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Identification and Toxicological Evaluation of Chlorinated Dibenzofuran and Chlorinated Naphthalene in Two Commercial Polychlorinated Diphenyls.

J.G. Vos.
Institute of Veterinary Pathology,
Biltstraat 172, Utrecht, The Netherlands.
J.H. Koeman and A.L. van der Heas.
Institute of Veterinary Pharmacology and Toxicology,
Biltstraat 172, Utrecht, The Netherlands.
M.C. ten Noever de Brauw.
R.H. de Vos.
Central Institute for Nutrition and Food Research,
TNO, Zeist, The Netherlands.

Running title:

Toxic impurities in technical PCB preparations.

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Abstract.

In a previous study (Vos & Koeman, 1970) a significant difference in toxicity was found between 3 commercial PCB preparations; high mortality, liver necrosis and chick oedema-like lesions occurred in 2 PCB's.

In the present study it was found, by means of column chromatography and gas chromatography, that in the 2,5 diethyl-ether fractions of these 2 PCB's polar compounds are present. A chicken embryo assay proved the high toxicity of this fraction. Subsequent mass spectrometric and microcoulometric analyses revealed the identity of these compounds; a.o. tetra- and pentachlorodibenzofuran. The significance of these impurities are discussed. It is concluded that evaluation of animalexperiments with PCB's is extremely difficult due to the presence of chlorinated dibenzofurans in some commercial PCB's.

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Introduction.

Polychlorinated biphenyls (PCB) have been identified in tissues of fish and wildlife (Jensen, 1966; Holmes, Simmons & Tatton, 1967; Holden & Marsden, 1967; Koeman, Ockhuip, Veen, Brouwer, Rooth, Zwart, van de Broek & van Genderen, 1967; Risebrough, Riecke, Peakall, Herman & Kirven, 1968; Koeman, ten Noever de Brauw & de Vos, 1969; Jensen, Johnels, Olsson & Otterlind, 1969), and have been recently been implicated in the mortality of sea birds in Britain (Bourne & Mead, 1969).

Commercial PCB preparations are extremely stable and only slightly soluble in water. They are used as lubricants, as heat transfer media, in protective coatings for wood meat metal and concrete, and in many other applications. They are compatible with paints, synthetic resins, varnishes and waxes, and improve such properties of these products as their chemical resistance, water resistance, adhesiveness and flexibility (Anonymus, 1960; Hubbard, 1964). However, it has not yet been established which of the materials treated with PCB has contributed to the present environmental contamination.

Experiments with rats and guinea pigs have shown that the ingestion of PCB may give rise to liver injury (Miller, 1944). Hydropericardium, occasionally accompanied by abdominal oedema, was found in chicks (McCune, Savage & O'Bell, 1962; Flick, O'Bell & Childs, 1965; Vos & Koeman, 1970) and Japanese quail (Koeman et al., 1969) after ingestion of PCB of American (Aroclor) and French (Phenoclor) origin, respectively. A marked resemblance between the gas chromatograms and mass spectra in mass spectrometer analyses of 3 commercial PCB preparations (Phenoclor DP6, Clophen A60, and Aroclor 1260) was found (Koeman et al., 1969).
The occurrence of "Chick oedema" like lesions in birds fed PCB (Koeman et al., 1969; McCune et al., 1962; Flick et al. 1965) was

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the reason for a recent comparative toxicological test with the 3 mentioned PCB preparations in chickens (Vos & Koeman, 1970). In this study a significant difference in toxicity between the 3 tested compounds was found; Clophen A60 and Phenoclor DP6 showing the highest, Aroclor 1260 showing the lowest mortality. Subcutaneous and abdominal oedema, and centrolobular liver necrosis was recorded only in those chicks fed with Clophen and Phenoclor. Hydropericardium was recorded in nearly all chicks fed with Clophen and Phenoclor and only occasionally in chicks fed with Aroclor. Chemical porphyria was found as a general PCB effect.

In the present study, the sequel to that mentioned above, we describe the identification and evaluation of compounds responsible for the difference in toxicity between the 3 commercial PCB preparations.

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Experimental.

The PCB preparations, which contain an average of 60% chlorine, were obtained from Prodelec, France (Phenoclor DP6), Bayer, Germany (Clophen A60, Lot no. 912454), and Monsanto, U.S.A. (Aroclor 1260, Lot no. Ak-3).

The screening method, used for detection of chick oedema factor (Anonymus, 1960a) was modified and used to isolate from the PCB preparations, compound(s) responsible for differences in toxicity. The clean-up step of this procedure was omitted. In the column chromatograph; 8g. activated florisil (column ∅ 10mm) was used instead of 15g. activated alumina. For fractionation redist. hexane was used as elution solvent instead of petroleum ether. Two mg PCB was dissolved in 2 ml hexane and fractionated on the florisil column. This column was eluted with hexane until nearly all PCB's were eluted (tested by gas chromatography). The 2nd fraction (5% diethylether in hexane) was also tested until no more PCB could be detected. The final fraction (fraction 3), eluted with 25% diethylether (reagent grade) in hexane, was evaporated to 1 ml. An aliquot of this concentrate was injected into a 204-1B Varian Aerograph gas chromatograph with electron capture detection. The Pyrex glass column (5 feet x 1/8 inch), filled with 10% DC 200 on Gaschrom Q (80-100 mesh) was operated at 200°C with nitrogen as carrier gas (about 50 ml/min.). The same prooodure was used to fractionate aliquots of about 550 mg of the PCB preparations in order to obtain sufficient quantities for a chicken embryo assay and for mass spectrometer analyses. The PCB was dissolved in 200 ml hexane. The column (∅ 32 mm) was filled with 180 g activated florisil. The more polar compounds were eluted in the final (3rd) fraction and, after evaporation, tested by gas chromatography.

A chicken embryo assay (Verrett, Carliac & McLaughlin, 1964) was used to determine the toxicity of the 5 PCB preparations,

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(dose: 3.5 µg/egg) dissolved and kept at 37°C in ethanol. Group 1 = Aroclor, group 2 = Phenoclor and group 3 = Clophen) and of the 3rd fraction of Clophen (dose: 3rd fraction obtained from 35, 3.5, and 0.35 and 0.035 µg. Clophen in groups 4, 5, 6 and 7 respectively). The samples, after dissolving in 0.05 ml ethanol were injected into the air cell of fresh White Leghorn eggs prior to incubation. The livers of all hatched chicks were removed for histological examination, fixed in 10% buffered formalin and processed in a conventional manner. Moreover, cryostat sections were studied in a fluorescence microscope.

Mass spectrometry was used to identify compounds present in the polar 3rd fractions. This analysis was carried out using a gas chromatograph-mass spectrometer combination. For this purpose a CH4 mass spectrometer (Varian Mat. G.m.b.H.) was coupled to a Varian Aerograph even, type A550D, with a Pyrex glass column (5 feet x 1/8 inch) filled with 10% DC200 on Gaschrom Q (60-100 mesh). Particular attention was paid to the design of the interface between the gas chromatograph and the mass spectrometer in order to obtain the best operating conditions (ten Noever de Brauw & Brunnée, 1967). High resolution mass spectra were taken on a double focus spectrometer type CH$_7$ (Varian Mat. G.m.b.H.). Exact mass measurements were done with the peak matching method (Measurements were also done from spectra which were recorded on photographic plates processed by Varian Mat. on a Leitz comparator SMM).

To obtain information about the quantities, microcoulometric analyses of the 3rd fractions were carried out. The equipment used was a Dohrmann microcoulometer, coupled to a Microtek MT 220 gas chromatograph, with a glass column (6 feet x ¼ inch). The same 10% DC 200 column packing was used. The column effluents were oxidized in a Dohrmann combustion furnace and the HCl, produced by chlorinated compounds, was detected and quantitatively measured [illegible]

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Results.

The resulting gas chromatogram of fraction 3, obtained with the small column (ø 10mm) revealed the presence of polar compounds in Phenoclor and Clophen (fig.1). These were not found in the Aroclor extract. The same results were found with fraction 3 obtained with the large column (ø 32 mm). The 3rd fraction 3, ~~obtained~~ of Clophen was subsequently used in the chicken embryo assays. This assay (table 1) confirmed the differences in toxicity between the 3 tested PCB preparations found in the previous study (Voo & Keenan, 1970). Mortality in group 1 (Aroclor) did not differ significantly (X^2 test) from mortality in group 9 (control). Mortality in group 4 and 5 is dose related; early mortality in the former, scattered mortality in the latter group. Corresponding mortality in group 3 (3.5 mg Clophen) and group 5 (3rd fraction from 3.5 mg Clophen) indicates strongly that the polar residue in the latter determines the toxicity of the commercial PCB. Dissection of all fresh embryos and of the one-day-old chicks revealed no abnormalities except for one embryo of group 3 which showed a severe subcutaneous oedema. Oedema and beak deformities are described after yolk sac injection of 25 µg and 10 mg aroclor 1242, resulting in respectively 100% and 95% embryonic mortality (McLaughlin, Marliac, Verrett, Hutchler & Fitzhugh, 1963). H.E. stained liver sections revealed no morphologic alterations. In the erythrocytic section of the liver of the only hatched weak chicken of group 3, a red fluorescence in some lobules was seen in the u.v. microscope. This fluorescence is indicative of the porphyrin biosynthetic disturbance found in the prior study (Voo & Keenan, 1970).

Mass spectrometric analyses revealed identical chlorinated compounds in fraction 3 from Phenoclor and Clophen in contrast with Aroclor. The results are given in table 2. Mass spectra of compounds with mass number 304 and 330, obtained from peak 1 and 4 (fig.1) are given in fig.2 and 3. From exact mass measurements it was found that the formulae of the molecule peaks 304 and 330 were $C_{\ldots}$

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$C_{12}H_4O^{35}Cl_4$ and $C_{12}H_5O^{35}Cl_5$. The formulae of the fragment ions 241 and 275 are $C_{11}H_4{}^{35}Cl_5$ and $C_{11}H_5{}^{35}Cl_4$. From this data it can be concluded that the parent ion has a preferential fragmentation for the loss of a single ClCO unit. Mass spectra were compared with the spectrum of a compound isolated from toxic fat (Hootton & Courchene, 1964). Subsequent study, using X-ray diffraction, has revealed that the identity of one of the toxic factors was 1, 2, 3, 7, 8, 9-hexachlorodibenzo-p-dioxin (Hootton & Courchene 1964). The mass-spectrum of this compound has a similar fragmentatio pattern i.e. loss of successive 2 mass units 63 suggests a loss of 2 ClCO units.

Microcoulometric analyses of the 3rd fraction of Clophen and Phenoclor revealed chromatograms with the same peak pattern as in fig.1. The "peak" of the tetrachlorodibenzofuran (mass number 304) is too marked by the proximity of the hexachloronaphthalene peak (peak no.2) to make quantitative calculations possible. For similar reason peak no.4 in the chromatogram, found to be a mixture of heptachlorobiphenyl and pentachlorodibenzofuran, could not be measured directly. However, when assuming that the whole peak is produced by the dibenzofuran compound alone, a certain maximum level can be indicated. This level was found to be 5 ppm pentachlorodibenzofuran in Clophen and 10 ppm in Phenoclor. Moreover the minimum level could be indicated with the estimated lower limit of detection of the mass spectrometric equipment. Under the conditions used this level was 1 ppm.

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Discussion.

Mass spectrometric results have not conclusively proved the identity of the chlorinated dibenzofurans. But synthesis of these compounds, necessary for comparison with the mass spectra obtained experimentally, was found extremely dangerous; a single oral dose of 0.5-1.0 mg/kg tri- and tetrachlorodibenzofuran caused severe and often lethal liver necrosis in rabbits (Bauer, Jenulz & Spiegelberg, 1961; Hofman, 1938). Application on the ear of a rabbit resulted in chloracne (Bauer et al., 1961). The related compound 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (following the description of Chemical Abstracts), which may appear in the synthesis of 2, 4, 5-trichlorphenol by hydrolysis (under pressure and with the presence of sodium hydroxide and methanol) of 1, 2, 4, 5-tetrachlorobenzene (Bauer et al., 1961), usually caused a lethal liver necrosis in the rabbit after a single oral dose of 0.05-0.1 mg/kg. Ear application, also in a 10 times lower dose compared with chlorinated dibenzofuran, resulted in chloracne (Bauer et al., 1961; Linn Jones & Krisch, 1962). 1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin, which can arise in the pyrolysis of 2,3,4,6-tetrachlorophenol (Higginbotham, Huang, Firestone, Verrett, Rose & Campbell, 1968), has been isolated and identified as one of the toxic substances of the Chick Oedema Factor (Cotton & Couronne, 1964; Anonymus, 1960b). The relationship between the toxic nature of PCB and the Chick Oedema Factor has already been discussed (Flick et al., 1965).

Air cell injection in chicken eggs of 0.05 ug hexachlorodibenzo-p-dioxin resulted in 100% embryonic mortality (Higginbotham et al., 1968). The experiment using an injection of the 3rd fraction obtained from 35 mg Clophen into the air cell resulted in 100% mortality (table 1). It can be calculated that the maximum

dose/egg is 0.2 ug pentachlorodibenzofuran (using a value of max. 5 ppm). This confirms the order of toxicity discussed above.

The combination of toxicological, pathological and chemical analytical data makes it almost certain that the compounds of mass numbers 304 and 338 are respectively tetra- and pentachlorodibenzofuran. The origin of these toxic factors in Clophen A60 and Phenoclor DP6 can possibly be explained by differences in manufacture. Particularly in the distillation procedure of crude PCB, in which sodium hydroxide can be used (Fay & Richards, 1947). PCB reacts with sodiumhydroxide at elevated temperatures to yield phenolic compounds, e.g. PCB is saponified into polychlorohydroxybiphenyls by means of sodium hydroxide in a polyhydric alcohol medium (Anonymus, 1957). A further loss of hydrochloric acid could produce dibenzofuran derivatives.

Chlorinated naphthalenes are far less toxic than chlorinated dibenzofurans (Hofmann, 1958). Therefore the latter may be assumed to determine the toxicity of fraction 5.

The occurrence of hydropericardium in chick fed with Aroclor (Flick et al., 1965), may indicate the presence of a very small quantity toxic factor.

From this study it is concluded that evaluation of PCB residues in wildlife animals, based on effects and residues in test animals, is extremely difficult due to differences in toxicity between commercial PCB preparations.

Work is in progress to detect and identify chlorinated dibenzofuran from wildlife specimens.

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Table 1. Chicken Embryo Assay of Chlorinated Biphenyls Aroclor 1260, Phenoclor DP 6 and Clophen A60 and the 25 per cent Diethylether Fraction of Clophen A60.

Group	Compound	Dose/egg[a]	Number	Number of Infertile Eggs	Per cent Hatch of Fertile Eggs	Remarks
1	Aroclor 1260	3.5 mg	15	0	60	
2	Phenoclor DP 6	3.5 mg	15	0	0	Most embryos died during first half of incubation period.
3	Clophen A 60	3.5 mg	20	1	5	Most embryos died during first half of incubation period.
4	Clophen A 60 3rd fraction of	35 mg	15	0	0	All embryos died during first four days of incubation perio
5	Clophen A 60 "	3.5 mg	15	0	7	Most embryos died during first half of incubation period.
6	Clophen A 60 "	0.35 mg	15	2	92	
7	Clophen A 60 "	0.035mg	15	2	100	
8	Ethanol		20	2	94	
9	Control		20	0	90	

[a]The samples, dissolved in 0.05 ml ethanol, were injected into the aircell of fresh eggs before incubation.

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Table 2. Retentions and Collected Mass Spectrometric Data of the Peaks in fig.1.

Peak no.	Relative retention[a]	Mass number(s)[b], and number of chlorine atoms/molecule	Identity of the compound(s).
1	1.40	304(4Cl)	tetrachlorodibenzofuran
2	1,59	332(6Cl)	hexachloronaphthalene
3	1.74	358(6Cl)	hexachlorobiphenyl
		392(7Cl)	heptachlorobiphenyl
4	2.42	338(5Cl)	pentachlorodibenzofuran
		392(7Cl)	heptachlorobiphenyl
5	3.48	366(7Cl)	heptachloronaphthalene

[a] Retention related to dieldrin = 1

[b] Mass number calculations based on Cl = 35

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