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Toxicity and 7-ethoxyresorufin O-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos

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Abstract. The toxicities of the coplanar polychlorinated biphenyls 3,3′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) and 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB) were compared in a 72-h study on chick embryos. The substances were injected into the air sacs of hens' eggs preincubated for 7 days. Mortality was measured 72 h later and corresponding LD_{50} values were calculated. The rank order of toxicity was PeCB > TCB > HCB. Using the same injection procedure, the potencies of these chlorobiphenyls with regard to their induction of hepatic 7-ethoxyresorufin *O*-deethylase activity were compared. The ranking order of the substances as inducers was the same as their order when ranked according to toxicity. The three coplanar chlorobiphenyls were considerably more toxic and potent as inducers than the nonplanar 2,2′,4,4′,5,5′-hexachlorobiphenyl. In a 2-week toxicity study, PeCB and HCB were injected into the yolks of hens' eggs preincubated for 4 days. PeCB was about 50-fold more potent than HCB in causing embryonic death. Both substances caused abnormalities, including edema, liver lesions, microphthalmia and beak deformities.

Key words: Chick embryo – Environmental pollutants – Enzyme induction – Polychlorinated biphenyls – Toxicity

Introduction

Polychlorinated biphenyls (PCBs) are widespread environmental contaminants, first recognized in biological samples by Jensen (1966). Technical preparations of PCBs are mixtures of individual chlorobiphenyls chlorinated at various positions and to various degrees. The PCBs chlorinated in the *para* positions and at least one of the *meta* positions of each ring, but lacking *ortho* chlorines, are considered to be most toxic (McKinney et al. 1976; Yoshimura et al. 1979; Silkworth and Grabstein 1982; Safe 1984). Since these most toxic PCBs, i.e. 3,3′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) and 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB), are unsubstituted in the *ortho* positions, they can exist in a coplanar conformation. The coplanar PCBs are structurally similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and bind to the soluble receptor referred to as the *Ah* receptor (TCDD receptor), albeit less avidly than TCDD. Most toxic effects of TCDD and its congeners are thought to be mediated via their binding to the *Ah* receptor. The receptor has a higher affinity for PeCB than for TCB, while the affinity for HCB has been hard to determine owing to the limited aqueous solubility of HCB (Poland and Glover 1977; Bandiera et al. 1982).

TCB, PeCB and HCB are present in technical PCB preparations and their high potency suggests that they are responsible for much of the toxicity of PCB mixtures (Kannan et al. 1987). Furthermore, these three coplanar PCBs have been detected in a wide variety of animal species, such as fish, marine mammals and terrestrial animals, including humans (Tanabe et al. 1986).

TCDD and related substances are well known inducers of certain cytochrome P-450-mediated enzyme activities (Nebert and Jensen 1979; Poland and Knutson 1982). Of the enzyme activities responding to induction by this class of compounds, the aryl hydrocarbon (benzo(*a*)pyrene) hydroxylase (AHH) axctivity is the most frequently studied. The 7-ethoxyresorufin-*O*-deethylase (EROD) activity is, however, considered to be more specifically induced by *Ah* receptor ligands (Phillipson et al. 1984).

In chick embryos, TCB is very toxic compared with the technical PCB preparation Aroclor 1248 (Brunström and Örberg 1982; Brunström and Darnerud 1983). TCB also induces hepatic AHH activity early during chick embryo development (Hamilton et al. 1983; Brunström 1986). In addition to TCB, TCDD and the TCDD congeners 3,3′,4,4′-tetrachloroazobenzene and 3,3′,4,4′-tetrachloroazoxybenzene have proved to be very toxic in chick embryos (Higginbotham et al. 1968; Schrankel et al. 1982).

In this study, TCB, PeCB and HCB were compared regarding their toxicity and potency as inducers of EROD in chick embryos. In addition, the toxicity and inducing potency of a nonplanar chlorobiphenyl, 2,2′,4,4′,5,5′-hexachlorobiphenyl (2-HCB), was studied.

Materials and methods

Eggs. Fertilized hens' eggs (White Leghorn, Shaver) were obtained from Linköpings Kontrollhönseri (Linköping, Sweden). They were incubated at 37.5–38.0° C and 60% relative humidity, and turned every 6 h.

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Chemicals. TCB, PeCB and HCB were synthesized by Dr Åke Bergman, Wallenberg Laboratory, University of Stockholm, Sweden. None of the coplanar PCBs contained TCDD or 2,3,7,8-tetrachlorodibenzofuran (TCDF). 2-HCB (containing <0.5 ppm TCDF) was obtained from Dr Göran Sundström of the National Swedish Environmental Protection Board, Special Analytical Laboratory, Stockholm.

7-Ethoxyresorufin was obtained from Pierce (Rockford, Ill., USA), and resorufin from Eastman Kodak Co. (Rochester, NY, USA). Bovine serum albumin (fraction V), NADPH and Rhodamine B were purchased from Sigma (St Louis, Mo., USA).

Toxicity study. The toxicity of the chlorobiphenyls in chick embryos was studied using two experimental procedures.

In the first procedure, the substances were injected into the air sacs of eggs preincubated for 7 days. Before injection, the eggs were candled, and infertile eggs as well as those containing dead or poorly developed embryos were discarded. Five different doses of each of the coplanar PCBs (3–22, 1–16 and 50–300 μg/kg egg for TCB, PeCB and HCB, respectively) were dissolved in peanut oil and injected into the air sacs through holes drilled in the shells (50 μl oil/egg, 20 eggs/group). A single dose of the non-planar 2-HCB (40 mg/kg egg) was also tested. Immediately after injection, the eggs were rotated to evenly distribute the oil. The eggs of the control group received peanut oil only. The holes were sealed with paraffin and the eggs were placed back into the incubator with the blunt end upward for 1 h, and were thereafter placed horizontally. The eggs were candled 72 h after injection, and the mortality rate was determined. LD_{50} values were estimated by probit analysis of the mortality rates using the Statistical Analysis System, procedure probit (SAS Institute Inc., Cary, NC, USA).

In the second experimental procedure, PeCB (0.2, 0.6 and 2.0 μg/kg egg) and HCB (10, 30 and 100 μg/kg egg), dissolved in an emulsion of peanut oil and lecithin in water, were injected into the yolks of eggs preincubated for 4 days (100 μl emulsion/egg, 20 eggs/dose) as previously described for injection of TCB (Brunström and Darnerud 1983). The eggs were candled every 2nd day after injection, and eggs containing dead embryos were opened for inspection of gross abnormalities in the embryos. The experiment was terminated 14 days after injection (day 18), when the mortality rate was noted and the embryos inspected for malformations and edema. In a separate experiment, 2-HCB was injected using the same procedure (50 mg/kg egg, 10 eggs).

Induction and assay for EROD activity. Chick embryos were treated with the chlorobiphenyls on day 7 of incubation using the procedure for air sac injection described above, and the hepatic EROD activity was determined 72 h later. For each substance, the highest concentration in the eggs was below the LD_{50} value estimated from the 72 h toxicity experiment. Each liver was homogenized in a Potter-Elvehjem homogenizer (glass/Teflon) in 350 μl (control and lowest dose) or 1000 μl (higher doses) of 150 mM Tris-HCl buffer. All enzyme activity determinations were performed in duplicate using fresh homogenate.

The EROD activity was determined essentially as described by Pohl and Fouts (1980). The reactions were carried out in 10 ml round-bottom centrifuge tubes, and the reaction mixture (total vol. 1.0 ml, pH 7.8) contained 0.36 μmol NADPH, 3.0 μmol $MgCl_2$, 1.6 mg bovine serum albumin, 100 μmol Tris-HCl and 0.1 ml liver homogenate. The amount of tissue in the reaction mixture ranged from 2 to 10 mg. The reaction was started by adding 1 nmol 7-ethoxyresorufin. The stock solution of 7-ethoxyresorufin (1 mM in DMSO) was diluted 1:2 with methanol followed by dilution 1:50 with Tris-HCl buffer. A 100 μl amount of this buffer solution of 7-ethoxyresorufin was used to start the reaction. The tubes were shaken in a water-bath at 37° C in air. The reaction time was 10 min for the controls and 1 min for the maximally induced livers. At intermediate doses the reaction time was 2 or 5 min. Under the conditions employed, the amount of product formed was proportional to the amount of tissue and to the reaction time. The reaction was stopped by adding 2.5 ml methanol. The tubes were left at 37° C in the water-bath for 10 min to facilitate precipitation of the proteins. After centrifugation, the fluorescence was determined at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. A solution of rhodamine B in ethylene glycol was used as a daily standard. This solution had previously been calibrated against resorufin.

Results

As a fast test for comparing the toxicity of the chlorobiphenyls, eggs were injected on day 7 and the mortality rate noted 72 h later (Table 1). By probit analysis of the mortality data, the LD_{50} value was calculated to be 8.6, 3.1 and 1.7×10^2 μg/kg egg (29, 9.4 and 4.8×10^2 nmol/kg egg) for TCB, PeCB and HCB, respectively. The single dose of 2-HCB administered, 40 mg (111 μmol)/kg egg, did not cause any mortality among the embryos.

The embryonic mortality by day 18 of incubation after injection of PeCB or HCB into the yolks of eggs preincu-

Table 1. Mortality rate in chick embryos 72 h after injection of 3,3',4,4'-tetrachlorobiphenyl (TCB), 3,3',4,4',5-pentachlorobiphenyl (PeCB), 3,3',4,4',5,5'-hexachlorobiphenyl (HCB) or 2,2',-4,4',5,5'-hexachlorobiphenyl (2-HCB) into the air sacs of eggs preincubated for 7 days.

Substance injected	Dose (μg/kg egg)	Embryonic mortality		LD_{50} value (μg(nmol)/kg egg)
		ratio	%	
Vehicle	–	2/29	7	–
TCB	3	0/20	0	
TCB	6	5/20	25	
TCB	10	14/20	70	8.6 (29)
TCB	16	17/20	85	
TCB	22	19/20	95	
PeCB	1	0/20	0	
PeCB	2	5/20	25	
PeCB	4	14/20	70	3.1 (9.4)
PeCB	8	19/20	95	
PeCB	16	20/20	100	
HCB	50	1/20	5	
HCB	100	1/20	5	
HCB	150	7/20	35	1.7×10^2 (4.8×10^2)
HCB	200	13/20	65	
HCB	300	18/20	90	
2-HCB	40 000	0/20	0	–

Table 2. Mortality rate in chick embryos 14 days after injection of 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) or 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB) into the yolks of eggs preincubated for 4 days.

Substance injected	Dose (μg/kg egg)	Embryonic mortality	
		ratio	%
Vehicle	–	3/20	15
PeCB	0.2	2/20	10
PeCB	0.6	6/20	30
PeCB	2.0	18/20	90***
HCB	10	5/20	25
HCB	30	7/20	35
HCB	100	16/20	80***

*** Significantly higher than the control value (χ^2-test, $p < 0.001$)

bated for 4 days is shown in Table 2. A dose of 2 μg (6.1 nmol) PeCB/kg egg caused 90% mortality among the embryos while 100 μg (277 nmol) HCB/kg egg caused 80% mortality. By day 10 the mortality rate was 50 and 60%, respectively, in these two groups. Liver lesions, hydropericardium, subcutaneous edema, microphthalmia and beak deformities were found in both PeCB- and HCB-treated embryos. 2-HCB did not cause any embryonic mortality or abnormalities when injected into the yolks at a dose of 50 mg (139 μmol)/kg egg.

The EROD activity was induced by all three coplanar chlorobiphenyls tested, PeCB being the strongest inducer and HCB the weakest (Fig. 1). It was assumed that *Ah* receptor ligands, such as these three PCBs, induce EROD activity by the same mechanism, and that the maximally induced activity is the same. The dose-response curves were therefore fitted to a common maximal mean value

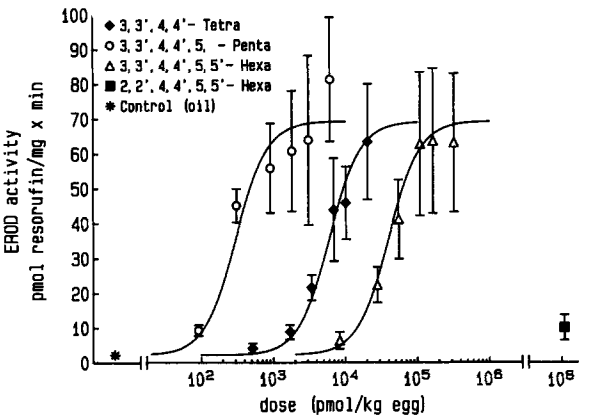

Fig. 1. Log dose-response curves for induction of hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in chick embryos by the coplanar chlorobiphenyls 3,3′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) and 3,3′,4,4′,5,5′-hexachlorobiphenyl (HCB). The activity after treatment with a single dose of the nonplanar chlorobiphenyl 2,2′,4,4′,5,5′-hexachlorobiphenyl (2-HCB) is also shown. The chlorobiphenyls (dissolved in peanut oil) were injected into the air sacs of hens' eggs preincubated for 7 days, and the EROD activity was determined 72 h later. The dose-response curves for the coplanar chlorobiphenyls are fitted to a common maximal value. Each point represents the mean from six livers, and variation is given by the standard deviation. The controls were injected with peanut oil only and their hepatic EROD activity (pmol resorufin/mg × min) was 2.2±0.4 (mean ± sd)

(about 30 times the control value), and the ED_{50} values thus estimated were 0.3, 6 and 40 nmol/kg egg for PeCB, TCB and HCB, respectively. After treatment with 111 μmol 2-HCB/kg egg, the enzyme activity was about 5 times that of the control.

Discussion

The three coplanar PCBs tested proved to be extremely toxic in chick embryos, and their rank order in the 72-h test was PeCB > TCB > HCB. In the 2-week study, PeCB was about 50-fold more toxic than HCB. When comparing TCB, PeCB and HCB injected into the yolks on day 4, TCB also appears to be intermediate between PeCB and HCB in toxicity (data on TCB from: Brunström and Darnerud 1983; Brunström 1988). In both kinds of tests the coplanar PCBs were much more toxic than the nonplanar 2-HCB. It has previously been found that TCB is more than four orders of magnitude more toxic than the nonplanar isomer 2,2′,4,5′-tetrachlorobiphenyl in chick embryos and about three orders of magnitude more toxic than the technical PCB preparation Aroclor 1248 (Brunström and Örberg 1982; Brunström and Darnerud 1983).

The higher toxicity of PeCB compared to TCB is in agreement with the finding by Bandiera et al. (1982) that the *Ah* receptor has a higher affinity for PeCB than for TCB. Millis et al. (1985) reported that the *Ah* receptor binds 3,3′,4,4′-tetrabromobiphenyl (TBB) more avidly than it binds 3,3′,4,4′,5,5′-hexabromobiphenyl (HBB). In contrast, HBB was considered more toxic than TBB in rats, since HBB caused greater thymic involution and more extensive histopathological changes to the liver than did TBB. Millis et al. suggested that the discrepancy between receptor affinity and toxicity was a result of the differential metabolism of these compounds. TBB was metabolized in vitro by rat hepatic microsomes whereas HBB was not, and TBB but not HBB rapidly disappeared from liver and adipose tissue in vivo.

Yoshimura et al. (1979) found PeCB to be more toxic than HCB in rats, while TCB was less toxic than HCB. In guinea pigs, HCB appears to be slightly more toxic than TCB after a single oral dose (McKinney et al. 1985). In the experiments by Millis et al. (1985), TCB was metabolized in vitro by rat hepatic microsomes, whereas HCB was not. It is thus probable that differences related to the metabolism of TCB and HCB can affect their relative toxicity.

In our chick embryo experiments, the metabolism and excretion of TCB were apparently of less importance in determining the relative toxicity of the three chlorobiphenyls than they were in the rodent studies referred to above. Differences in the capacity to metabolize TCB may explain the discrepancy between chick embryos and rodents, but the manner of administration of the substances and the duration of the experiments could also have been important.

The ranking order of the substances as inducers of EROD was the same as their order when ranked according to toxicity. PeCB proved to be extremely potent, the lowest concentration tested in the eggs (92 pmol/kg) giving an enzyme activity about 4-fold that of the controls. EROD activity was also induced by the single dose of 2-HCB (111 μmol/kg), although only to about 5 times the control value. This induction might have been due to contamination of the 2-HCB preparations with trace amounts of TCDF.

It is notable that TCB was only 3-fold less toxic than PeCB in the chick embryos while it was 20 times less potent as an inducer of EROD activity. It cannot be excluded that TCB metabolites of high toxicity, but acting as relatively weak inducers, could have formed in the chick embryos. TCB is metabolized by rat and mouse liver microsomes to reactive metabolites that covalently bind to proteins (Shimada and Sawabe 1983; Shimada 1987).

In the 2-week study, PeCB- and HCB-treated embryos exhibited the kinds of abnormalities previously seen after TCB treatment (Brunström and Darnerud 1983; Brunström 1988). Hydropericardium, subcutaneous edema and liver lesions occurred frequently, and a shortening of the upper beak and microphthalmia were also noted.

Tanabe et al. (1986) suggested that PeCB may be a greater threat than TCDD to humans and wildlife. The results from the present study emphasize the high toxicity of the coplanar PCBs, especially PeCB. The occurrence of coplanar PCBs in the environment and their effects on various animal species need to be more thoroughly investigated.

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