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# Effects of PCBs on Plasma Enzymes, Testosterone Level, and Hepatic Xenobiotic Metabolism in the Grey Partridge, *Perdix perdix*

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The hepatic cytochrome P-450-dependent monooxygenase (MO) system functions in oxidative biotransformation of a wide variety of both endogenous and exogenous (xenobiotic) compounds in many animal species. Of particular interest, environmental pollutants such as polycyclic aromatic hydrocarbons, some organochlorine insecticides and polychlorobiphenyls (PCBs) were shown to be inducers of MO activities in mammalian species. The inducing effects of PCBs are also well established in a variety of more primitive vertebrates, e. g., fish or birds. However, most of the previous studies were carried out with a narrow range of species and investigations on wild species are lacking. In birds, there have been reports of induction of cytochrome P-450 and MO activities with PCBs in several species, but results have not been entirely consistent, induction being found in Japanese quail (Bunyan and Page 1978; Rivière et al. 1985) and owl (Rinsky and Perry 1981), but not in buzzard (Rivière et al. 1985).

In this report, we describe the effects of a commercial mixture of PCBs (DP5) on the hepatic MO activities of the grey partridge (*Perdix perdix*). To more thoroughly investigate the inducing effects of DP5, we used two series of homologous substrates, alkylresorufins (Burke et al. 1985) and alkoxycoumarins (Kamataki et al. 1980; Matsubara et al. 1982), and an endogenous compound, testosterone (Wood et al. 1983; Waxman et al. 1983), which were shown in mammals to differentiate between different forms of cytochrome P-450. Furthermore, to more carefully assess the effects of DP5, we also measured the activity of two plasma marker enzymes, alanine transpeptidase (ALAT) and gamma-glutamyl transferase (gamma-GT), and the plasmatic concentration of testosterone.

## MATERIALS AND METHODS

Polychlorobiphenyls were DP5 (Prodelec, France). Alkoxycoumarins were synthesized from the alkyl iodides and umbelliferone by the method of Matsubara et al. (1982). Alkylresorufins, $NADP^+$, glucose 6-phosphate (G 6-P) and glucose 6-phosphate dehydrogenase

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(G 6-P-DH) were obtained from Boehringer, France. Testosterone (T) and androstenedione (AD) were obtained from Sigma (La Verpillière, France). 16β-hydroxytestosterone (16β-OH-T) was purchased from Steraloids Inc. (USA). 7α- and 16β-hydroxytestosterones (7α-OH-T and 16β-OH-T, respectively) were kindly provided from the Steroid Reference Collection of the Medical Research Council, London, UK, by Pr DN Kirk. Solvents were HPLC grade, while all other chemicals were reagent-grade.

Partridges were reared outside in wire-mesh cages. Food and water were supplied *ad libitum*. In the first experiment, male birds (one-year old) were fed a commercial diet containing various levels of DP5. The compound was dissolved in acetone, mixed with the diet and the solvent evaporated. The levels were 5, 25, and 125 mg/kg (ppm). Controls were fed the same diet without added DP5. After the diet had been fed for 15 days, animals were sacrificed for assays of hepatic enzyme activities. In a second experiment, male birds (one-year old) were fed once with DP5 in gelatine capsules. The dosage level was 150 mg/kg body wt. Animals were sacrificed 4 days after.

Livers were homogenized in 3 vol of 0.15 M KCl, 50 mM phosphate buffer, pH 7.4, using a Potter-Elvehjem homogenizer with three passes of a motor-driven Teflon™ pestle. The tissue homogenates were centrifuged for 15 min at 10,000 *g* at +4°C. The supernatant was then recentrifuged for 60 min at 105,000 *g* at +4°C. The microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4 containing 1 mM EDTA, 20% glycerol and stored in small aliquots at -80°C. When maintained at this temperature, there was no loss of enzymatic activities until assay.

The monooxygenase activities were alkylresorufin O-dealkylases (ethylresorufin, EROD; pentylresorufin, PROD; benzylresorufin, BROD), alkoxycoumarin O-dealkylases (methoxycoumarin, MCOD; ethoxycoumarin, ECOD; propoxycoumarin, PCOD; butoxycoumarin, BCOD) and coumarin 7-hydroxylase (Cou 7-OH). All activities were assayed in a final volume of 1 ml containing 100 mM phosphate buffer (pH 7.4), 0.5 mM $NADP^+$, 5 mM G 6-P, 1 unit G 6-P-DH, microsomal protein, and substrate. The concentrations of substrates were saturating : alkoxycoumarins (in 10 µl dimethylsulfoxide) 500 µM, coumarin (in 100 µl of water) 100 µM and alkylresorufins (in 10 µl methoxyethanol) 1 µM. Reactions were stopped by 2 ml acetone (alkylresorufins), or 100 µl trichloracetic acid (20% in water; coumarin and alkoxycoumarins). The fluorescence of 7-hydroxy-coumarin was measured (excitation wavelength : 380 nm; fluorescence wavelength : 480 nm) after extraction of the product by ethyl acetate (4 ml) and mixing 1 ml of the organic phase with 1 ml of ethanol and 1 ml of glycine buffer (pH 10.4). The fluorescence of resorufin was measured according to the method of Rifkind and Muschick (1983). Fluorometer was standardized by 7-hydroxycoumarin or resorufin. Activities were measured at 42° C. All reaction rates were linear with respect to time and protein concentration.

Testosterone metabolism was assayed for 15 min in 100 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 5 mM G 6-P, 1 unit G 6-P-DH, 0.5 mM $NADP^+$, microsomal protein and 0.26 mM testosterone in a final volume of 1.0 ml. The incubate was extracted with 5 ml methylene chloride. The organic phase was removed, evaporated to dryness under a $N_2$ stream, and resuspended in 200 µl methanol. Metabolites were analyzed by HPLC using a Beckman™ liquid chromatograph fitted with a 150 x 4.6 mm (inner diameter) reverse phase column. Elution of metabolites was achieved at room temperature by a modification of the method of Halperin-Walega and Greene (1985). The mobile phase was 75% solution B (2 vol water: 1 vol acetonitrile) and 25% solution A (methanol) from 0 to 4 min, followed by a linear gradient of 75 to 60% solution B (15 min), then 60 to 40% solution B for an additional 5 min. The flow rate was 0.8 ml/min from 0 to 16 min, then increased to 1.2 ml/min by a linear gradient for 8 min. A second chromatography system was utilized to separate 6β- and 7α-OH-T and consisted of a silica gel column eluted isocratically with isopropanol:tetrahydrofuran: hexane (5:15:80) as the mobile phase (Shaikh et al. 1979). Column effluents were monitored at 254 nm. Metabolites were quantitated by measurements of their peak areas. Some metabolites were also identified by mass spectrometry after TMS derivatization on a Ribermag R10/10 GC/MS apparatus. Testosterone was determined in plasma after extraction by ethyl ether with a commercial kit ($^3$H-Testostérone RIA-Kit, BioMérieux, France).

Plasma enzyme activities were determined with commercial kits (BioMérieux, France). Cytosolic glutathione S-transferase activity towards CDNB (1-chloro-2,4-dinitrobenzene) and DCNB (1,2-dichloro-4-nitrobenzene) was assayed at 42°C according to Baars et al. (1978). Total cytochrome P-450 content was measured according to Estabrook and Werringloer (1978). Protein was determined by the method of Hartree (1972) with bovine serum albumin as a standard.

All analyses were performed in duplicate. Statistical analysis of results was performed by comparing treated and control groups with the Student's *t*-test.

## RESULTS AND DISCUSSION

In male partridges, activities of plasma marker enzymes and testosterone concentration were not modified after 2 weeks of continuous feeding of the DP5-containing diet (Table 1), but levels of hepatic microsomal alkylresorufin O-dealkylase activities were significantly elevated (Fig. 1). Maximal induction of enzymatic activities was obtained with 25 ppm of DP5, resulting in a 33.4-, 4.8-, and 6.1-fold increase in EROD, PROD and BROD activities respectively, then a significative decrease in activity was observed, while maximal induction (3-fold) of cytochrome P-450 was obtained at the highest dietary level (125 ppm) of DP5. In contrast, the treatment by DP5 (125 ppm) did not modify ECOD activity, and significantly decreased Cou 7-OH activity to one-tenth of the control value. Administration *per os* of a single large dose to male birds produced similar effects (Table 2).

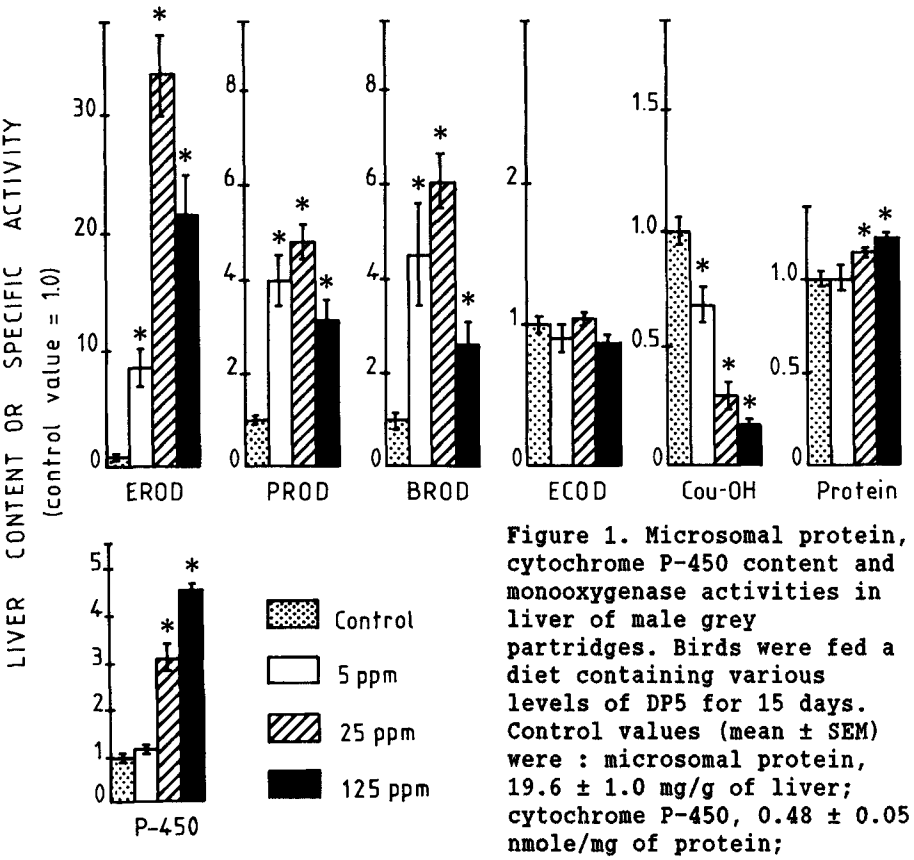


Figure 1. Microsomal protein, cytochrome P-450 content and monooxygenase activities in liver of male grey partridges. Birds were fed a diet containing various levels of DP5 for 15 days. Control values (mean ± SEM) were : microsomal protein, 19.6 ± 1.0 mg/g of liver; cytochrome P-450, 0.48 ± 0.05 nmole/mg of protein; EROD, 35 ± 2 pmole/mg/min; PROD, 0.90 ± 0.09 pmole/mg/min; BROD, 11 ± 2 pmole/mg/min; Cou 7-OH, 0.66 ± 0.04 nmole/mg/min and ECOD, 3.9 ± 0.3 nmole/mg/min. Results are mean ± SEM (bars), significantly different from control at * $p < 0.05$ ($n = 8$).

Table 1. Plasma enzyme activities and testosterone concentration from male grey partridge fed a diet containing various levels of DP5 for 15 days.

| | ALAT[a] | gamma-GT[a] | testosterone[b] |
|---|---|---|---|
| control | 12.5 ± 1.8[c] | 3.0 ± 0.4 | 0.53 ± 0.07 |
| 5 ppm | 16.8 ± 3.9 | 5.8 ± 2.0 | 0.44 ± 0.03 |
| 25 ppm | 12.8 ± 4.5 | 3.8 ± 0.5 | 0.55 ± 0.07 |
| 125 ppm | 12.0 ± 5.0 | 3.8 ± 0.4 | 0.54 ± 0.07 |

[a] IU/l, [b] ng/ml, [c] mean ± SD ($n = 4$).

Table 2. Effect of DP5 (150 mg/kg) on alkoxycoumarin O-dealkylase, alkylresorufin O-dealkylase and glutathione S-transferase activities in hepatic microsomes from male grey partridge.

| | control | DP5-treated | fold-induction |
|---|---|---|---|
| liver weight[a] | 5.97 ± 0.82[f] | 6.06 ± 0.53 | 1.02 |
| cytochrome P-450[b] | 0.39 ± 0.05 | 2.34 ± 0.12* | 6.1 |
| microsomal protein[c] | 20.5 ± 2.2 | 22.9 ± 0.8 | 1.12 |
| EROD[d] | 35.2 ± 10.8 | 1030 ± 180* | 29.2 |
| PROD[d] | 1.3 ± 0.6 | 4.3 ± 0.4* | 3.2 |
| BROD[d] | 7.3 ± 4.3 | 57.6 ± 6.4* | 7.9 |
| MCOD[e] | 5.4 ± 1.4 | 4.1 ± 0.9 | 0.76 |
| ECOD[e] | 3.4 ± 0.7 | 2.5 ± 0.4 | 0.74 |
| PCOD[e] | 0.17 ± 0.07 | 0.56 ± 0.08* | 3.3 |
| BCOD[e] | 0.24 ± 0.04 | 0.40 ± 0.09* | 1.7 |
| DCNB[e] | 4.00 ± 0.82 | 5.15 ± 0.52 | 1.28 |
| CDNB[e] | 3590 ± 720 | 5260 ± 600* | 1.47 |

[a] g, [b] nmole/mg protein, [c] mg/g, [d] pmole/mg/min, [e] nmole/mg/min. [f] mean ± SD. Significantly different from control at * $p < 0.05$ ($n = 4$).

Alkylresorufins are a homologous series of heterocyclic ether substrates for which constitutive and induced forms of rat liver cytochrome P-450 show differing selectivity. In control rat, EROD was the highest activity, followed by BROD and PROD. Administration of Aroclor 1254 (a mixture of PCBs identical to DP5) to rats resulted in a considerable increase (61-fold) in EROD activity and lower increases in PROD and BROD activities, 22- and 30-fold, respectively (Burke et al. 1985). We observe a similar pattern of inducibility in partridge liver. In terms of dose-response relationship, DP5 is an inducer at a low level in the diet, but a fall was observed at the highest dose. EROD activity is the most sensitive index to detect induction by these pollutants.

The second experiment was expanded to include the O-dealkylation of alkoxycoumarins, an other series of homologous substrates. With these substrates, there is a general trend to observe a decreasing activity by increasing the chain length of the alkyl group, but some differences were observed depending on the source of microsomes. Partridges are characterized by a higher specific activity toward 7-methoxycoumarin than toward 7-ethoxycoumarin and a very low activity toward 7-propoxycoumarin and 7-butoxycoumarin (Table 2). In contrast, ECOD activity is higher than MCOD activity in rat liver microsomes, and changing the alkyl group does not result in so marked alterations in the O-dealkylase activities (Kamataki et al. 1980; Matsubara et al. 1982). DP5 treatment increased PCOD and BCOD and slightly decreased MCOD and ECOD

Table 3. Oxidation of testosterone catalyzed by hepatic microsomes from control grey partridges. Results are expressed as percent of total metabolites.

| | 6β-OH-T | 16α-OH-T | 1 | AD |
|---|---|---|---|---|
| control | 53.5 | 6.7 | 4.7 | 29.9 |
| DP5-treated | 51.2 | 4.7 | 6.2 | 25.8 |

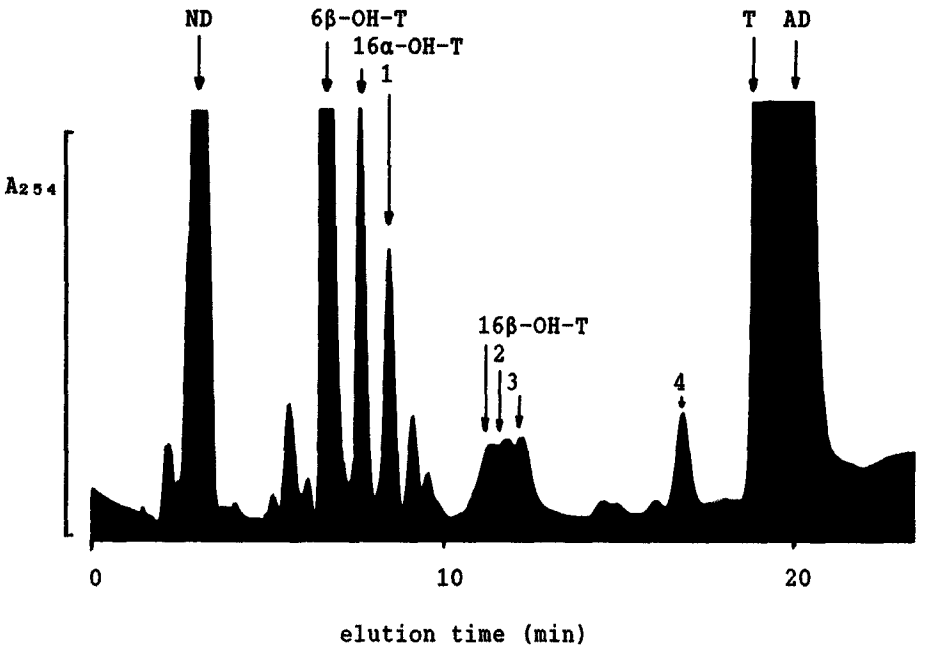


Figure 2. HPLC profile of testosterone metabolites formed by hepatic microsomes from untreated partridges. Conditions and abbreviations as described in Materials and Methods (ND = not determined).

activities in partridge liver microsomes (Table 2), while a similar treatment of male rats (150 mg/kg i.p., 4 days before sacrifice) resulted in induction (4.1 to 7.1-fold) of these four activities (Rivière, data not shown). Thus, according to our results, rats and partridges respond similarly to DP5 treatment when alkylresorufins were used as substrates. However, differences between these two sources of cytochromes P-450 are clearly demonstrated by using alkoxycoumarins as enzymatic probes.

Previous studies have shown that rat liver cytochromes P-450 can oxidize testosterone at several different positions. The profile of generated metabolites have proved useful in characterizing individual cytochrome P-450 isozymes (Wood et al. 1983; Waxman et al. 1983). The profile of testosterone metabolites formed by partridge liver microsomes under our experimental conditions is illustrated in Fig. 2. Several metabolites were identified by comparison of retention times with known standards. The chromatography system used in this study failed to distinguish 7α-OH-T from 6β-OH-T. By the use of a different column, it was subsequently shown that the main metabolite was 6β-OH-T, 7α-OH-T being formed in only small quantities. Compound 1 was identified (GC/MS, data not shown) as an hydroxylated oxo-derivative and should be hydroxylated AD. On the basis of their order of elution (Halperin-Walega and Greene 1985), compound 2 and 3 were tentatively identified as 2α- and 2β-OH-T, respectively. Compound 4 was not a steroid. In untreated birds, 6β-OH-T, 16α-OH-T, and AD represented 53.5, 6.7, and 29.9%, respectively, that is *ca* 90% of total metabolites (Table 3). In untreated rats, Wood et al. (1983) found that 6β-OH-T, 7α-OH-T and AD represented 68, 12 and 8% of total identified metabolites. The predominence of the 6β-OH-T metabolite was a common characteristic for microsomes of partridges and rats, but the former species had a greater ability to form AD. PCBs (Aroclor 1254) treatment of male rats was associated with an increase in the formation of 16α-OH-T and 16β-OH-T, a minor decrease in AD and marked decreases in other metabolites. Compared to control microsomes, the overall rate of testosterone metabolism (per nmol of cytochrome P-450) decreased but increased when expressed per mg of protein (Wood et al. 1983). By contrast, treatment of birds with DP5 did not result in dramatic changes in the profile of testosterone metabolites, nor in the rate of hepatic testosterone metabolism.

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