Bull. Environ. Contam. Toxicol. (1987) 38:962–968

Bulletin of Environmental Contamination and Toxicology

On the Absence of Polychlorinated Dibenzodioxins and Dibenzofurans After Lactoperoxidase-Catalyzed Transformation of Chlorophenols

Lars G. Öberg[1],* and Stephen E. Swanson[2]

Departments of [1]Physiological and [2]Organic Chemistry, University of Umeå,
S-90187 Umeå, Sweden

Polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) have been detected in many species and environments (Rappe 1986), their bioresistance and toxicity being of great concern (Ayres et al. 1985; Sci. Rev. Com. 1985). PCDDs and PCDFs, or the predioxins and -furans, are formed from chlorophenols (CPs) by burning and pyrolysis (Zoller and Ballschmiter 1986), by arcing (Safe 1986), and by photolysis (Choudhry et al. 1985). PCDDs and PCDFs have also been found in emissions from automobiles, municipal waste incinerators, and nickel and copper smelting (Rappe 1986). They are by-products from industrial chemical synthesis, e.g. 2,4,5-trichlorophenol production, and may thus enter the biosphere as impurities of pesticides and other chemicals (Ryan et al. 1985). Forest fires do not cause any emission (Safe 1986).

Peroxidases (POs), a group of heme-proteins, are found in many organs and organisms. They are exceptional enzymes because of low substrate specificity and multiple reaction mechanisms (Dunford and Stillman 1976; Neidleman and Geigert 1986). Organic substrates are commonly oxidized by hydrogen peroxide in two one-electron steps:

$$\text{PO} + H_2O_2 \longrightarrow \text{cpd-I} \qquad (1)$$
$$\text{cpd-I} + AH_2 \longrightarrow \text{cpd-II} + HA\cdot \qquad (2)$$
$$\text{cpd-II} + AH_2 \longrightarrow \text{PO} + HA\cdot \qquad (3)$$

where cpd-I and cpd-II denote oxidized forms of the enzyme. This enzyme-catalyzed free radical reaction resembles reactions in pyrolysis, arcing, and photolysis. Halogenated phenols are among the peroxidase substrates (Öberg and Paul 1985), and phenolic substrates have been found to yield dibenzodioxin- and dibenzofuran-

---

Send reprint requests to Lars Öberg at the above adress.

like products (Subrahmanyam and O'Brien 1985; Kovar and El-Bolkiny 1986). The question then arose whether CP's in peroxidase-mediated reactions could yield chlorinated dibenzodioxins and dibenzofurans.

Since no-one has yet reported a biological formation of PCDDs and PCDFs we have analyzed the product mixture from the lactoperoxidase-catalyzed oxidation of some chlorophenols.

MATERIALS AND METHODS

Bovine lactoperoxidase (LP, EC 1.11.1.7), as a representative of mammalian peroxidase, was prepared (Paul et al. 1980) with the DEAE-cellulose chromatography step left out. Pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (TeCP), 2,4-dichlorophenol (DCP), and water were purified as described (Öberg and Paul 1985). Other chemicals were of analytical grade.

The starting material was analyzed for PCDF and PCDD impurities. A 19.8 µmol solution of 2,4-dichlorophenol in 8 ml water and 39.4 µmol 2,3,4,6-tetrachlorophenol in 250 ml 10% ethanol (v/v) both had less than 1 ppb of PCDDs and PCDFs; 308 µmol pentachlorophenol in 1010 ml 21% ethanol (v/v) contained less than 40 ppb of hexa-, hepta-, and octachlorodibenzofuran (Table 1).

The 5.8 ml reaction mixtures consisted of: 100 mM sodium phosphate buffer at pH 7.0, sterile-filtered through a 0.2 µm Sartorius membrane and deoxygenated with N_2-gas; 200 nM LP for PCP and TeCP, and 600 nM LP for DCP; 10% ethanol (v/v) to increase the solubility of PCP and TeCP; 150 µM PCP, 200 µM TeCP, or 300 µM DCP; 200, 250, and 300 µM H_2O_2 for PCP, TeCP, and DCP respectively. Samples were weighed out. Stoppered tubes and flasks were used throughout. The temperature was held at 25°C. The reactions were performed in the dark. LP was fully active at the ethanol concentrations used. After one hour all CP had reacted while some LP still was active {DCP inactivates LP (Öberg and Paul 1985)}, and there was H_2O_2 in excess. A 45 ml phosphate buffer aliquot was added to each sample to make sample-handling during clean-up easier.

The extraction techniques for PCDDs and PCDFs were based on methods described by USEPA (1979). Burdick & Jackson "Distilled in Glass" solvents were used throughout. The samples were adjusted to a pH-value greater than 12 and extracted with dichloromethane. The extracts were dried over anhydrous sodium sulfate, reduced to less than 1 ml by rotary evaporation, and taken to dryness under a gentle stream of dry N_2-gas. The samples were solvent-exchanged into n-hexane, fol-

Table 1. Analysis of polychlorinated dibenzodioxins and dibenzofurans in chlorophenol substrate (S) and reaction mixtures (RM).

	DCP and TeCP		PCP	
	S	RM	S	RM
Homolog	(ng/g)[a]		(ng/g)[a]	
TetraCDF	<1[b]	<1[bc]	<1[b]	<1[bc]
TetraCDD	<2	<2	<2	<2
PentaCDF	<1	<2	<1	<2
PentaCDD	<5	<8	<5	<8
HexaCDF	<3	<5	<3	<5
HexaCDD	<3	<5	<3	<5
HeptaCDF	<10	<10	<20	<30
HeptaCDD	<10	<10	<20	<30
OctaCDF	<20	<20	<40	<50
OctaCDD	<10	<10	<20	<30

a) Detection limits for each isomer are given.
b) No value was above the actual detection limit.
c) RM has higher detection limits solely because of smaller sample.

lowed by a three-column clean-up procedure. First the concentrated extracts, and two 1-ml hexane rinses, were passed through a pair of columns which oxidized and removed interfering compounds from the enzymatic reaction mixture. The top column was packed with 0.25 g of activated silica gel and 1.5 g of concentrated sulfuric acid on silica gel (40% w/w). The bottom column was packed with 1.1 g of activated basic alumina. From the first column the effluent flowed directly into the second. The column pair was eluted with an additional 7ml of hexane, and the total eluate was saved. Then the silica column was removed and the alumina column was eluted with 10 ml of dichloromethane/hexane (1:1, v/v). After concentration to less than 1 ml, this eluate was applied, together with two 1-ml rinses, to the third clean-up column which separated the planar PCDD and PCDF molecules from other chlorinated compounds. This third column was packed with 0.75 g of 18% carbopack C (Supelco) on 80/100 mesh celite 545, which had been activated for 6 hours at 130 °C and kept desiccated. The column was washed with 2 x 5 ml of toluene, 2 x 2ml of dichloromethane, 2 x 2 ml of n-hexane, and eluted with 3 ml of dichloromethane/n-hexane (1:1, v/v). This eluate was saved. Finally, the column was eluted with 20 ml of toluene. The toluene fraction was concentrated and analyzed as described below.

Table 2. Polychlorinated dibenzodioxin and dibenzofuran quantification standard.

1368-TetraCDF
2378-TetraCDF*
3467-TetraCDF
2378-TetraCDD*
13468-PentaCDF
12378-PentaCDF*
23468/12469-PentaCDF
12469-PentaCDF
23467-PentaCDF
12378-PentaCDD*

123468-HexaCDF
123478/123479-HexaCDF
123678-HexaCDF*
123789-HexaCDF
234678-HexaCDF
123478-HexaCDD*
1234678-HeptaCDF
1234789-HeptaCDF*
1234678-HeptaCDD*
OctaCDF*
OctaCDD*

12378-^{13}C-PentaCDF* (Internal Standard)

* Isomers used for quantification.

Extracts were analyzed by High Resolution capillary Gas Chromatography with Mass Spectrometry detection (HRGC/MS) using a Finnigan model 4500 quadrapole Mass Spectrometer or a VG model 70-250 double focusing Mass Spectrometer. The following HRGC/MS analysis parameters were used:

Column: 60 m x 0.25 mm fused-silica wall-coated with SP-2330 (Supelco)
Column Temp: 100°C (2 min hold) to 180°C at 20°C/min then to 260°C at 3°C/min (20 min hold)
Injector: Grob-type splitless
Injector Temperature: 260°C
Carrier gas: He at 40 psig
Nominal Mass Resolution: unit resolution: Finnigan
5000 resolution: VG.

The gas chromatographic retention indices for each of the tetra- through octaCDDs and CDFs were determined using a mixed standard which contained all of the tetra- through octa- isomers (not shown). In Table 2 the isomers in the quantitation standard are listed. The Negative Chemical Ionization mode was used on the Finnigan spectrometer, with methane as the reagent gas. On the VG spectrometer, the Electron Impact Ionization mode was used. Thus, together with Selected Ion Response (SIR) scanning, maximum sensitivity was achieved. In the SIR scanning mode, the mass spectrometer only monitors two ions from the molecular ion cluster for each PCDD and PCDF homolog (Table 3). Detection limits in the range of sub-picograms per injection resulted in the levels shown in Table 1. The PCDD and PCDF isomers

Table 3. Masses monitored for polychlorinated dibenzodioxin and dibenzofuran analysis.

Homolog	Masses Monitored	Theoretical ratio
TetraCDF	303.902/305.899	76/100
TetraCDD	319.896/321.894	76/100
PentaCDF	337.863/339.860	61/100
PentaCDD	353.858/355.855	61/100
HexaCDF	371.824/373.823	51/100
HexaCDD	387.819/389.816	51/100
HeptaCDF	405.785/407.782	43/100
HeptaCDD	421.799/423.777	43/100
OctaCDF	441.746/443.740	87/100
OctaCDD	457.738/459.735	87/100

Table 4. Compounds in surrogate fortification.

2378-^{13}C-TetraCDF
2378-^{13}C-TetraCDD
12378-^{13}C-PentaCDD
123478-^{13}C-HexaCDF
123678-^{13}C-HexaCDD
1234678-^{13}C-HeptaCDD
^{13}C-OctaCDD

were identified by the match of retention times and correct ratios for the intensities of the two molecular ion cluster ions monitored (Table 3).

To monitor the extraction efficiency and the possible loss of analyte during clean-up, each sample was fortified, prior to extraction, with 50 pg of the ^{13}C-labeled surrogate compounds listed in Table 4. Hence, each sample became both an unknown and a quality assurance sample. The average recovery for these surrogate compounds was 70% to 80% with a range of 60% to 120%.

The medium resolution quadrapole mass spectrometer was used and the results were confirmed on the high resolution double-focusing mass spectrometer (resolution 5000). The detection level was good compared to levels reported for similar samples. This was achieved by the combination of sensitive instrument responses and good surrogate recoveries. For a peak to be identified as a PCDD or PCDF two criteria had to be met: the peak had to appear for both the M+ and M+2 ions at the established theoretical ratio (Table 3), and it had to match the relative retention time established by the retention time standard which contained all tetra- through octa- CDD and CDF congeners.

RESULTS AND DISCUSSION

PCDFs and PCDDs are widespread in nature: in mothers milk, herring gull eggs, Baltic herring, ringed seal, salmon, sediment off the Swedish west coast, crab, turtle, et cetera. One would therefore expect these compounds to be formed, unintentionally, in great quantities from precursors, and to be present in many technical applications widely used. To date only a few technical procedures have been identified as generators of PCDDs and PCDFs, and they do not account for what is detected. The formation is a high-energy process which has led to the conclusion that metabolism could not be involved. The biological formation of PCDDs and PCDFs has not as yet been demonstrated. Nevertheless, at least one enzyme system exists which is capable of the like reactions: the peroxidases. The oxidation-reduction potentials of cpd-I/cpd-II and cpd-II/PO•FeIII are about 1.2 V (Hayashi and Yamazaki 1979).

Table 1 summarizes the results from the analysis of the three enzyme-treated samples, corrected for ^{13}C-surrogate recoveries. In the product mixture from LP-catalyzed oxidation of PCP, 2,3,4,6-TeCP, or 2,4-DCP no PCDDs or PCDFs were detected, at a detection limit less than 50 ppb. The results may suggest that peroxidases are not the biological catalysts of PCDD or PCDF formation. Though, there exist additional biochemical ways: other enzyme systems, other peroxidases with different substrate profiles, other substrates. Peroxidases may also be involved at another level: they are common in the marine milieu where they function as halogenating systems. Polychlorination of pre-formed dibenzodioxins and furans is feasible.

Acknowledgments. We thank KG Paul for fruitful discussions and C Rappe for kindly reading the manuscript. P-I Ohlsson is acknowledged for providing the lactoperoxidase. LÖ thanks the following for financial support: Jubileumsklinikens i Umeå Forskningsfond - Lions projects 273/83 and 340/84, JC Kempes Minnes Stipendiefond, and the Medical Faculty at Umeå University.

REFERENCES

Ayres SM, Webb KB, Evans RG, Mikes J (1985) Is 2,3,7,8-TCDD (dioxin) a carcinogen for humans? Environ Health Persp 62:329-335

Choudhry GG, van der Wielen FWM, Barrie Webster GR, Hutzinger Otto (1985) Photochemistry of halogenated benzene derivatives. Part VI. Photoreactions of tetra- and pentachlorophenols in water-acetonitrile mixtures. Can J Chem 63:469-475

Dunford HB, Stillman JS (1976) On the function and mechanism of action of peroxidases. Coord Chem Revs 19:187-251

Hayashi Y, Yamazaki I (1979) The oxidation-reduction potentials of compound I/compound II and compound II/ferric couples of horseradish peroxidases A_2 and C. J Biol Chem 254:9101-9106

Kovar K-A, El-Bolkiny MN (1986) Reaction of 4-hydroxyphenylacetic acid with hydrogen peroxide in the presence of peroxidase. Arch Pharm (Weinheim) 319:280-282

Neidleman SL, Geigert J (1986) Biohalogenation: principles, basic roles and applications. John Wiley & Sons, Toronto

Öberg LG, Paul KG (1985) The transformation of chlorophenols by lactoperoxidase. Biochim Biophys Acta 842:30-38

Paul KG, Ohlsson PI, Henriksson A (1980) The isolation and some liganding properties of lactoperoxidase. FEBS Lett 110:200-204

Rappe C (1986) at Dioxin 86., Fuokoka, Japan, September

Ryan JJ, Lizotte R, Sakuma T, Mori B (1985) Chlorinated dibenzo-p-dioxins, chlorinated dibenzofurans, and pentachlorophenol in Canadian chicken and pork samples. J Agric Food Chem 33:1021-1026

Safe SH (1986) Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Ann Rev Pharmacol Toxicol 26:371-399

Scientific Review Committee (1985) Commentary on 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD). J Toxicol Clin Toxicol 23:191-204

Subrahmanyam VV, O'Brien PJ (1985) Phenol oxidation product(s), formed by a peroxidase reaction, that bind to DNA. Xenobiotica 15:873-885

USEPA (1979) U.S. Environmental Protection Agency waste water analysis method: 2,3,7,8-tetrachlorodibenzo-p-dioxin. Method 613. Fed Reg 44:69464

Zoller W, Ballschmiter K (1986) Formation of polychlorinated dibenzodioxins and dibenzofurans by heating chlorophenols and chlorophenates at various temperatures. Fresenius Z Anal Chem 323:19-23

Received January 31, 1987; accepted March 9, 1987.