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

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Abstract—In a previous study (Vos & Koeman, *Toxic. appl. Pharmac.* 1970. In press) a significant difference in toxicity was found between three commercial polychlorinated biphenyl (PCB) preparations, high mortality, liver necrosis and chick oedema-like lesions being associated with only two of the compounds tested. In the present study, column and gas chromatography demonstrated the presence of polar compounds in the 25% diethyl ether fraction of each of these two PCB preparations, and a chick-embryo assay confirmed the high toxicity of this fraction. The polar compounds were not found in the corresponding fraction of the third PCB preparation; neither did this preparation have a significantly toxic effect on the chick embryo. Subsequent mass spectrometric and microcoulometric analyses indicated the identity of the polar compounds, which included tetra- and pentachlorodibenzofuran. The significance of these impurities is discussed. It is concluded that evaluation of animal tests on PCB is made extremely difficult by the presence of chlorinated dibenzofurans in some commercial PCB preparations.

INTRODUCTION

Polychlorinated biphenyls (PCB) have been identified in tissues of fish and wildlife (Jensen, 1966; Holmes, Simmons & Tatton, 1967; Holden & Marsden, 1967; Koeman, Oskamp, Veen, Brouwer, Rooth, Zwart, van de Broek & van Genderen, 1967; Risebrough, Rieche, Peakall, Herman & Kirven, 1968; Koeman, ten Noever de Brauw & de Vos, 1969; Jensen, Johnels, Olsson & Otterlind, 1969), and have 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, metal and concrete, and in many other applications. They are compatible with paints, synthetic resins, varnishes and waxes, and improve the chemical resistance, water resistance, adhesiveness, flexibility and other properties of these products (Monsanto Chemical Co. 1960; Hubbard, 1964). However, it has not yet been established which of the materials treated with PCB has contributed to the present environmental contamination.

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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'Dell, 1962; Flick, O'Dell & 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 of three commercial PCB preparations (Phenoclor DP6, Clophen A60, and Aroclor 1260) was found (Koeman *et al.* 1969). The occurrence of lesions resembling those of 'chick-oedema' in birds fed PCB (Koeman *et al.* 1969; McCune *et al.* 1962; Flick *et al.* 1965) prompted a recent comparative toxicity test of the three named PCB preparations in chickens (Vos & Koeman, 1970). In this study a significant difference in toxicity between the three compounds was found, Clophen A60 and Phenoclor DP6 showing the highest and Aroclor 1260 the lowest mortality. Subcutaneous and abdominal oedema and centrilobular liver necrosis was recorded only in those chicks fed Clophen or Phenoclor. Hydropericardium was recorded in nearly all chicks fed Clophen or Phenoclor but only occasionally in chicks fed 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 the compounds responsible for the differences in toxicity between the three commercial PCB preparations.

EXPERIMENTAL

Materials. The PCB preparations, which contained an average of 60% chlorine, were obtained from Prodelec S.A., France (Phenoclor DP6), Farbenfabriken Bayer AG, Leverkusen, Germany (Clophen A60; Lot no. 912434), and the Monsanto Chemical Co., St. Louis, USA (Aroclor 1260; Lot no. AK-3).

Fractionation of samples. The screening method used for the detection of the chick oedema factor (*Journal of the Association of Official Analytical Chemists*, 1968) was modified and used to isolate from the PCB preparations the compound(s) responsible for differences in toxicity. The clean-up step of this procedure was omitted. In the column chromatography, 8 g activated florisil (column diameter 10 mm) was used instead of 15 g activated alumina, and for fractionation redistilled hexane was used as the elution solvent instead of petroleum ether. PCB (2 mg) was dissolved in 2 ml hexane and fractionated on the florisil column, which was eluted with hexane until nearly all the PCB compounds were eluted (as demonstrated by gas chromatography). The second fraction (5% diethyl ether in hexane) was also tested until no more PCB could be detected. The final fraction (fraction 3), eluted with 25% diethyl ether (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 ft × 1/8 in.), 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 procedure was used to fractionate aliquots of about 550 mg of the PCB preparations in order to obtain sufficient quantities for a chick-embryo assay and for mass spectrometric analyses. The PCB was dissolved in 200 ml hexane. The column (diameter 32 mm) was filled with 180 g activated florisil. The more polar compounds were eluted in the final (third) fraction and, after evaporation, were tested by gas chromatography.

Toxicity study. A chick-embryo assay (Verrett, Marliac & McLaughlin, 1964) was used to determine the toxicity of the three PCB preparations and of the third fraction of Clophen.

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The three PCB preparations were dissolved and kept at 37°C in ethanol and in each case doses of 3·5 mg/egg in 0·05 ml ethanol were injected into the air cell of fresh White Leghorn eggs prior to incubation. The third fraction of Clophen was tested in the same way at four dose levels, the third fractions obtained from 35, 3·5, 0·35 and 0·035 mg Clophen being dissolved in 0·05 ml ethanol as before. The livers of all hatched chicks were removed for histological examination, fixed in 10% buffered formalin and processed in a conventional manner. In addition, cryostat sections were studied in a fluorescence microscope.

Analysis of third fractions. Mass spectrometry was used to identify compounds present in the polar third fractions. This analysis was carried out using a gas chromatograph–mass spectrometer combination. For this purpose a CH4 mass spectrometer (Varian MAT GmbH, Bremen) was coupled to a Varian Aerograph oven, type A550B, with a Pyrex glass column (5 ft × 1/8 in.) filled with 10% DC 200 on Gaschrom Q (80–100 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 SM_1 (Varian MAT). Exact mass measurements were made by the peak matching method and measurements were also made from spectra which were recorded on photographic plates processed by Varian MAT on a Leitz comparator SAM1.

To obtain quantitative information, microcoulometric analyses of the third fractions were carried out. The equipment used was a Dohrmann microcoulometer, coupled to a Microtek MT 220 gas chromatograph, with a glass column (6 ft × 1/4 in.). 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 measured quantitatively in a T-300-S titration cell.

RESULTS

The gas chromatograms of the third fractions obtained with the small column (diameter 10 mm) revealed the presence of polar compounds in Phenoclor and Clophen (Fig. 1), but

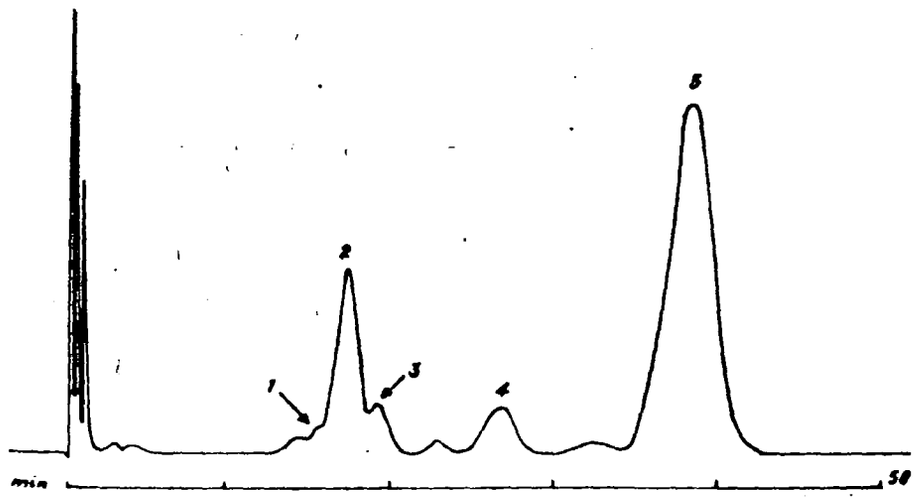

FIG. 1. Gas chromatogram of third fraction of Clophen A60 (showing pattern identical to that for third fraction of Phenoclor DP6). Data for peaks 1–5 are given in Table 2.

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not in the Aroclor extract. The same results were found with the third fractions obtained with the large column (diameter 32 mm). Fraction 3 from Clophen was subsequently used in the chick-embryo assay, which confirmed (Table 1) the differences in toxicity between the three PCB preparations found in the previous study (Vos & Koeman, 1970). The mortality in group 1 (Aroclor) did not differ significantly (chi-square test) from that in group 9 (control). The mortalities in groups 4 and 5 (the two highest doses of fraction 3 from

Table 1. *Chick-embryo assay of the chlorinated biphenyls, Aroclor 1260, Phenoclor DP6 and Clophen A60, and the 25% diethyl ether fraction (third fraction) of Clophen A60*

Test compound	Group no.	Dose* (mg/egg)	No. of eggs treated	No. of infertile eggs	Percentage hatch (% of fertile eggs)	Remarks
Aroclor 1260	1	3·5	15	0	80	
Phenoclor DP6	2	3·5	15	0	0	Most embryos died during first half of incubation period
Clophen A60	3	3·5	20	1	5	
Clophen A60 (third fraction)	4	35	15	0	0	All embryos died during first 4 days of incubation period
	5	3·5	15	0	7	Most embryos died during first half of incubation period
	6	0·35	15	2	92	
	7	0·035	15	2	100	
—	8	0	20	2	94	Ethanol control
—	9	—	20	0	90	Untreated control

*The doses, each dissolved in 0·05 ml ethanol, were injected into the air cell of fresh eggs before incubation.

Clophen) were dose related, with early mortality in the former group and scattered mortality in the latter. There was a close similarity between the mortality levels in groups 3 (3·5 mg Clophen) and 5 (third fraction from 3·5 mg Clophen).

Dissection of all fresh embryos and of the one-day-old chicks revealed no abnormalities except for one embryo in group 3 which showed a severe subcutaneous oedema. Oedema and beak deformities have been described after yolk-sac injection of 10 and 25 mg Aroclor 1242, resulting in, respectively, 95 and 100% embryonic mortality (McLaughlin, Marliac, Verrett, Mutchler & Fitzhugh, 1963). Liver sections stained with haematoxylin and eosin showed no morphological changes. In the cryostat section of the liver of the only weak chick that hatched in group 3, a red fluorescence in some lobules was seen in the ultraviolet microscope. This fluorescence is indicative of the disturbance of porphyrin biosynthesis found in the prior study (Vos & Koeman, 1970).

Mass spectrometric analyses revealed that identical chlorinated compounds were present in fraction 3 from Phenoclor and from Clophen but not in that from Aroclor. The data are shown in Table 2. The mass spectra of compounds with mass numbers 304 and 338, obtained from peaks 1 and 4 (Fig. 1), are given in Figs 2 and 3. From exact mass measurements it was found that the formulae of the molecule peaks 304 and 338 were $C_{12}H_4O^{35}Cl_4$ and $C_{12}H_3O^{35}Cl_5$. The formulae of the fragment ions 241 and 275 were $C_{11}H_4{}^{35}Cl_3$ and $C_{11}H_3{}^{35}Cl_4$. 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

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Table 2. *Retentions and collected mass spectrometric data of peaks shown in Fig. 1*

Peak no.	Relative retention*	Mass nos† and no. of chlorine atoms/mol	Identity of compounds
1	1·40	304 (4 Cl)	Tetrachlorodibenzofuran
2	1·58	332 (6 Cl)	Hexachloronaphthalene
3	1·74	358 (6 Cl)	Hexachlorobiphenyl
		392 (7 Cl)	Heptachlorobiphenyl
4	2·42	338 (5 Cl)	Pentachlorodibenzofuran
		392 (7 Cl)	Heptachlorobiphenyl
5	3·48	366 (7 Cl)	Heptachloronaphthalene

*Retention related to dieldrin = 1.
†Mass no. calculations based on Cl = 35.

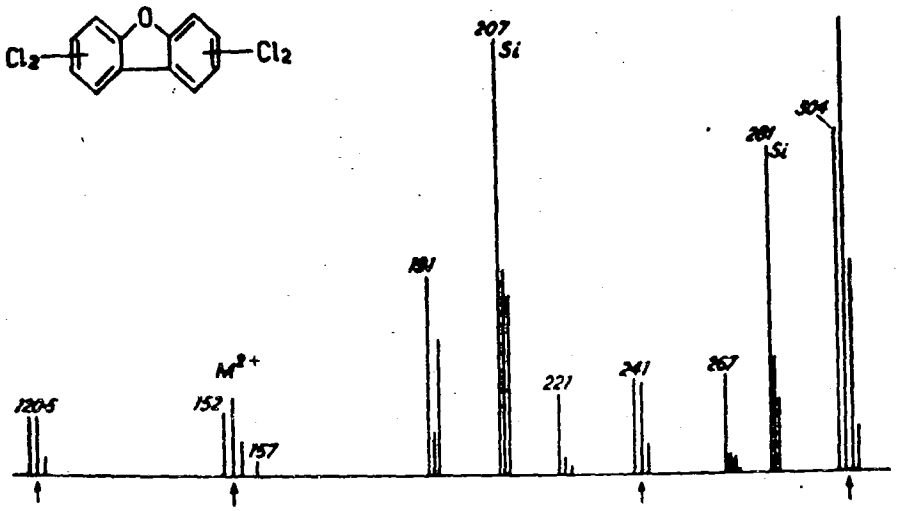

FIG. 2. Mass spectrum of tetrachlorodibenzofuran (mass no. 304) obtained from third fractions of Clophen A60 and Phenoclor DP6 (peak 1 in Fig. 1).

compound isolated from toxic fat (Wootton & 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 (J. C. Wootton, personal communication, 1968). The mass spectrum of this compound has a similar fragmentation pattern, i.e. the loss of two successive mass units of 63 suggests a loss of two ClCO units.

Microcoulometric analyses of the third fraction of Clophen and Phenoclor revealed chromatograms with the same peak pattern as in Fig. 1. The peak of the tetrachlorodibenzofuran (mass number 304) was too masked by the proximity of the hexachloronaphthalene peak (peak no. 2) to make quantitative calculations possible. For a similar reason peak no. 4 in the chromatogram, found to be a mixture of heptachlorobiphenyl and pentachlorodibenzofuran, could not be measured directly. However, assuming that the whole peak was produced by the dibenzofuran compound alone, a certain maximum level could be indicated. This level was found to be 5 ppm pentachlorodibenzofuran in Clophen and 20 ppm in Phenoclor. The minimum level could be indicated with the estimated lower limit of

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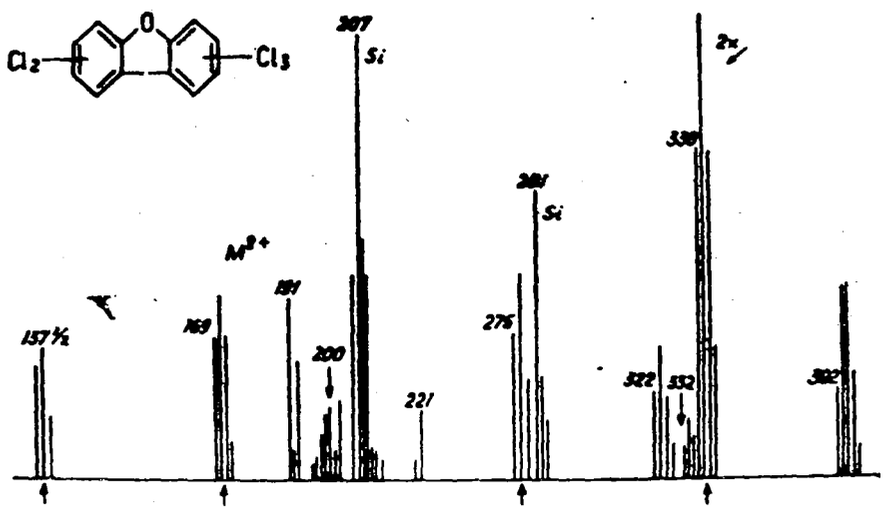

FIG. 3. Mass spectrum of pentachlorodibenzofuran (mass no. 338) obtained from third fractions of Clophen A60 and Phenoclor DP6 (peak 4 in Fig. 1). The spectrum of heptachlorobiphenyl (mol wt 392) is also present.

detection of the mass spectrometric equipment. Under the conditions used, this level was 1 ppm.

DISCUSSION

Mass spectrometric results have not proved conclusively the identity of the chlorinated dibenzofurans, but synthesis of these compounds, necessary for comparison with the mass spectra obtained experimentally, was found extremely dangerous. Tri- and tetrachlorodibenzofuran in a single oral dose of 0·5–1·0 mg/kg caused severe and often lethal liver necrosis in rabbits (Bauer, Schulz & Spiegelberg, 1961; Hofmann, 1958), and application to the ear of a rabbit resulted in chloracne (Bauer *et al.* 1961). The related compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which may appear in the synthesis of 2,4,5-trichlorophenol by hydrolysis of 1,2,4,5-tetrachlorobenzene (under pressure and in the presence of sodium hydroxide and methanol) (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. Application to the ear, again in a dose 10 times lower than that found to be effective in the case of chlorinated dibenzofuran, resulted in chloracne (Bauer *et al.* 1961; Linn Jones & Krizek, 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, Ress & Campbell, 1968), has been isolated and identified as one of the toxic components of the chick oedema factor (J. C. Wootton, personal communication, 1968; *Nutrition Reviews* 1968). The relationship between the toxic nature of PCB and the chick oedema factor has already been discussed (Flick *et al.* 1965).

Injection of 0·05 μg hexachlorodibenzo-*p*-dioxin into the air cell of chicken eggs resulted in 100% embryonic mortality (Higginbotham *et al.* 1968). Injection of the third fraction obtained from 35 mg Clophen into the air cell also resulted in 100% mortality (Table 1). It can be calculated that the maximum dose/egg is 0·2 μg pentachlorodibenzofuran (taking 5 ppm as the maximum value). This confirms the order of toxicity discussed above.

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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 a consideration of their manufacture, and particularly of the procedure for the distillation of crude PCB, in which sodium hydroxide can be used (Fay & Richards, 1947). PCB react with sodium hydroxide at elevated temperatures to yield phenolic compounds, and may, for example, be saponified to polychlorohydroxybiphenyls by sodium hydroxide in a polyhydric alcohol medium (Progil SA, 1957). A further loss of hydrochloric acid could produce dibenzofuran derivatives.

The similarity between the mortality levels in groups 3 and 5 (Table 1) provides a strong indication that the polar residue in the latter determines the toxicity of the commercial PCB. Chlorinated naphthalenes are far less toxic than chlorinated dibenzofurans (Hofmann, 1958) and the latter may therefore be assumed to determine the toxicity of fraction 3.

The occasional occurrence of hydropericardium in chicks fed Aroclor (Flick *et al.* 1965) may indicate the presence of a very small quantity of a toxic factor in this preparation.

From this study one may conclude that the evaluation of PCB residues in wild animals on the basis of effects and residues in test animals is extremely difficult, because of differences in toxicity between various commercial PCB preparations. Work is in progress to detect and identify chlorinated dibenzofuran in specimens of wildlife.

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Identification et évaluation toxicologique du dibenzofuranne chloré et du naphtalène chloré dans deux biphényles polychlorés commerciaux

Résumé—Une étude précédente (Vos et Koeman, *Toxic. appl. Pharmac.* 1970. Sous presse) a établi des différences significatives entre les toxicités de trois préparations commerciales de biphényle polychloré (BPC); une mortalité élevée, une nécrose du foie et des lésions ressemblant à l'oedème du poussin n'étant observées seulement qu'avec deux des produits examinés. Les recherches décrites ici ont démontré par chromatographie sur colonne et en phase gazeuse la présence de composés polaires dans la fraction à 25% d'éther diéthylique de chacun de ces deux produits et un essai sur embryon de poulet a confirmé la haute toxicité de cette fraction. Ces composés polaires n'ont pas été rencontrés dans la fraction correspondante du troisième produit et ce produit n'a pas, non plus, exercé d'effet toxique significatif sur l'embryon de poulet. Les analyses ultérieures (spectrométrie de masse et microcoulométrie) ont établi l'identité des composés polaires, qui comprennent du tétra-et du pentachlorodibenzofuranne. La signification de ces impuretés est discutée. L'article conclut que l'évaluation toxicologique du BPC par des essais sur des animaux est rendue extrêmement difficile par la présence de dibenzofurannes chlorés dans certaines préparations commerciales de BPC.

Identifizierung und toxikologische Untersuchung von chloriertem Dibenzofuran und chloriertem Naphtalin in zwei polychlorierten Biphenylen

Zusammenfassung—Bei einer vorangehenden Untersuchung (Vos und Koeman, *Toxic. appl. Pharmac.* 1970. Im Druck) wurde ein wesentlicher Toxizitätsunterschied zwischen drei PCB-Präparaten des Handels festgestellt, da hohe Mortalität, Lebernekrose und hühnerödemähnliche Läsionen bei nur zwei der untersuchten Verbindungen auftraten. Bei der jetzigen Untersuchung zeigten Flüssigkeits- und Gaschromatographie die Anwesenheit polarer Verbindungen in der 25%-Diäthyläther fraktion jedes dieser beiden PCB-Präparate an, und ein Hühnerembryonenversuch bestätigte die hohe Toxizität dieser Fraktion. Die polaren Verbindungen wurden in der entsprechenden Fraktion des dritten PCB-Präparats nicht gefunden, und dieses Präparat hätte

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auch keine wesentliche toxische Wirkung auf den Hühnerembryo. Anschliessende massenspektrometrische und mikrocoulometrische Analysen gaben über die Identität der polaren Verbindungen Aufschluss, zu denen Tetra- und Pentachlordibenzofuran gehörten. Die Bedeutung dieser Verunreinigungen wird besprochen. Es wird der Schluss gezogen, dass die Auswertung von Tierversuchen mit PCB durch die Anwesenheit von chlorierten Dibenzofuranen in einigen kommerziellen PCB-Präparaten äusserst erschwert wird.

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