer	Swiss* BUS	USEPA*	German** BGA	Suggested by this study
2378-TCDD	1	1	1	1
12378-PeCDD	0.1	0.2	0.1	0.4***
123678-HxCDF	0.1	0.01	0.1	0.1
23478-PeCDF	0.1	0.1	0.1	0.4
12348-PeCDF	0.1	0.001	0.01	<0.0003
12378-PeCDF	0.1	0.1	0.1	0.01

* Barnes [5]; ** BGA [6]; *** Data kindly provided by M.Suter [7]

Toxicokinetics may also be an important factor for the toxicity of the individual isomers. For example, the different potency of the two toxic penta-congeners could in part be due to the different accumulation of these compound in the body of the rat: The highly toxic 23478-PeCDF accumulates in liver approximately 30 times more than the less toxic 12378-PeCDF.

Few data are available on the toxic effects of combinations of isomers. Competitive effects of TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDF) on enzyme induction and antibody production was reported in a study using rats [8], but when cleft palate induction in mice was used as the toxic endpoint, TCDD and TCDF in combination contributed additively to the overall toxicity of the mixture [9]. Body and thymus weight as well as AHH-induction of rats, treated once with a mixture of several toxic PCDF-isomers was influenced to a degree which would suggest an additive action of the individual compounds [10]. On the other hand, feeding for 5 1/2 weeks a mixture of PCDDs and PCDFs, obtained by extraction of particulate matter from a municipal incinerator, produced considerably less severe effects than expected [11].
In the present study, the concentration of TCDD-equivalents in the mix low and high diet are calculated to be 1.5 and 15 ppb, respectively, using TEFs based on this and on an analogous study (12378-PeCDD, [7]). The degree of toxic lesions caused by the low mix diet (1.5 ppb TCDD equivalents) and the TCDD diet (2 ppb) were comparable. Likewise, in the high mix groups (15 ppb TCDD equivalents) bodyweight depression and thymic atrophy were rather similar to those produced by the 20 ppb TCDD or the 123678-HxCDF high diet (20 ppb TCDD equivalents). These data indicate that the single compounds additively contribute to the overall toxicity of the mixture. The TEFs used seem to reflect satisfactorily the toxic action of isomers when present in a mixture. The subchronic studies of four single isomers showed also that the TEFs should preferably be based on feeding studies.

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