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Toxicology Letters 106 (1999) 191–200

Toxicology Letters

Peroxidase-catalyzed in vitro formation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans from chlorophenols

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Received 5 January 1999; received in revised form 10 March 1999; accepted 17 March 1999

Abstract

Chlorophenols (CP) are transformed in vitro to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) by a peroxidase-catalyzed oxidation. This is shown for 2,4,5-tri-, 2,3,4,6-tetra- and pentachlorophenol with plant horseradish peroxidase and with myeloperoxidase recovered from human leukocytes, each in the presence of hydrogen peroxide. The yield, the reaction and the PCDD/F-pattern found are dependent on the CP. The amounts of PCDD/F formed within 4 or 24 h are in the μmol/mol-range for all substrates and both peroxidases. The experiments suggest that biochemical formation of PCDD/F from precursors such as CPs can take place in the human body and that this metabolic pathway may lead to a higher inner exposure to PCDD/F than up to now assumed based on intake data for PCDD/F. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Peroxidase; Horseradish peroxidase; Myeloperoxidase; Hydrogen peroxide; Enzymatic formation; Polychlorinated dibenzo-*p*-dioxins; Polychlorinated dibenzofurans; Octachlorodibenzo-*p*-dioxin; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Chlorophenols; Pentachlorophenol; 2,3,4,6-Tetrachlorophenol; 2,4,5-Trichlorophenol

1. Introduction

Several mass balance studies on humans (Moser et al., 1996; Schrey et al., 1998) and studies on cattle, which were in contact or fed with pentachlorophenol (PCP) treated wood, (Fries et al., 1996; Fries et al. 1997) indicate a higher fecal excretion of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F), especially higher chlorinated PCDD, in relation to the amount ingested. The results point to the possible biochemical formation of PCDD/F in the organisms from precursors such as chlorophenols (CP) or the known contaminants of technical-grade CP,

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PII: S0378-4274(99)00066-1

i.e. chlorinated phenoxyphenols. Biochemical formations of PCDD/F from chlorophenols and other precursors have been observed in sewage sludge (Öberg et al., 1992; Schramm et al., 1996), compost (Öberg et al., 1992, 1993; Schäfer et al., 1993), in in vitro studies catalyzed by horseradish peroxidase (HRP) (Svenson et al., 1989a; Svenson et al. 1989b; Öberg et al., 1990; Wagner et al., 1990), bovine lactoperoxidase (LP) (Öberg et al., 1990) or more complex enzymatic systems such as whey or culture filtrates from fungi (Wagner et al., 1990). The formation of octachlorodibenzo-*p*-dioxin from reagent or technical grade pentachlorophenol and from nonachloro-2-phenoxyphenol, a contaminant of technical PCP, has been observed in rats (Feil and Tiernan, 1997; Huwe et al., 1998).

Peroxidases are omnipresent bi-substrate enzymes in nature. Within the catalyzed reactions the presence of hydrogen peroxide is necessary because it is used as the electron accepting substrate. In the present study we investigated the in vitro formation of PCDD/F from the commercial significant CPs 2,4,5-trichlorophenol (2,4,5-TrCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and pentachlorophenol catalyzed by plant HRP and by myeloperoxidase (MYP) from human leukocytes, each in the presence of hydrogen peroxide.

In the present study the MYP catalyzed formation of PCDD/F from different chlorophenols, from chlorophenol substrate mixtures and a comparison with the catalyzation by HRP are described for the first time. The experiments with HRP were repeated here for better comparison because formations of PCDD/F might be influenced by impurities of the substrates.

2. Materials and methods

2.1. Enzymes and substrates

HRP Type II (P 8250/Lot 16H9522) was obtained from Sigma. MYP recovered from human leukocytes and lyophylized from 0.02 M sodium acetate buffer at pH 6.0 (MPO; EC 1.11.1.7, M 6908/Lot 126H9402) was purchased from Sigma. 2,4,5-TrCP (99.9% chemically pure, certified, Dr Ehrenstorfer) and 2,3,4,6-TeCP (97.2% chemically pure, certified, Dr Ehrenstorfer) were obtained from Promochem (Wesel, Germany) and PCP (certified reference material) was from International Physical Laboratory (UK). Methanolic solutions of the chlorophenols were made with concentrations of 2, 5 or 15 mg/ml.

2.2. Composition and incubation conditions of the experiments

The reactions with HRP were incubated over 24 h in a total volume of 10 ml of a dimethyl succinic acid (DSA) buffer at pH 4.0 at room temperature as described by Wagner et al. (1990). All experiments with myeloperoxidase were carried out in a potassium dihydrogen phosphate buffer (pH 5.4) with a total volume of 2.0 ml in 20 ml glass test tubes at 37°C. The incubation time was 4 h in these experiments. The incubation was started with the addition of hydrogen peroxide. All series were performed with two blank controls containing either the chlorophenol substrate or the peroxidase while all other additions were left unchanged. The detailed lists of composition are shown in Tables 1 and 2.

2.3. Analysis

After incubation 100 μl of a standard solution containing 17 $^{13}C_{12}$-labelled PCDD/F congeners (2.5 or 5.0 pg/μl) in toluene, 3 ml saturated ammonium sulphate solution and 3 ml ethanol were added to an 2-ml aliquot of the HRP incubations or to the total amount of the MYP incubations. The samples were extracted three times with 4 ml hexane. The hexane extracts were dried over sodium sulphate and purified using three column clean up steps: a combination of neutral, acidic and basic silica gels, alumina and activated charcoal (Nygren et al. 1988; Höckel and Hagenmaier, 1995). After addition of 2 μl dodecane as keeper the cleaned extracts were evaporated to dryness using a nitrogen stream and 10 μl toluene containing 2.5 pg/μl 1,2,3,4-[$^{13}C_{12}$] tetraCDD were added as recovery standard.

The analytical instrument system consisted of a VG AutoSpec high-resolution mass spectrometer and a Hewlett-Packard 5890 series II gas chromatograph equipped with a Gerstel KAS 2 vaporization system (MS-parameters: single ion recording mode; resolution 8000–10 000 at 10%; electron impact ionization at 40 eV; perfluorokerosene lock mass check; observation of two ions

Table 1
List of experiments with HRP (total volume: 10 ml)

Experiment No.	DSA buffer (mM)	HRP (U)[a]	CP (mM)	Hydrogen peroxide (mM)
H0 (CP-blank)	10	2.0	–	2.0
2,4,5-TrCP				
H1 (blank)	10	–	0.1 (2,4,5-TrCP)	2.0
H2	10	2.0	0.1 (2,4,5-TrCP)	2.0
2,3,4,6-TeCP				
H3 (blank)	10	–	0.1 (2,3,4,6-TeCP)	2.0
H4	10	2.0	0.1 (2,3,4,6-TeCP)	2.0
PCP				
H5 (blank)	10	–	0.1 (PCP)	2.0
H6	10	2.0	0.1 (PCP)	2.0

[a] Units as specified by the manufacturer.

Table 2
List of experiments with MYP (total volume: 2 ml)

Experiment No.	KH_2PO_4 buffer (mM)	MYP (U)[a]	CP (mM)	Hydrogen peroxide (mM)
M0 (CP-blank)	0.375	1.0	–	0.5
2,4,5-TrCP				
M1 (blank)	0.375	–	1.0 (2,4,5-TrCP)	1.0
M2	0.375	1.0	1.0 (2,4,5-TrCP)	1.0
M3	0.375	1.0	0.1 (2,4,5-TrCP)	0.1
2,3,4,6-TeCP				
M4 (blank)	0.375	–	1.0 (2,3,4,6-TeCP)	1.0
M5	0.375	1.0	1.0 (2,3,4,6-TeCP)	1.0
PCP				
M6 (blank)	0.375	–	1.0 (PCP)	1.0
M7	0.375	1.0	0.1 (PCP)	0.1
M8	0.375	1.0	1.0 (PCP)	0.5
M9	0.375	1.0	0.1 (PCP)	1.0
M10	0.375	1.0	1.0 (PCP)	1.0
M11	0.375	1.0	1.0 (PCP)	2.0
Equimolar multisubstrate experiments				
M12	0.375	1.0	1.0 (2,4,5-TrCP), 1.0 (PCP)	1.0
M13	0.375	1.0	0.1 (2,4,5-TrCP), 0.1 (PCP)	0.1
M14	0.375	1.0	1.0 (2,3,4,6-TeCP), 1.0 (PCP)	1.0
M15	0.375	1.0	0.1 (2,3,4,6-TeCP), 0.1 (PCP)	0.1
M16	0.375	1.0	1.0 (2,4,5-TrCP), 1.0 (2,3,4,6-TeCP), 1.0 (PCP)	1.0

[a] Units as specified by the manufacturer.

Table 3
HRP- or MYP-catalyzed formation of PCDD/F from PCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.	OctaCDD (pg)	ΣPCDD/F (pg)	OCDD (μmol/mol $_{PCP}$)[a]	ΣPCDD/F (μmol/mol $_{PCP}$)[a]
H5 (bl)	64	150	–	–
H6	6800	6910	14.7	14.9
M6 (bl)	140	155	–	–
M7	1600	1660	15.9	16.4
M8	3500	3670	3.65	3.88
M9	1600	1600	15.9	16.0
M10	10 000	10 400	10.7	11.1
M11	6100	6350	6.48	6.80

[a] Blank (experiment H5 or M6) corrected values.

Table 4
HRP- or MYP-catalyzed formation of PCDD/F from 2,4,5-TrCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.		PentaCDD	HexaCDD	HeptaCDD	PentaCDF	HexaCDF	ΣPCDD/F
H1 (bl)	pg	<0.5	<0.5	1.2	4.9	0.76	50.3
H2	pg	560	510	9.6	62	140	1340
	μmol/mol$_{TrCP}$[a]	1.57	1.30	0.02	0.17	0.37	3.45
M1 (bl)	pg	<0.5	9.2	17	< 0.5	1.9	83.2
M2	pg	150	140	74	89	310	782
	μmol/mol $_{TrCP}$[a]	0.21	0.17	0.07	0.13	0.41	0.99
M3	pg	24	30	7.4	18	53	138
	μmol/mol $_{TrCP}$[a]	0.34	0.27	0.00	0.26	0.68	1.55

[a] Blank (experiment H1 or M1) corrected values.

each for native and labelled isomers; setting of five time windows; GC-parameters: column: J&W Scientific, DB-5, 60 m, 0.1 μm film thickness; temperature program: 180°C (3 min), 5°C/min, 220°C (16 min), 5°C/min, 235°C (7 min), 5°C/min, 280°C (15 min); injector program: 60°C (60 s), 12°C/s, 330°C (10 min), split off (1 min); split on (2 min); injection volume: 4 μl).

The PCDD/F concentrations were calculated considering the recovery rates of the internal standard compounds and response factors.

3. Results and discussion

The results of the PCDD/F homologue groups of major interest and the sum of the PCDD/F are given in the Tables 3–6 for the experiments with PCP, 2,4,5-TrCP, 2,3,4,6-TeCP and the multisubstrate experiments.

The PCDD/F concentrations in the chlorophenol free blanks (exp. H0 and M0) were near to the detection limit for all congeners and very small amounts were detected in the HRP or MYP free blank samples (exp. H1, H3, H5, M1, M4 and M6). In all other samples a significant formation of PCDD/F—2,3,7,8-chlorosubstituted as well as non-2,3,7,8-chlorosubstituted congeners—was observed. The formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was only observed in the experiments using 2,4,5-TrCP.

3.1. Monosubstrate experiments with PCP

The monosubstrate experiments with PCP and both HRP or MYP showed a predominant formation of OctaCDD (Table 3). The amounts formed were in the same order of magnitude for both peroxidases. The amount of OctaCDD built by

MYP showed a tendency to increase with the amount of the substrate. But highest formations were observed with the lower PCP concentrations (exp. M7 and M9), whereas the hydrogen peroxide concentration only showed an influence on the PCDD/F formation in the higher dosed (1 mM) (exp. M8, M10, M11) but not in the lower dosed (0.1 mM) (exp. M7 and M9) experiments.

3.2. Monosubstrate experiments with 2,4,5-TrCP and 2,3,4,6-TeCP

In comparison to the experiments with PCP the formation of PCDD/F by HRP or MYP was lower in the monosubstrate experiments with 2,4,5-TrCP (Table 4) and 2,3,4,6-TeCP (Table 5) and showed different homologue patterns. The main homologue groups were the PentaCDD, followed by HexaCDD and HexaCDF (2,4,5-TrCP, HRP), the HexaCDF, PentaCDD, HexaCDD, and PentaCDF (2,4,5-TrCP, MYP), the OctaCDD, HeptaCDD and HexaCDD (2,3,4,6-TeCP, HRP) or the HeptaCDD, OctaCDD and HexaCDD (2,3,4,6-TeCP, MYP). A comparison of the amounts of PCDD/F formed on a molar basis for the HRP catalyzed monosubstrate experiments with 0.1 mM concentrations (exp. H2, H4 and H6) is given in Fig. 1 and for the MYP catalyzed monosubstrate experiments with 1 mM concentrations (exp. M2, M5 and M10) in Fig. 2.

Table 5
HPR- or MYP-catalyzed formation of PCDD/F from 2,3,4,6-TeCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.		HexaCDD	HeptaCDD	OctaCDD	ΣPCDD/F
H3 (bl)	pg	2.7	14	23	189
H4	pg	43	180	2000	2530
	μmol/mol $_{TeCP}$[a]	0.10	0.39	4.30	5.12
M4 (bl)	pg	17	220	65	1320
M5	pg	260	2200	1100	4610
	μmol/mol $_{TeCP}$[a]	0.31	2.33	1.13	3.77

[a] Blank (experiment H3 or M4) corrected values.

Table 6
MYP-catalyzed formation of PCDD/F from CP mixtures (detailed information on the experiments are given in Table 2)

Experiment No.		PentaCDD	HexaCDD	HeptaCDD	OctaCDD	HexaCDF	ΣPCDD/F
M12	pg	280	680	13 000	1400	880	16 000
	μmol/mol$_{\Sigma CP}$	0.20	0.43	7.64	0.76	0.59	9.70
M13	pg	120	100	1300	140	200	1830
	μmol/mol$_{\Sigma CP}$	0.84	0.64	7.64	0.76	1.33	11.3
M14	pg	12	150	3800	9700	450	14 800
	μmol/mol$_{\Sigma CP}$	0.01	0.10	2.23	5.27	0.30	8.34
M15	pg	0.29	7.0	84	310	29	492
	μmol/mol$_{\Sigma CP}$	0.00	0.04	0.49	1.69	0.19	2.78
M16	pg	36	1100	3000	910	890	6760
	μmol/mol$_{\Sigma CP}$	0.02	0.47	1.18	0.33	0.40	2.70

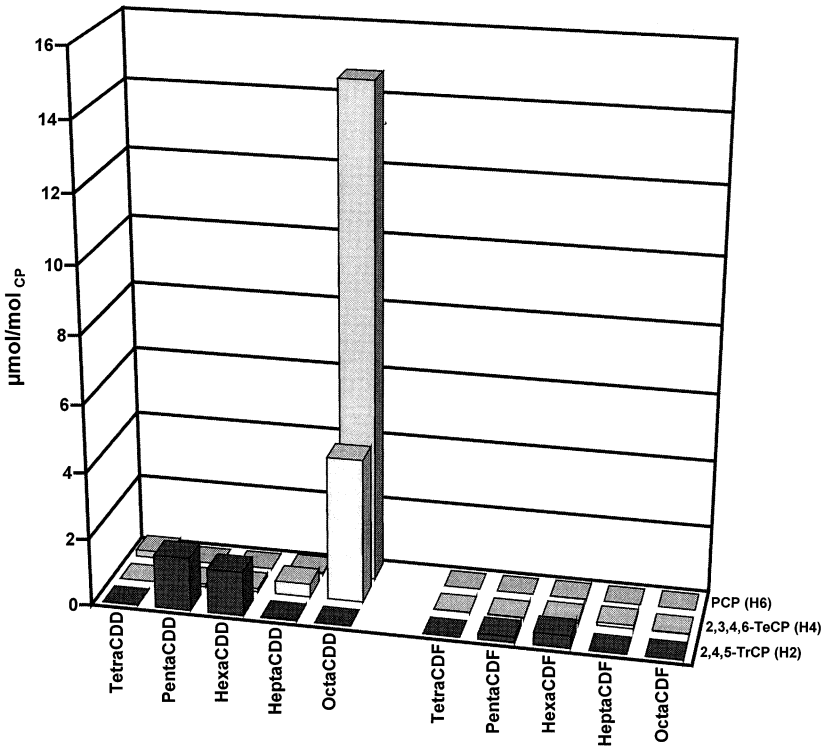

Fig. 1. PCDD/F formation of the 0.1 mM monosubstrate experiments H2, H4 and H6 with HRP (detailed information on the experiments are given in Table 1)

3.3. MYP-catalyzed multisubstrate experiments with mixtures of 2,4,5-TrCP, 2,3,4,6-TeCP and PCP

Within the MYP catalyzed experiments series (Table 6) the combination of equimolar mixtures of 2,4,5-TrCP and PCP resulted in the formation of HeptaCDD as the major component, whereas OctaCDD, followed by HeptaCDD, were the main components in the experiments with equimolar mixtures of 2,3,4,6-TeCP and PCP. Hepta- and HexaCDD were the main components in the experiment containing all three chlorophenols. The results of the experiments M12, M14 and M16 are shown in Fig. 3.

3.4. Comparison with other in vitro studies

A comparison of the experiments with HRP and MYP with studies of other authors is given in Table 7. All experiments with PCP gave OctaCDD as the main component. The formations observed in the studies with LP and HRP published by Öberg et al. (1990) and Öberg and Rappe (1992) were two to four times higher as in our own experiments with HRP and MYP showing both about 15 µmol/mol_{PCP}. The PCDD/F pattern found in the experiments with 2,4,5-TrCP is different between authors. The differences observed could be an effect of the experimental conditions, especially the different peroxidase and hydrogen peroxide concentrations used. In addition the discrepancies may be attributed to different analytical procedures and to different quality grades of the CPs used.

3.5. Comparison with in vivo studies

First in vivo studies with rats fed with 0.1 mg/day of purified PCP over 14 days showed no higher PCDD/F levels in the liver in comparison to a control group. Administration of reagent

grade PCP and technical grade PCP showed the formation of higher chlorinated PCDD (Feil and Tiernan, 1997). The main component was OctaCDD with levels 2.6 or 1042 times higher in comparison to the control group. In other experiments the formation of OctaCDD from nonachloro-2-phenoxyphenol, which is a known contaminant of technical PCP, was observed in rats (Huwe et al., 1998). Both studies confirm the formation of PCDD/F in vivo from PCP impurities.

3.6. Assessment and conclusion for human exposure

All observed formations in the in vitro studies are in the μmol/mol range and so—at first glance—they seem to be of minor importance, but the estimated intake of pentachlorophenol for unexposed persons in Germany is in the range of 1–2 μg/day (Butte and Heinzow, 1995; HBM-Kommission, 1997) and in the mass balance studies on humans (Schrey et al., 1998) the fecal excretion of OCDD of adults was on average 2300 pg/day higher than the dietary intake. Chlorophenols can also be significant metabolites of other chlorinated substances such as chlorobenzenes and chlorocyclohexanes. Typical serum concentrations of pentachlorophenol for unexposed persons from Germany range up to 20 μg/l and occupational exposed individuals show CP concentrations 10 to 1,000 times higher (HBM-Kommission, 1997). From this background and under consideration of the observed amounts formed in the in vitro experiments additional PCDD/F formation in the pg/day range would be expected, although kinetic data are not available at the moment.

It should be noted, that in all cases the presence of hydrogen peroxide as second substrate is neces-

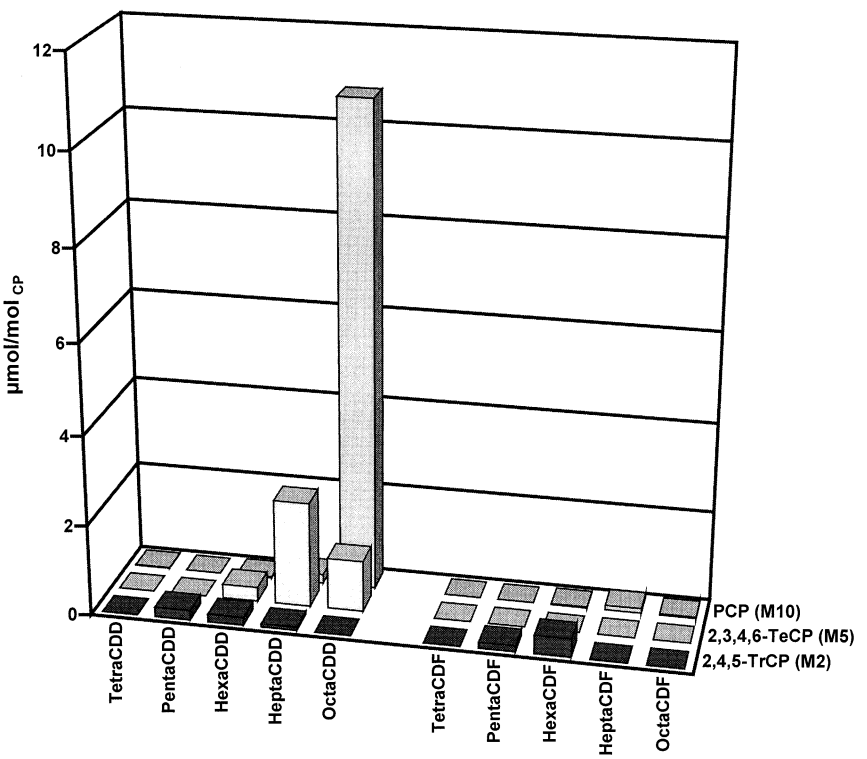

Fig. 2. PCDD/F formation of the 1 mM monosubstrate experiments M2, M5 and M10 with MYP (detailed information on the experiments are given in Table 2)

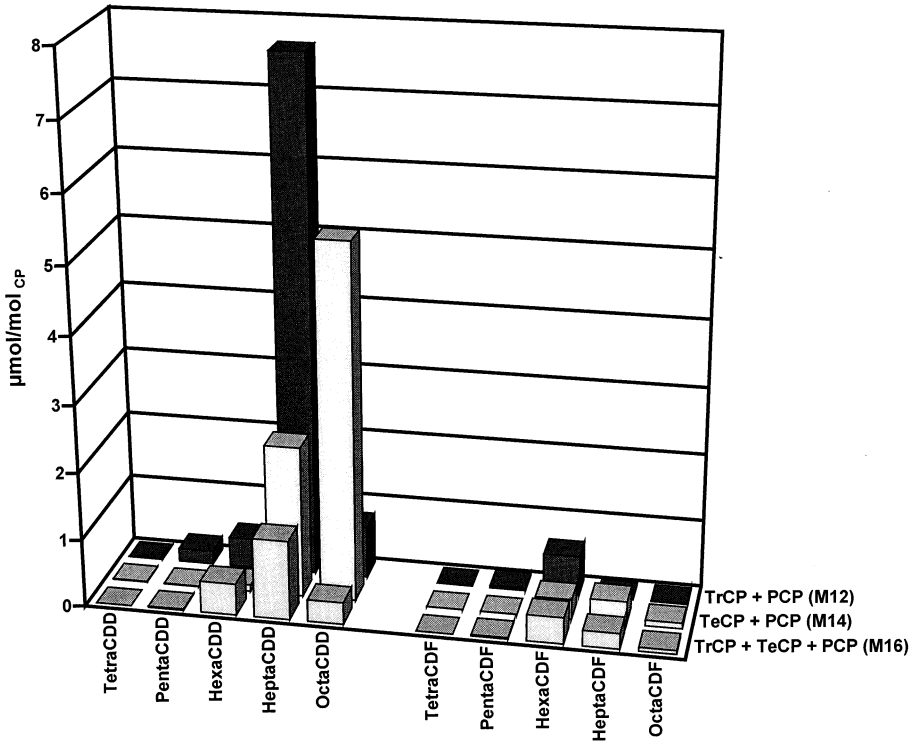

Fig. 3. PCDD/F formation of the multisubstrate experiments M12, M14 and M16 with MYP (detailed information on the experiments are given in Table 2)

Table 7
Comparison with data from literature

Parameter	Öberg et al. (1990) and Öberg and Rappe (1992)		Svenson et al. (1989b) HRP	This study	
	LP	HRP	HRP	HRP	MYP
Experimental conditions					
2,4,5-TrCP (mM)	0.1	0.1	0.1	0.1	0.1
PCP (mM)	0.1	0.1	–	0.1	0.1
Peroxidase (U/ml)	1.3–1.7	1.3–1.7	1.3–1.7	0.2	0.5
Hydrogen peroxide (mM)	0.2	0.2	9.0	2.0	0.1
Formation of PCDD/F from 2,4,5-TriCP[a]					
PentaCDD (µmol/mol $_{TrCP}$)	3.60	1.27	3.37	1.57	0.34
HexaCDD (µmol/mol $_{TrCP}$)	1.01	0.86	5.88	1.30	0.27
PentaCDF (µmol/mol $_{TrCP}$)	0.37	0.06	0.37	0.17	0.26
HexaCDF (µmol/mol $_{TrCP}$)	0.04	0.03	0.75	0.37	0.68
ΣPCDD/F (µmol/mol $_{TrCP}$)	5.73	2.43	10.5	3.45	1.55
Formation of PCDD/F from PCP					
OctaCDD (µmol/mol $_{PCP}$)	34.8	57.9	–	14.7	15.9

[a] Wagner et al. (1990) found a formation of 5,7 $\mu g_{\Sigma\ PCDD/F}/g_{2,4,5\text{-}TrCP}$. The formation on a molar basis cannot be calculated due to missing data for the several homologue groups.

sary. Cellular and subcellular hydrogen peroxide concentrations at steady state are in the range 1–100 nM (Chance et al., 1979). The formations of PCDD/F in the human body can first takeplace in the digestive tract, where peroxidases can be present as part of microorganisms, vegetable or animal food (Wagner et al., 1990). Anyway, different peroxidase systems can be found in many organs and cells. The MYP used in this study is a component of human neutrophile granulocytes. The content of MYP is up to $5\%_{\text{dry matter}}$ in the peripheral cells, whereas the content in growing granulocyte cells in bone marrow can be higher (Marquardt and Schäfer, 1994). This shows that there are many locations where the formation of PCDD/F by peroxidase systems can take place.

It must be mentioned that the CPs used in the present study were of high purity and the influence of minor impurities in the CPs on the formation of PCDD/F was not examined. It can not be excluded that specific impurities are in particular responsible for the observed formation of PCDD/F.

4. Conclusion

The in vitro studies confirm the suspicion that the biochemical formation of PCDD/F from precursors such as chlorophenols can take place in the human body and that this metabolic pathway may lead to a higher inner exposure to PCDD/F than up to now estimated by food analyses or duplicate studies. Thus, part of the observed higher excretion rates of higher chlorinated PCDD/F in relation to dietary intake found in the mass balances studies (Moser et al., 1996; Schrey et al., 1998) might be explained by peroxidase-catalyzed metabolic transformations of chlorophenols.

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