59277

Ref 0202

TOXICOLOGY AND APPLIED PHARMACOLOGY 36, 65–80 (1976)

Toxicological Assessment of Hexachlorobiphenyl Isomers and 2,3,7,8-Tetrachlorodibenzofuran in Chicks

I. Relationship of Chemical Parameters

J. D. McKinney, K. Chae, B. N. Gupta, J. A. Moore and J. A. Goldstein[1]

National Institute of Environmental Health Sciences, Environmental Biology and Chemistry Branch, Research Triangle Park, North Carolina 27709

Received July, 14 1975; accepted November 6, 1975

Toxicological Assessment of Hexachlorobiphenyl Isomers and 2,3,7,8-Tetrachlorodibenzofuran in Chicks. I. Relationship of Chemical Parameters. McKinney, J. D., Chae, K., Gupta, B. N., Moore, J. A. and Goldstein, J. A. (1976). *Toxicol. Appl. Pharmacol.* 36, 65–80. Five hexachlorobiphenyl (HCB) isomers and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were fed to chicks for 21 days. Liver weights increased, with the smallest increase produced by 2,3,6,2′,3′,6′-HCB and the largest by 2,4,6,2′,4′,6′-HCB. Weight gain was decreased by 2,3,4,2′,3′,4′-, 2,3,6,2′,3′,6′-, and 2,4,5,2′,4′,5′-HCB, while 1μg/kg of TCDF did not affect body weight or liver weight. Pathological changes in the liver were greatest in the 2,4,6,2′,4′,6′-HCB group, moderate in the 2,3,4,2′,3′,4′- and 2,3,6,2′,3′,6′-HCB groups, and mildest in the 3,4,5,3′,4′,5′-, 2,4,5,2′,4′,5′-HCB, and 5μg/kg TCDF groups, with no significant effect for the 1μg/kg TCDF group. Thymic involution and edema were observed with 3,4,5,3′,4′,5′ HCB and TCDF. 3,4,5,3′,4′,5′-HCB and 5μg/kg of TCDF were lethal. HCB retention indices from gas chromatography correlated well with adipose-tissue concentration, with the exception of the 2,4,6,2′,4′,6′-isomer, which had the smallest retention index but relatively high tissue accumulation. Extraction *p*-values correlated poorly, but the 2,3,6,2′,3′,6′-HCB gave the smallest *p*-value, whereas the 2,4,6,2′,4′,6′-HCB *p*-value was next to the highest. The HCB retention indices generally correlated with their overall biological response and were highest in isomers with 3,4-substitution.

The commercial formulations of polychlorinated biphenyls (PCBs) encompass a very complex heterogeneous series of chemical compounds in which 210 isomers and congeners are theoretically possible. A great deal of work has been done in the past few years on the chemistry (Hutzinger *et al.*, 1974) and biological effects (Vos, 1972) of the PCBs and related materials, revealing untoward biological effects and wide distribution in the environment. Even though PCBs are a very inert and stable class of compounds, the lower chlorinated homologs are less abundant in biological and environmental samples (Bailey and Bunyan, 1972; Grant *et al.*, 1971), indicating their more rapid metabolism and degradation to more polar compounds (Schulte and Acker, 1974). Therefore, the mixtures formulated commercially, which man may inadvertently

[1] Environmental Protection Agency, National Environmental Research Center, Research Triangle Park, North Carolina 27711.

Copyright © 1976 by Academic Press, Inc.
All rights of reproduction in any form reserved.
Printed in Great Britain

9203

consume, do not represent the mixtures present in biological samples. Although realistic, the data obtained from mixtures do not lend to unequivocal interpretation. Recent studies (Hansell and Ecobichon, 1974; Street *et al.*, 1972) have dealt with the biological effects of pure isomers and homologs instead of the commercial PCB mixtures. Biological studies have been undertaken in our laboratories utilizing individually a series of purified symmetrical hexachlorobiphenyl (HCB) isomers. Although some of the compounds synthesized are not likely to be present in commercial mixtures, they are representative hexachloro isomers and enable a systematic study of both chemical and biological properties of PCBs of higher chlorine content with varying substitution.

The chick was used in these initial studies since we felt that it might be a sensitive indicator (Vos and Koeman, 1970) of relative biological activity for individual HCBs via development of hydropericardium (chick edema disease), and permit rapid screening of ineffective isomers prior to initiating studies with mammalian species. The dose was set at 400 ppm in the diet since this dose of some PCB mixtures produces a variety of toxic signs in chicks (Vos and Keoman, 1970), and limited quantities of the HCBs did not permit use of more than one dose level, with the exception of the 2,4,5,2′,4′,5′-HCB. 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was included for comparative measurements, since evidence has been presented that some of the toxic effects of PCB preparations may be due to chlorodibenzofuran impurities (Vos *et al.*, 1970).

METHODS

Synthesis, Chemical Purity Analysis, and GC Retention Index Determinations

The 2,4,6,2′,4′,6′- and 2,3,4,2′,3′,4′-HCBs were obtained from Analabs, Inc., and the rest were synthesized in our laboratory according to the usual procedures adapted from the literature (Tas and Kleipool, 1972; Tas and de Vos, 1971; Van Roosmalen, 1934). 2,4,5,2′,4′,5′-HCB and 2,3,6,2′,3′,6′-HCB were obtained by coupling of the appropriate trichloroiodobenzenes with activated copper. Electron capture-gas chromatography failed to detect (minimum detectability, less than 10 pg) any 2,3,7,8-TCDF as a critical by-product in our Ullman synthesis of 2,4,5,2′,4′,5′-HCB (Moron *et al.*, 1973). 2,4,5-Trichloroiodobenzene was obtained by diazotization of the commercially available trichloroaniline. 2,3,6-Trichloroiodobenzene was obtained by diazotization of the trichloroaniline which was uniquely prepared (Shioiri *et al.*, 1972) from purified commercially available 2,3,6-trichlorobenzoic acid. 3,4,5,3′,4′,5′-HCB was synthesized by chlorination of benzidine ,which is known to afford hepta (~30%) and penta (~4%) contaminants as ultimate products of diazotization and chlorine substitution (Ayres, 1972). Pure 3,4,5,3′,4′,5′-HCB (>99%) was later synthesized via the Ullman synthesis from 3,4,5-trichloroiodobenzene and activated copper dust. The appropriate iodobenzene was prepared from 3,4,5-trichloroaniline via diazotization and treatment with potassium iodide. The aniline had been prepared from commercially available 2,6-dichloro-*p*-nitroaniline according to a published procedure (Johary *et al.*, 1952). Melting points were in agreement with those previously reported.

2,3,7,8-TCDF was synthesized (Kende *et al.*, 1974) in other laboratories. The major contaminant was the pentachloro homolog.

All gas chromatography (GC) was done on a Varian Aerograph Series 2100 instrument using a flame ionization detector. A 6 ft × 1/4 in o.d. × 2 mm i.d. glass column containing the mixed phase (Henly *et al.*, 1966) 11% (QF-1 + OV-17) on 80/100 mesh gas chrom Q was used. The column temperature was maintained at 210°C, the detector at 225°C, and the injector at 220°C. The carrier gas was nitrogen at a rate of 60 ml/min. The reported retention indices on this column are the fractional carbon numbers (Rohrschneider, 1966) based on the C-24, C-26, C-28, and C-30 homologous *n*-alkanes. Retention parameters and peak areas were determined by an Autolab System IV computing integrator.

Extraction p-Value Determinations

p-Values were determined for all the HCBs by partitioning their hexane solutions (1.0 μg/ml) with an equal volume (0.5 ml) of acetonitrile, and represent the ratio of HCB remaining in hexane after partitioning divided by the starting amount. The concentrations were measured with a differential uv detector set at 254 nm with a Waters Associates ALC 202 liquid chromatograph equipped with a Porasil A-60 column operated at approx 23°C, with 5% methylene chloride as solvent and a 1.5 to 3.5 ml/min flow rate.

Nuclear Magnetic Resonance (NMR) and Mass Spectra (MS) Analysis

The NMR studies were done with a Varian Fourier Transform XL-100 spectrometer. The ^{13}C natural abundance spectra required averages of 500 to about 10,000 transients and concentrations of samples as previously reported (Wilson and Anderson, 1974; Wilson, 1975). Mass spectra were obtained previously (Oswald *et al.*, 1974) using a Finnigan 1015C quadrupole electron impact spectrometer interfaced to a Finnigan Model 9500 gas chromatograph and all-glass transfer interface line with single-stage glass jet separator. The spectrometer was controlled by a System 150 data collection system.

Experimental Animals

The diet, specially formulated for chick edema bioassay, was obtained from Nutritional Biochemicals, Cleveland, Ohio. One-day-old White Leghorn cockerels were obtained from Central Carolina Farmers, Durham, N.C. A premix was prepared by dissolving the HCBs in acetone and mixing with 200 g of diet. After evaporation of the acetone in a hood, the premix was thoroughly mixed with diet at a concentration of 400 ppm HCB (also 100 ppm for 2,4,5,2′,4′,5′-HCB). Diet without HCB supplementation was used for controls. TCDF was dissolved in a minimum amount of acetone and diluted with corn oil (1:7). TCDF (1 and 5 μg/kg) was administered daily by gastric intubation in 0.1 ml. Control birds received an equal volume of acetone–corn oil. The chicks for the TCDF studies received unsupplemented diets.

Chicks were housed in a temperature controlled room and kept in wire-floored cages. Room temperature was maintained at 25°C. The temperature in the cages was about 30°C initially as a result of local heating in the back of each cage. The temperature was lowered at the beginning of Week 3 by disconnecting the heating circuit. Food and tap water were provided *ad libitum* for a 3-week period. Excrement was collected in the bottom of the cages over the entire 20- to 21-day period, air-dried, and saved for future

TABLE 1

MORTALITY AND EFFECTS ON ORGAN WEIGHTS AND RELATIONSHIP OF TISSUE HEXACHLOROBIPHENYL CONCENTRATIONS TO GAS CHROMATOGRAPHIC RETENTION INDICES

Compound (400 ppm in diet)	Number of birds determined	Mortality %	Mortality Mean time to death (days)	Terminal body weights (g)[a]	Organ weight ratios (× 100)[a] Liver/body	Organ weight ratios (× 100)[a] Spleen/Body	GC retention index value	Conc. (ppm)[b] Adipose tissue	Conc. (ppm)[b] Liver
Control (diet)	9	0	—	193.1 ± 7.3	3.79 ± 0.13	0.129 ± 0.010	—	—	—
2,4,6,2′,4′,6′-HCB	10	0	—	175.8 ± 9.0	6.90 ± 0.22[c]	0.146 ± 0.009	2346	4172	24
2,3,6,2′,3′,6′-HCB	9	10	17[d]	159.0 ± 9.0[c]	5.05 ± 0.19[c]	0.142 ± 0.008	2478	2893	9
2,4,5,2′,4′,5′-HCB	9	10	3[d]	150.0 ± 9.0[c]	6.33 ± 0.19[c]	0.109 ± 0.006[e]	2543	3921	59
2,3,4,2′,3′,4′-HCB	8	0	—	166.5 ± 5.0[c]	6.63 ± 0.13[c]	0.135 ± 0.013	2678	3998	44
3,4,5,3′,4′,5′-HCB	—	100	11	—	—	—	2820	4609	203
Control (Corn oil and diet)	3	0	—	183.3 ± 6.4	4.16 ± 0.17	0.168 ± 0.016[c]	—	—	—
2,3,7,8-TCDF (1 μg/kg)	6	16	19[d]	177.8 ± 4.8	4.46 ± 0.31	0.066 ± 0.010	2620	—[f]	—[f]
2,3,7,8-TCDF (5 μg/kg)	—	100	11.5	—	—	—			

[a] Values represent means ± SE for combined weights from chicks killed on Days 20 and 21, for all treatment groups except TCDF and its control which are for birds killed on Day 21 only.

[b] GC determinations based on weight of wet tissue or feed. Pooled tissue samples from all chicks sacrificed or for 3,4,5,3′,4′,5′-HCB that died or were moribund. For 100 and 300 ppm pure 3,4,5,3′,4′,5′-HCB, the adipose tissue concentrations were 1210 and 4912, respectively.

[c] Significantly ($p < 0.05$) different from controls.

[d] These values are the day of death for one chick.

[e] Significantly ($p < 0.10$) different from controls.

[f] Not determined.

examination of HCB and metabolite content. The chicks were observed daily and food consumption was recorded weekly. Average body weights were recorded twice a week.

A later study compared 100 and 300 ppm of both the impure (66%) and pure (>99%) preparations of the 3,4,5,3′4′,5′-HCB along with a control group (10 chicks per group) under identical experimental conditions.

Necropsy Examination

All surviving chicks were killed with CO_2 inhalation on Days 20 and 21. Body weight and weights of liver and spleen were recorded (Table 1).

Tissue samples from liver, spleen, kidney, lung, heart, thymus, lymph nodes, adrenal glands, testes, skin, esophagus, crop, proventriculus, gizzard, duodenum, ileum, cecum, pancreas, and striated muscles from hind legs were collected from most of the chickens and fixed in buffered neutral 10% formalin for histopathologic evaluation. All tissue specimens were paraffin-embedded, sectioned 6-μm thick, and stained with Harris' hematoxylin and eosin Y (H & E).

Blood Examination

Prior to sacrifice, blood was collected from the brachial vein for packed cell volume determination. Cellulose acetate electrophoresis (Microzone electrophoresis system, Beckman) was used for serum protein fractionation, and total serum protein was determined by biuret method (Gornall *et al.*, 1949).

Tissue HCB Residue Analysis

Subcutaneous adipose tissue was removed near the thoracic inlet and pooled for each treatment group. Adipose tissue and liver were homogenized with two 25-ml portions of benzene, and were centrifuged. Benzene was substituted for hexane normally used (Grant *et al.*, 1971), since some of the HCBs were only partially soluble in hexane. The combined extracts were concentrated, and 0.2 g of the extractable lipids was redissolved in 3 ml of benzene. To this benzene solution was added 1 ml of concd. H_2SO_4, and the resulting mixture was shaken for 5 min. The aqueous acid layer was extracted twice with two 2-ml portions of benzene. The combined benzene extracts were neutralized, dried with 2 g of anhydrous sodium sulfate–sodium carbonate (3:1), and analyzed by GC, as described above. Diet concentrations were also analyzed. Pesticide grade solvents were used throughout these studies.

Statistical Methods

To achieve an overall experimental error rate of 0.05 (or in one instance, 0.10), among group differences were examined using the Kruskal–Wallis test. If significant differences were found, then pairwise comparisons were made by two-sided Mann–Whitney *U* tests (Hollander and Wolfe, 1973).

RESULTS

GC and Spectral Analyses

The individual HCBs had been previously characterized by mass spectrometry and nuclear magnetic resonance spectroscopy to confirm the number and positions of the chlorine atoms. NMR was chosen in preference to other spectroscopic methods as it is more definitive.

Retention indices (RIs, *n*-paraffin fractional carbon number × 100) of all HCBs and TCDF are given in Table 1. The RI of the 2,3,4,5,3′,4′,5′-heptachloro contaminant which constituted 30% of the 3,4,5,3′,4′,5′-HCB mixture was 2880. These RIs were obtained on a mixed silicone GC phase [11% (OV-17 + QF-1), QF-1/OV-17 ratio = 1.30] known (Henly *et al.*, 1966) to separate a wide range of closely related chlorinated hydrocarbon pesticides. The HCBs were clearly separable on this phase as indicated by the RI values. In addition, the elution order for these HCBs has been observed to be the same on all columns tested (OV-17 + QF-1, OV-17, 210, 101, and K-350 and CHDMS), confirming the value of GC in determining relative polarizability in this isomeric series.

The concentrations of each HCB in adipose tissue and liver are shown in Table 1. There were clearly differences in tissue PCB concentrations in chicks fed identical dietary levels of isomers with different chlorine patterns. The results of analyses of diet concentrations were in good agreement with those formulated and confirmed the doses given and the efficiency of the extraction method in removing these HCBs from fatty and proteinaceous material. No attempt was made to quantitate TCDF since the total amount and the number of birds to which it was administered were small.

Toxicity Indicators

Hexachlorinated biphenyl isomers. 3,4,5,3′,4′,5′-HCB was the most toxic isomer studied causing death in all birds at an average of 11 days. Average food intake of these birds was always lower than that of controls. Death was preceded by a 1- to 2-day period of unthrifty appearance and apparent depression as denoted by closed eyelids, lack of activity or response to sudden audible stimulus. At time of necropsy, these birds evidenced fecal soiling of the cloacal area, subcutaneous edema, ascites, hydropericardium, and loss of subcutaneous and visceral adipose tissue. Marked involution of the thymus was also observed. Microscopically, the lymphoid elements in thymus and spleen were greatly depleted. Congestion, mild necrosis, and marked fatty infiltration were observed in the liver. The mucosal surface of small and large intestines was congested.

Except for the 3,4,5,3′,4′,5′-HCB group mentioned above, the average food consumption of all other HCB groups approximated that of controls throughout the study. Significant reduction in weight gain occurred during Weeks 2 and 3 of the study in all HCB groups, except the 2,4,6,2′,4,′6′-HCB where reduced weight gain occurred only during the 14- to 21-day period. At sacrifice, body weight was significantly reduced ($P < 0.05$) in the 2,4,5,2′,4′,5′; 2,3,6,2′,3′,6′; and 2,3,4,2′,3′,4′ groups, but not the 2,4,6,2′,4′,6′-group (Table 1). Liver enlargement was observed in all groups, but was least for 2,3,6,2′,3′,6′-HCB and greatest for 2,4,6,2′,4′,6′-HCB. The effects of 2,4,6,2′,4′,6′-, 2,3,4,2′,3′,4′-, and 2,4,5,2′,4′,5′-HCB were significantly greater than 2,3,6,2′,3′,6′-HCB. Spleen weights were significantly decreased ($P < 0.10$) by 2,4,5,2′,4′,5′-HCB. Atrophy of the spleen has been previously observed in chicks fed PCB mixtures (Vos and Koeman, 1970).

A single bird in the 2,3,6,2′,3′,6′-HCB group died on Day 17 of the study. The pathological changes in the liver of the chicks in this group was characterized by moderate-to-marked fatty infiltration, focal areas of coagulative necrosis, and giant cell formation due to apparent coalescing of hepatocytes. Occasionally, edema in cardiac muscles was also observed.

At necropsy, large black-colored spots ranging from 1 to 5 mm in diameter were observed under the capsule and on the cut surface of the liver of those birds that received 2,4,6,2′,4′,6′-HCB. Microscopically, these areas represented foci where sinusoids were markedly dilated and contained blood (Fig. 1). Focal areas of coagulative necrosis and hemorrhage were also observed (Fig. 2). In some instances, several hepatocytes appeared to coalesce, forming multinucleated giant cells. These foci of

FIG. 1. Liver of chicken receiving food contaminated with 400 ppm of 2,4,6,2′,4′,6′-HCB showing an area with marked dilatation of sinusoids containing blood. H and E stain; ×150.

dilated sinusoids had no relation to any particular zone within the liver lobules. Marked fatty infiltration and hyalinization of hepatocytes was present throughout the liver. Occasional areas of congestion and hemorrhage in the heart and thymus were also observed. Slight thymic changes as manifested by loss of lymphoid elements were also seen.

Chickens treated with 2,3,4,2′,3′,4′-HCB had moderate degenerative liver changes such as hyalinization of the cytoplasm of hepatocytes, single-cell necrosis of hepatocytes, and occasional giant cell formation. In some cases, the endocardium was markedly edematous. Some loss of thymic lymphoid cells had occurred.

The packed cell volume did not significantly vary from those of the controls. Total serum protein values were extremely variable within groups and also somewhat variable between groups. The mean albumin content of serum was found to be markedly reduced in those birds that were fed 3,4,5,3′,4′,5′-HCB.

The death of one bird on Day 3, in the group fed 2,4,5,2′,4′,5′-HCB, was not felt to be compound-related. Slight-to-moderate fatty infiltration and degenerative liver changes were present in the chicks of this group; single-cell necrosis of hepatocytes and occasional hemorrhagic foci also occurred.

FIG. 2. Photomicrograph of liver of a chicken receiving food contaminated with 400 ppm of 2,4,6,2′,4′,6′-HCB. Note the necrosis of cord cells and hemorrhage in the liver. H and E stain; ×150.

In the later study comparing 100 and 300 ppm of both impure (66%) and pure (>99%) 3,4,5,3′,4′,5′-HCB, no significant differences between the impure and pure preparations at the 100- and 300-ppm dose levels were observed as all birds died at an average of 13.3 days. In addition to the presence of edema and the effects on weight gain, food consumption, liver, thymus, and spleen previously described, consistent histopathologic changes were observed in gizzard and bursa. Microscopic changes in the gizzard were characterized by marked degeneration, necrosis, fibrinoid and cystic changes, purulent exudation, and the presence of numerous bacterial colonies in the mucosal layer. Necrosis, cystic changes filled with mucus, and proliferation of reticulo-endothelial cells in the lymphoid follicles of the bursa were also observed in most chicks. Porphyrin accumulation was indicated as red fluorescence for gizzard, flat and long bones (sternum, femur, and skull), liver, and ceca. The fluorescence was most readily visualized in the mucosal layer of gizzard and bones in the early stages of the disease.

2,3,7,8-Tetrachlorodibenzofuran. Birds that received 5μg/kg/day of TCDF died at an average of 11.5 days. General unthriftiness and apparent depression preceded death. Food consumption in the 5μg/kg group was reduced during the 7- to 14-day period. One of six chicks in the 1μg/kg/day TCDF group died on Day 19. Body weight gain and food consumption were also decreased in the 1μg/kg groups during the 14- to 21-day observation period. There was no significant effect on liver weight, and mild liver

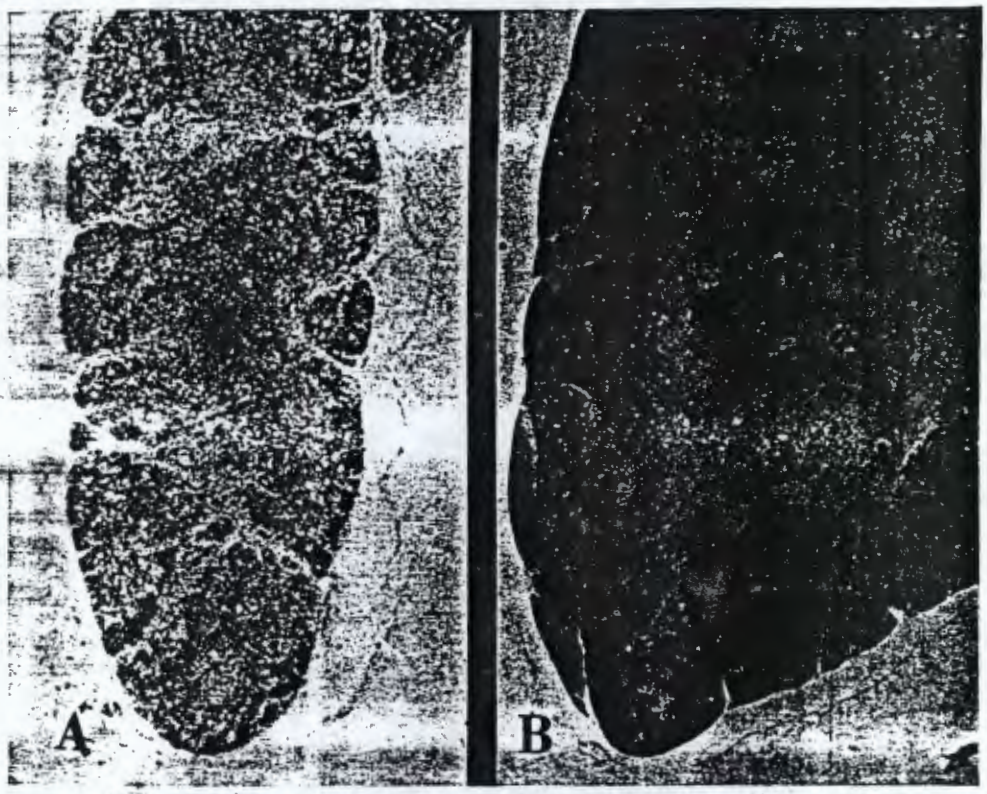

FIG. 3. (A) Thymus of a chicken dosed daily with 5μg/kg of TCDF. Notice the marked depletion of thymocytes and loss of demarcation between the cortex and medulla, and compare with the normal control thymus (B). H and E stain; ×150.

pathological changes only occurred with the high dose. The most striking gross pathologic change observed was the accumulation of clear fluid as evidenced by marked subcutaneous edema, ascites, and hydropericardium, with the severity of fluid accumulation greater at the 5μg/kg dose. The hearts of these birds also appeared enlarged and flabby (rounded). The thymus was markedly involuted. Histologically, there was marked depletion of lymphocytic cell types in the spleen and thymus (Fig. 3). A significant reduction in spleen weight was found for the 1μg/kg TCDF group. The blood vessels in the thymus also appeared dilated, with the surrounding connective tissue appearing edematous. Acute-to-subacute inflammatory changes were also observed on the pericardial surface of the heart which were characterized by the presence of regenerating

fibroblasts, lymphocytes, and heterophils (Fig. 4). The endocardium was also edematous in a few cases. Inflammatory exudate in the lung, edema, congestion, and hemorrhage in the gastrointestinal tract were also observed. Total serum protein values were reduced in the TCDF group, and there was a marked reduction in albumin content of the serum.

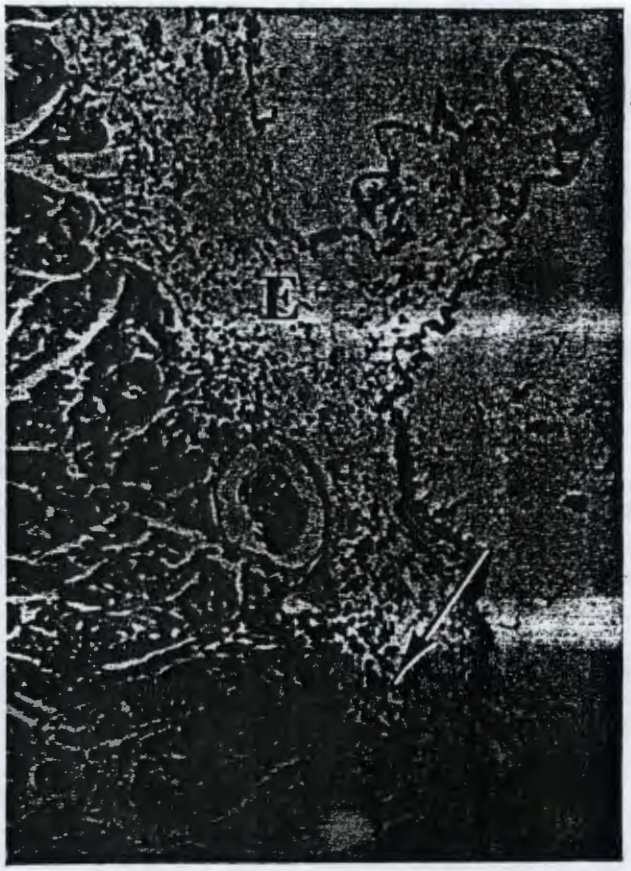

FIG. 4. Photomicrograph of the heart of a chicken dosed daily with 1 μg/kg of TCDF showing the edema (E) and infiltration of lymphocytes and heterophils (arrow) on the pericardial surface. H and E stain; ×150.

A summary of the major pathologic changes in the chicken given different HCB isomers and TCDF is found in Table 2. Specific effects of these compounds on parameters of hepatic drug metabolism and porphyrin accumulation in chicks are described in a separate report (Goldstein *et al.*, 1976). Metabolism of at least two of the HCBs (2,4,5,2′,4′,5′- and 2,4,6,2′,4′,6′-) does occur in the chick to some extent, since metabolites have been identified by gas chromatography–mass spectral analysis of extracts of group pooled excreta (unpublished data).

TABLE 2

SUMMARY OF MAJOR PATHOLOGIC CHANGES IN THE CHICKEN GIVEN DIFFERENT ISOMERS OF HCB OR TCDF

Compound	Thymic involution	Major pathologic changes		
		Spleen	Liver	Edema
2,4,5,2′,4′,5′-HCB	Slight	None	Moderate; single-cell necrosis	No
2,3,6,2′,3′,6′-HCB	Slight	None	Moderate; fatty infiltration necrotic foci, giant cell formation	No
2,4,6,2′,4′,6′-HCB	Slight	None	Marked; black spots grossly due to dilatation of sinusoids containing blood, marked fatty infiltration, necrosis, and giant cell formation	No
2,3,4,2′,3′,4′-HCB	Slight	None	Moderate; hyalinization and single-cell necrosis of hepatocytes	Endocardium
3,4,5,3′,4′,5′-HCB	Marked	Depletion of lymphocytes	Mild; fatty infiltration	Subcutaneous, ascites, hydropericardium
2,3,7,8-TCDF (1 μg/kg)	Marked	Depletion of lymphocytes	Not significant	Marked subcutaneous edema, ascites, hydropericardium
2,3,7,8-TCDF (5 μg/kg)	Marked	Depletion of lymphocytes	Mild; passive congestion	Similar to above but more pronounced

DISCUSSION

Toxicity as measured by mortality is highest for the 3,4,5,3′,4′,5′-HCB and the high-dose (5μg/kg) TCDF groups (Table 1). The toxicity of TCDF reflects a much more specific mechanism of action than for PCBs and is not explicable on the basis of lipophilicity alone. Edema has been reported (Vos, 1972) to be minimal with 400 ppm of a 60% chlorinated Aroclor, but chick edema was noted at low feeding levels for lower chlorinated Aroclors. However, these mixtures were contaminated with low levels of dibenzofurans. We find that both 3,4,5,3′,4′,5′-HCB and TCDF produce general edema. Hydropercardium was not a sensitive indicator for relative toxicity determination, possibly due to difficulties in quantitating the effect. Clearly, 3,4,5,3′,4′,5′-HCB produces edema in the absence of dibenzofuran contamination. However, it is possible that a dibenzofuran or prefuran could have been formed as a metabolite, albeit in low yields. Since there is essentially no *ortho* effect in the 3,4,5,3′,4′,5′-HCB, coplanarity of the two rings may contribute to transannular participation of vacant *ortho* positions in metabolism. Only 3,4,5,3′,4′,5′-HCB and TCDF produced appreciable thymic involution, while neither produced appreciable pathological changes in the liver (Table 2). In contrast, pathological changes in the liver were marked for 2,4,6,2′,4′,6′-HCB and moderate for 2,3,4,2′,3′,4′-HCB. The hepta-contaminant does not account for the higher toxicity and edema seen with the impure 3,4,5,3′,4′,5′-HCB, since essentially identical results were seen with 100 and 300 ppm of pure 3,4,5,3′,4′,5′-HCB subsequently synthesized in our laboratory by an alternate method to eliminate the hepta-contaminant. In addition, TCDF did not produce porphyria, and the effects on hepatic drug-metabolizing enzymes were quantitatively less and qualitatively different from those of HCBs (Goldstein *et al.*, 1976). These results suggest that effects on thymus, liver pathological changes and liver weight, edema, and liver enzymes may permit differentiation between HCB and 2,3,7,8-TCDF by bioassay.

We compared the biological activity of the five HCBs on several biological parameters (Tables 1 and 2): decreases in growth rate, increases in liver to body weight ratios, pathological changes in the liver and mortality. The low mortality in the 2,4,5,2′,4′,5′- and 2,3,6,2′,3′,6′-HCB groups does not appear to be related to compound treatment and was not considered in this comparison. From these data, the increasing order is 2,3,6,2′,3′,6′ $\ll$ 2,4,5,2′,4′,5′; 2,3,4,2′,3′,4′ $\leqslant$ 2,4,6,2′,4′,6′ $\ll$ 3,4,5,3′,4′,5′.

In explaining these differences (both qualitative and quantitative), it is important to consider compound purity, lipophilicity, and metabolism. Since all were of high purity as confirmed by chromatographic and spectral analyses, the differences found can be related directly to the varying substitution pattern as the degree of chlorination is constant. Preliminary studies of metabolism (unpublished data) have indicated the importance of both C-4 and C-2 (or C-6) substitution with a vacant C-3 (or C-5) position in affording detectable amounts of metabolites. Since the 2,3,6,2′,3′,6′-HCB showed relatively little, if any, metabolism and the lowest tissue accumulation, metabolism alone does not appear to account for the low accumulation of this HCB; however, metabolism may have affected the liver concentrations of the 2,4,5,2′,4′,5′- and 2,4,6,2′,4′,6′-isomers.

Lipophilicity as used in this context is meant to include hydrophobic effects of hydrocarbons and induction of those effects due to factors inherent in both the solute (HCB)

and solvent (adipose tissue) structures. Lipophilicity may relate directly to the potential for molecular aggregation (McKinney, 1974) which is a function of molecular polarity. Adipose tissue accumulation may reflect lipophilicity. Increases in liver to body weight ratios, cytochrome P-450, and drug metabolizing enzymes (Goldstein *et al.*, 1976) correlated reasonably well with increasing adipose concentrations of HCB. Possibly, lipophilicity is a factor in biological effectiveness. Earlier work (Albro and Fishbein, 1972b) has indicated that appreciable differences in intestinal absorption do not occur for PCBs and are not likely to account for the differences seen in tissue accumulation.

The NMR data previously obtained (Wilson, 1975) clearly indicate that proton magnetic resonance (PMR) and carbon magnetic resonance (CMR) are useful adjuncts to other techniques for structure elucidation in this series of related isomers and congeners. The observation of trends in NMR parameters suggests the importance of variations in σ charge distribution in determining the effects of varying chlorine substitution in biphenyls. Chlorine effects can be transmitted through several covalent bonds, and in the case of a C-4 substituent, the effect can be maximally transmitted through eight covalent bonds (to C-4′). A C-4 substituent effect also appears to be symmetrically distributed to the *ortho* positions as well. Such transmissions would impart varying patterns and degrees of polarizability to HCBs. Polarity as used in this context can be defined as the summation of the individual group dipoles resulting from varying chlorine patterns. The effect of chlorine at C-4 appears to be more a matter of degree, whereas at C-3 it may be more a matter of the number of affected group dipoles (Wilson. 1975). From these data, one would expect the HCBs with both C-4 and C-3 (or C-5) substitution to have greater relative polarity.

Retention indices (Table 1) on the mixed liquid phase used for gas chromatography were considered as an index of the net relative polarizability (summation of individual group dipoles both permanent and inducible) since other factors such as Van der Waal's forces are apparently nearly equal in this isomeric series. High RIs were generally associated with 3,4-substitution, as predicted from NMR data. Except for 2,4,6,2′,4′,6′-HCB, increasing indices for this isomeric series correlated exactly with increasing adipose tissue accumulation regardless of what the basis for differences in RIs may be. The 2,4,6,2′,4′,6′-isomer has a pronounced *ortho* substitution effect which is thought to alter the RI (Albro and Feishbin, 1972a) by twisting of the two rings out of coplanarity. However, the highly symmetric structure is still quite polar and lipophilic. The 2,3,6,2′,3′,6′-isomer has an equal *ortho* effect but is dissymmetric and lacks 4,4′-substitution and, therefore, is considerably less polar and lipophilic.

Extraction *p*-values using hexane and acetonitrile as immiscible solvents could be a measure of hydrophobicity. The values obtained for this isomeric series of biphenyls were very similar and not particularly useful for this purpose. The 2,3,6,2′,3′,6′-HCB had the smallest (0.55) *p*-value (least hydrophobic), but the rest with common substitution in the 4 and 4′ positions had *p*-values ranging from 0.60 (2,3,4,2′,3′,4′) to 0.83 (2,4,5,2′,4′,5′). The two isomers accumulated most in adipose tissue, 2,4,6,2′,4′,6′- and 3,4,5,3′,4′,5′-, had *p*-values of 0.78 and 0.70, respectively. The *p*-values would be expected to be influenced more by permanent group dipoles than inducible ones. It is interesting that the least hydrophobic HCB was one of the least metabolized (unpublished data), perhaps because less compound is presented to metabolizing organs.

The toxicity of PCBs varies not only with the degree of chlorination but also with the substitution pattern as indicated by this study with pure HCB isomers. The relationships between structure and accumulation factors are only beginning to be assessed (Sugiura *et al.*, 1975). Our work does appear to show a trend in tissue accumulation of HCBs which correlates with molecular polarizability as measured by chromatographic and spectroscopic techniques. Increasing tissue HCB concentrations were generally associated with increased biological response. The role of metabolism in toxicity is difficult to assess since determination of the degradation rates and activities of the terminal and intermediary metabolites would be necessary.

According to previous work (Sissons and Welti, 1971; P. W. Albro, unpublished observations), the most abundant chlorine substitution patterns in Aroclor 1254 (predominantly pentachlorobiphenyls with smaller amounts of tetra- and hexa-congeners), for example, are 2,5-,3,4-,2,3,4-, 2,3,6-,2,4,5-, and 2,3,4,5. The 2,4,6-pattern rarely occurs, and such patterns as 3-, 3,5-, and 2,3- occur infrequently. In Aroclor 1260 (predominantly hexachlorobiphenyls with significant levels of penta- and hepta-), the most frequent substitution patterns are similar to those found in Aroclor 1254.

The prevalence of patterns containing 3,4-, 4,5-, and 3,4,5- substitutions in Aroclor is remarkable. It is perhaps not so coincidental that these same substitution patterns are found in the more toxic HCB isomers in this study. Biological responses may be related to greater accumulation of biphenyls with these substitution patterns in tissue and possibly formation of small amounts of highly toxic metabolites. A relationship between substitution pattern and persistence in the avian system has been previously indicated in studies with an Aroclor mixture (Bush *et al.*, 1974). The alarm over environmental PCB contamination may be justified since the more abundant and apparently more stable isomers and congeners of commercial mixtures may include the more toxic ones, at least on a chronic basis. The role of chlorinated dibenzofurans as toxic components or metabolites of PCBs will require further study.

ACKNOWLEDGMENTS

Dr. L. A. Levy generously supplied synthetic hexachlorobiphenyls. Dr. J. Haseman kindly performed the statistical analysis of the data. We also thank Drs. J. Vos and P. Albro for their assistance and advice.

REFERENCES

ALBRO, P. W. AND FISHBEIN, L. (1972a). Quantitative and qualitative analysis of PCB's by gas–liquid chromatography and flame ionization detector. I. One to three chlorine atoms. *J. Chromat.* **69**, 273–283.

ALBRO, P. W. AND FISHBEIN, L. (1972b). Intestinal absorption of PCBs in rats. *Bull. Environ. Contam. Toxicol.* **8**, 26–31.

AYRES, D. C. (1972). Critical by-products in the synthesis of PCB's. *Nature (London)* **240**, 161–162.

BAILEY, S. AND BUNYAN, P. J. (1972). Interpretation of persistence and effects of PCB's in birds. *Nature (London)* **236**, 34–36.

BUSH, B., TUMASONIS, C. F. AND BAKER, F. D. (1974). Toxicity and persistence of PCB homologs and isomers in the avian system. *Arch. Environ. Contam. Toxicol.* **2**, 195–212.

GOLDSTEIN, J., MCKINNEY, J. D., LUCIER, G. W., HICKMAN, P., BERGMAN, H. AND MOORE, J. A. (1976). Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachloro-

dibenzofuran in chicks. II. Effects on drug metabolism and porphyrin accumulation. *Toxicol. Appl. Pharmacol.* **36**, 81–92.

GORNALL, A. G., BARDAWILL, C. J. AND DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751–766.

GRANT, D. L., PHILLIPS, W. E. J. AND VILLENEUVE, D. C. (1971). Metabolism of a PCB (Aroclor 1254) mixture in the rat. *Bull. Environ. Contam. Toxicol.* **6**, 102–112.

HANSELL, M. M. AND ECOBICHON, D. I. (1974). Effects of chemically pure PCB's on the morphology of rat liver. *Toxicol. Appl. Pharmacol.* **28**, 418–427.

HENLY, R. S., KRUPPA, R. F. AND SUPINIA, W. R. (1966). Mixed-phase silicone packing for pesticide analysis by gas chromatography. *J. Agr. Food Chem.* **14**, 667–668.

HOLLANDER, M. AND WOLFE, D. A. (1973). *Nonparametric Statistical Methods.* John Wiley and Sons, New York.

HUTZINGER, O., SAFE, S. AND ZITKO, V. (1974). *The Chemistry of PCB's.* CRC Press, Cleveland.

KENDE, A. S., WADE, J. J., DECAMP, M., RIDGE, D. AND POLAND, A. (1974). 167th National Meeting, American Chemical Society, Los Angeles, California (Abstr. ORGN 130).

JOHARY, N. S., GUHA, S. S. AND GUHA, P. C. (1952). Preparation of 3,4-dichloro and 3,4,5-trichloroanilines. *J. Ind. Inst. Sci.* **34**, 287–291.

MCKINNEY, J. D. (1974). Lecithin reversed micelle formation and interactions with *p,p'*-DDA and kelthane. NMR observations. *Chem. Phys. Lipids* **13**, 249–259.

MORON, M., SUNDSTROM, G. AND WACHTMEISTER, C. L. (1973). 2,3,7,8-Tetrachlorodibenzofuran, a critical byproduct in the synthesis of 2,2',4,4',5,5'-HCB by Ullman Reaction. *Acta Chem. Scand.* **27**, 3121–3122.

OSWALD, E. O., LEVY, L., CORBETT, B. J. AND WALKER, M. P. (1974). Differentiation and characterization of isomeric PCB's by gas–liquid chromatography coupled with electron impact and chemical ionization mass spectrometry. *J. Chromat.* **93**, 63–90.

ROHRSCHNEIDER, L. (1966). A method for the characterization of gas chromatography stationary liquids. *J. Chromat.* **22**, 6–22.

SCHULTE, E. AND ACKER, H. (1974). Identification and metabolizability of PCB's. *Naturwissenschaften* **61**, 79–80.

STREET, J. C., GEER, R. D., URRY, F. M., BELUND, F. A. AND BLAU, S. E. (1972). Induction of rat liver monooxygenase activity by selected PCB's. Symposium on PCB's in the environment, Division of Water, Air, and Waste Chemistry, 164th Meeting, American Chemical Society, New York.

SHIOIRI, T., NINOMIYA, K. AND YAMADA, S. (1972). Diphenylphosphoryl azide. A new convenient reagent for a modified Curtius reaction and for peptide synthesis. *J. Amer. Chem. Soc.* **94**, 6203–6204.

SISSIONS, D. AND WELTI, D. (1971). Structural identification of PCB's in commercial mixtures by gas–liquid chromatography, nuclear magnetic resonance and mass spectrometry. *J. Chromat.* **60**, 15–32.

SUGIURA, K., HATTORI, M., BABA, M. AND GOTO, M. (1975). Accumulation and excretion of PCBs in the mouse. *Chemosphere* **4**, 181–188.

TAS, A. C. AND DE VOS, R. H. (1971). Characterization of four major components in a technical PCB mixture. *Environ. Sci. Technol.* **5**, 1216–1218.

TAS, A. C. AND KLEIPOOL, R. J. C. (1972). Characterization of the components of technical PCB mixtures. *Bull. Environ. Contam. Toxicol.* **8**, 32–37.

VAN ROOSMALEN, F. L. W. (1934). Chloro- and bromo-derivatives of biphenyls. *Rec. Trav. Chim.* **53**, 359–386.

VOS, J. G., KOEMAN, J. H., VAN DER MAAS, H. L., TEN NOEVER DE BRAU, M. C. AND DE VOS, R. H. (1970). Identification and toxicological evaluation of chlorinated dibenzofuran and chlorinated naphthalene in two commercial PCB's. *Fd. Cosmet. Toxicol.* **8**, 625–632.

VOS, J. G. AND KOEMAN, J. H. (1970). Comparative toxicologic study with PCB's in chickens with special reference to porphyria, edema formation, liver necrosis, and tissue residues. *Toxicol. Appl. Pharmacol.* **17**, 656–668.

VOS, J. G. (1972). Toxicology of PCB's for mammals and birds. *Environ. Health Perspect.* **1**, 105–118.

WILSON, N. K. AND ANDERSON, M. (1974). Carbon-13 and proton magnetic resonance studies of chlorinated biphenyls. In *Mass Spectrometry and NMR in Pesticide Chemistry* (R. Haque and F. Biros, eds.), Environmental Sci. Res. Series Vol. 4, pp. 197–218. Plenum Publishing Co., New York

WILSON, N. K. (1975). Carbon-13 nuclear magnetic resonance. ^{13}C Shieldings and spin-lattice relaxation times in chlorinated biphenyls. *J. Amer. Chem. Soc.* 97, 3573–3579.