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Polychlorinated Biphenyls as Inducers of Hepatic Porphyria in Japanese Quail, with Special Reference to δ-Aminolevulinic Acid Synthetase Activity, Fluorescence, and Residues in the Liver

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Polychlorinated Biphenyls as Inducers of Hepatic Porphyria in Japanese Quail, with Special Reference to δ-Aminolevulinic Acid Synthetase Activity, Fluorescence, and Residues in the Liver. Vos, J. G., Strik, J. J. T. W. A., van Holsteÿn, Catherina W. M., and Pennings, J. H. (1971). *Toxicol. Appl. Pharmacol.* 20, 232–240. Male Japanese quail were orally dosed, daily for 7 days, with 1000, 500, 250, 100, 50, and 0 mg PCB (Aroclor 1260) per kg body weight. The birds receiving 1000 mg/kg died. A dose-related increase in liver and liver:body weight ratio was found. Macroscopic and microscopic examination under ultraviolet light were used for the detection of porphyrin fluorescence. Mitochondrial ALA synthetase activity demonstrated a large increase (20-fold at the 500 mg/kg level; 10-fold at the 50 mg/kg level). The doubling of mitochondrial protein, the increase of ALA synthetase activity, the liver enlargement, and the pyroninophilic staining of the hepatic nucleoli suggest an increased protein synthesis.

In a second experiment, 5 groups of female quail received 100, 10, 1, 0.1, and 0 mg PCB per kg body weight. ALA synthetase activity was increased about 20-fold in the 100 mg/kg group. This increase, together with the high PCB content of the livers (mean 478 ppm) and the incidence of fluorescence, indicating porphyrins, were correlated with the loss of weight in this group (mean 8%). Mobilization of PCB from storage fat is considered to be responsible for the increased porphyria.

The dose-response relationship and liver residue relationship of PCB and ALA synthetase activity are given.

Since the discovery of polychlorinated biphenyls (PCB) in the Swedish environment (Jensen, 1966), a wide variety of samples including birds, fish, sediment, and water were found to be contaminated with PCB (Holmes *et al.*, 1967; Holden and Marsden, 1967; Koeman *et al.*, 1967, 1969; Risebrough *et al.*, 1968; Jensen *et al.*, 1969., Duke *et al.*, 1970). PCB's have also been demonstrated in human adipose tissue samples (Biros *et al.*, 1970), in vegetable oils and in some foods of animal origin (Westöö *et al.*, 1970). Ingestion of rice bran oil contaminated with PCB produced human poisoning in Japan, with a total of 600 patients (quoted by Nishizumi, 1970).

The wide spectrum of application, the persistence and the toxicologic data were recently discussed by Gustafson (1970) and by Peakall and Lincer (1970).

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In a previous study (Vos and Koeman, 1970), it was found that PCB could induce chemical porphyria in chickens, Japanese quail, and rats. Several tissues of chickens that died during the experiment were strongly fluorescent under ultraviolet light. In addition, a significant difference in toxicity was found between 3 commercial PCB preparations. In the following studies it was shown that the most toxic preparations contained trace amounts of chlorinated dibenzofurans, considered to be responsible for most of the toxic effects (Vos *et al.*, 1970) but not for the porphyrogenic action (Vos and Beems, 1971).

This study presents a further investigation of the PCB-evoked porphyria. The increase in the activity of δ-aminolevulinic acid synthetase (ALA synthetase) in liver mitochondria was taken as a sensitive parameter for hepatic porphyria. The effect level of PCB as measured by ALA synthetase activity and tissue fluorescence was determined and correlated with residues in the liver. This experiment was made with a PCB preparation which, according to our analytical procedure, was free from contamination with chlorinated dibenzofurans.

METHODS

The PCB sample (average content 60% chlorine) was obtained from Monsanto (U.S.A.) (Aroclor 1260. Lot. No. Ak-3).

In a preliminary experiment 12 male Japanese quail (*Coturnix coturnix japonica*) (6 wk old; 88–116 g body weight) were divided at random into 6 pairs. The separate pairs were orally dosed, with a syringe, daily for 7 days with 1000, 500, 250, 100, 50, or 0 mg Aroclor 1260 per kg body weight, respectively. The PCB mixture was dissolved in arachis (peanut) oil (180 mg PCB/ml); the control birds received 0.25 ml arachis oil daily.

The animals were killed after 1 wk. The body and liver weights were determined, and the tissues were visually examined in uv light. Cryostat sections of liver were studied under the fluorescence microscope (Zeiss, exciting filters BG 38/2.5 and BG 12/4, barrier filters 53 and 33, Osram super pressure lamp HBO 200 W). For light microscopic examination, liver tissue was fixed in Carnoy fluid and embedded in Paraplast. Sections were stained with hematoxylin-eosin and with methylgreen–pyronin.

The livers of each pair were pooled and homogenized in ice-cold sucrose, and mitochondria were isolated according to Hogeboom (1962). ALA synthesis in liver mitochondria was measured by the method of Wada *et al.* (1968). The reaction was terminated by adding trichloroacetic acid. The ALA in the aliquot of deproteinized supernatant was determined by the method of Urata and Granick (1963). The protein content of the mitochondrial fraction was determined by the biuret method (Robinson and Hogden, 1940).

In a second experiment 25 female Japanese quail (12 wk old and weighing 117–162 g) were divided at random into 5 groups. The separate groups were treated in the same way as in the first experiment, daily for 1 wk with, respectively, 100, 10, 1, 0.1, and 0 mg Aroclor per kg body weight.

The PCB sample was dissolved in arachis oil in different concentrations, resulting in a final dose of 5 ml of solvent per kg body weight. The same parameters were studied as already mentioned, with exception of the light microscopic examination of liver

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sections. In addition, a microsomal fraction was obtained from the 10,000 *g* supernatant fraction by centrifugation for 60 min at 100,000 g. Cytochrome P-450 was determined by measuring the CO-difference spectrum of a dithionite-reduced microsome suspension (Omura and Sato, 1964). This method was found to be rather insensitive using Japanese quail. Values below 4.3 mμmoles P-450/g liver could not be measured. For this reason statistical evaluation was not made. The PCB concentration in the livers was determined by gas chromatography. The livers were dried with anhydrous sodium sulfate and extracted with petroleum ether in a Soxhlet apparatus. After concentration, cleanup of the samples was carried out by column chromatography using alumina, deactivated with 5% water, according to Holden and Marsden (1969). The amount of PCB present in the post cleanup solution was determined by gas chromatography (204-1B Varian *Aerograph with electron capture detector). The Pyrex glass column (5 ft × 1/8 in),* filled with 10% DC 200 on Gaschrom (80–100 mesh) was operated at 200°C with nitrogen as carrier gas (about 50 ml/min). Only an approximate quantitative determination of the residue from the chromatogram of the liver extracts could be made, since the composition of the residue was not necessarily identical with the PCB preparation used in the treatment. For quantification 1 peak (r_x = 1.45 relative to dieldrin), considered to be representative, was selected. The approximate amount of total PCB-residue was calculated by peak-height comparison.

The significance of differences between the treated and the control groups was calculated, on a two-tailed significance level, using the Wilcoxon test for 2 unrelated samples (van der Waerden, 1957).

RESULTS

First Experiment

The 2 male Japanese quail receiving the highest dose of PCB (1000 mg/kg) died after 2 and 5 days, respectively. The other birds survived the experimental period. The birds treated with 500 mg PCB/kg demonstrated loss of weight (10%).

At necropsy, a dose-related increase in liver weight and liver:body weight ratio was found (Table 1). Visual examination in uv light revealed intense red fluorescence of all tissues, indicating porphyrins, in the birds that died (1000 mg/kg group); bone and kidney fluorescence was found in the quail treated with 500 mg/kg. Fluorescent microscopy of the livers of these 2 birds showed an intense red fluorescence. A weak fluorescence was noted in a liver of one bird receiving 100 mg PCB/kg. The porphyria induced by PCB at the 1000 mg/kg dose was found to concur with a large increase (20-fold) in ALA synthetase activity. Even a dose of 50 mg/kg demonstrated a 10-fold increase in the activity of ALA synthetase (Table 1). Mitochondrial protein was about doubled at the higher doses. Microscopic examination of H-E stained liver sections showed enlargement of nucleoli and nuclei in the 500 mg/kg group and to a lesser extent in the group treated with 250 mg/kg. The enlarged nucleoli stained partly pyroninophilic using the methylgreen-pyronin stain.

Second Experiment

A weight loss of 8% was noted in the group treated with 100 mg/kg. The results are given in Table 2. Liver weights in each group showed considerable variation. A slight

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TABLE 1

FORMATION OF δ-AMINOLEVULINIC ACID BY LIVER MITOCHONDRIA, PROTEIN CONTENT OF THE MITOCHONDRIAL FRACTION, LIVER WEIGHTS, AND TISSUE FLUORESCENCE IN MALE JAPANESE QUAIL ORALLY DOSED WITH PCB FOR 7 DAYS

Aroclor 1260 (mg/kg body weight)	ALA formed (mμmoles ALA/g liver/hr)[a]	Protein content mitochondrial fraction (mg/g liver)[a]	Liver (g)[b]	Liver (g/100 g body weight)[b]	Tissue fluorescence incidence	
					Macroscopic	Microscopic (liver)
0	12.2	14.8	3.14	2.93	0/2	0/2
50	115.7	26.5	3.80	3.17	0/2	0/2
100	169.7	31.0	4.02	3.60	0/2	1/2
250	206.8	28.7	4.30	3.95	0/2	0/2
500	272.7	24.2	4.14	4.06	2/2	2/2
1000[c]	—	—	—	—	2/2	—

[a] The figures are comprised of pooled samples each of 2 birds.
[b] Mean value of 2 birds.
[c] Died on day 2 and day 5, respectively.

TABLE 2

FORMATION OF δ-AMINOLEVULINIC ACID BY LIVER MITOCHONDRIA, CYTOCHROME P-450, PROTEIN CONTENT OF THE MITOCHONDRIAL FRACTION, LIVER WEIGHTS, LIVER RESIDUES, AND TISSUE FLUORESCENCE IN FEMALE JAPANESE QUAIL ORALLY DOSED WITH PCB FOR 7 DAYS[a]

Aroclor 1260 (mg/kg body weight)	ALA formed (mμmoles ALA/g liver/hr)	Cytochrome P-450 (mμmoles/ g liver)	Protein content mitochondrial fraction (mg/g liver)	Liver (g)	Liver (g/100 g body weight)	PCB Content liver (ppm)	Tissue fluorescence incidence	
							Macroscopic	Microscopic (liver)
0	6.46 ± 1.65	< 9.1	19.1 ± 5.3	4.43 ± 0.51	3.13 ± 0.13	0.15[c]	0/5	0/5
0.1	8.76 ± 4.06	< 6.9	17.7 ± 1.8	4.31 ± 0.62	3.09 ± 0.51	0.54 ± 0.27	0/5	0/5
1	10.50 ± 1.21[b]	< 4.3	20.9 ± 3.7	4.22 ± 0.76	2.76 ± 0.35	1.41 ± 0.67	0/5	0/5
10	17.3 ± 6.4[b]	14.0	17.8 ± 2.5	5.09 ± 1.19	3.52 ± 0.65	27.0 ± 9.4	0/5	0/5
100	119.9[c]	19.7[c]	29.1[c]	4.73 ± 0.82	3.89 ± 0.63	478 ± 294	3/5	2/5

[a] Mean values ± SD, 5 birds per group.
[b] Significantly different from controls, $P < 0.01$.
[c] Pooled samples.

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but not significant increase in liver weight and liver: body weight ratio was found after treatment with 10 and 100 mg/kg. Macroscopic fluorescence was found in 3 birds of the 100 mg/kg group. The microscopic examination of 2 out of 3 livers with macroscopic fluorescence, demonstrated diffuse fluorescence of the parenchymal cells. ALA synthetase activity was significantly ($P < 0.01$) increased in the 1 and 10 mg/kg groups.

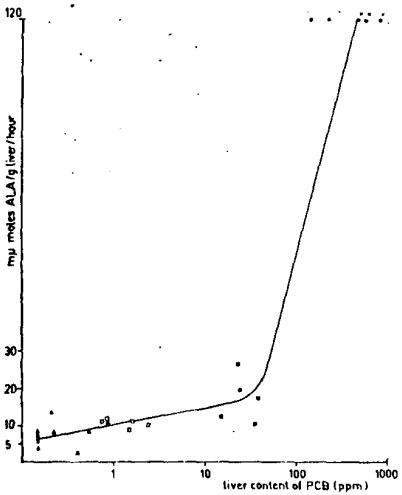


FIG. 1. Correlation between the liver content of PCB (in ppm) and the mitochondrial ALA synthetase activity (μmoles ALA/g liver/hr). △, control animals; ▲, 0.1 mg/kg; □, 1 mg/kg; ■, 10/mg/kg; ●, 100 mg/kg; × ▲ birds showing fluorescence under ultraviolet light. Liver samples were pooled in the control group for the determination of the PCB content, and in the 100 mg/kg group for the determination of the ALA synthetase activity.

The pooled liver mitochondria sample of the 100 mg/kg group showed a 20-fold increase in activity. Mitochondrial protein/g liver was not altered. The amount of the microsomal hemoprotein cytochrome P-450 varied considerably within the groups. It tended to increase. PCB content in the livers showed considerable variation within each group. The residue of 0.15 ppm PCB in the pooled sample of the livers from the control animals can be explained by the presence of PCB in the arachis oil. This solvent appeared to contain 0.55 ppm PCB.

The highest PCB concentrations in the 100 mg/kg group were found in the livers of 3 birds showing macroscopic fluorescence. The relationship between the PCB content

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in the livers, the mitochondrial ALA synthetase activity and the macroscopic fluorescence is given in Fig. 1.

DISCUSSION

It is evident, that the experimental results indicate that the porphyrogenic effect of PCB is closely associated with an increase of mitochondrial ALA synthetase.

PCB-evoked porphyria has much in common with hexachlorobenzene (HCB)-induced porphyria. This opinion is supported by the finding that the excretion of coproporphyrin is increased more than that of protoporphyrin after treatment with both PCB and HCB (Vos and Koeman, 1970; Vos and Beems, 1971; Vos *et al.*, 1971). Fluorescence microscopy of liver sections demonstrated diffuse fluorescence of the parenchymal cells in the case of PCB (Vos and Koeman, 1970) and HCB (Pathak and Burnett, 1964; Vos *et al.*, 1971). The picture of this fluorescence is different from the microscopic fluorescence produced by treatment with another well known hepato-porphyrogenic compound, diethyl-1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxy-lase (DDC). DDC causes deposition of protoporphyrin crystals in and around bile ducts and ductules (Magnus *et al.*, 1969).

PCB tended to increase the content of cytochrome P-450, HCB did increase this cytochrome at a very high dose level (Wada *et al.*, 1968). DDC treatment, on the contrary, results in a decreased P-450 content (Wada *et al.*, 1968). These findings suggest that the porphyria caused by PCB is due to increase of ALA synthetase, followed by overproduction of porphyrins. It does not seem likely that in the case of PCB, shortness of heme or inhibition of iron incorporation into protoporphyrin are involved in the induction of the ALA synthetase and porphyrin overflow, as is postulated for the porphyrogenic action of DDC (Magnus *et al.*, 1969; Onisawa and Labbe, 1963).

The morphological changes in liver tissue, other than porphyria, have certain features in common with the morphological effects of the porphyrogenic chemicals, HCB and allylisopropylacetamide (AIA). The enlargement of nucleoli and nuclei was also found after AIA (Moses *et al.*, 1970) and HCB (Vos *et al.*, 1971) treatment, together with prominent liver enlargement. The staining of the nucleoli with pyronin (RNA stain), the doubling of mitochondrial protein in the male quail, and the liver enlargement suggest a stimulated de novo protein synthesis including the ALA synthetase. This is supported by the finding that PCB enhances microsomal enzyme activity (Street *et al.*, 1969; Risebrough *et al.*, 1968). The increase in hepatic enzyme activity was correlated with an increase in cytoplasmic RNA by Lincer and Peakall (1970). An electron micro-scopic study of PCB in mice and monkeys demonstrated an increase of the smooth endoplasmic reticulum in hepatocytes (Nishizumi, 1970).

It is worth mentioning that, in the present study, fluorescence was found in 3 birds showing the highest liver content of PCB (Fig. 1). These findings can possibly be explained by the mobilization of storage fat from adipose tissue, since 2 of these birds showed a weight loss of 15 and 18%, resulting in increased blood and liver concentrations of the lipophilic PCB. This could also account for the high incidence of fluorescence and the high PCB contents of livers in chicks that died from PCB, as was found in a previous study (Vos and Koeman, 1970).

No evidence was obtained of the presence of a threshold in the dose- or residue-response relationship of PCB and ALA synthetase activity. This, however, does not

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mean that there is not a dose range where no effect of PCB on ALA synthetase activity is present. It is of interest to note that the curve of Fig. 1 has a sharp bend, which may indicate the presence of 2 processes which are separately influenced by PCB. The second influence clearly is absent at dose levels below 10 mg/kg (residue levels below 27 ppm). According to the usual evaluation of toxicity tests in terms of a no-effect level, the no-effect level in this case (Wilcoxon test) is 0.1 mg/kg (mean liver residue 0.45 ppm).

The PCB-induced porphyria is the result of the treatment with a commercial preparation. It is not yet known which component(s) are responsible for the porphyria. Work will be continued with some pure and representative compounds of the PCB mixture.

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