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Induction of both 3-methylcholanthrene- and phenobarbitone-type microsomal enzyme activity by a single polychlorinated biphenyl isomer

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Commercial mixtures of polychlorinated biphenyls (PCBs) are inducers of hepatic microsomal drug-metabolizing enzymes [1]. These enzymes, which include the microsomal mono-oxygenases, are involved in the metabolism of endogenous and exogenous chemicals and in metabolically mediated cellular toxicosis.

Chemicals which induce the microsomal mono-oxygenases have been divided into two categories: one is typified by phenobarbitone (PB) which induces the formation of cytochrome P-450 hemoproteins and the other by 3-methylcholanthrene (3-MC) which induces the formation of cytochrome P-448 hemoproteins [2, 3]. The two induced microsomal mono-oxygenase enzyme systems exhibit different spectral properties, substrate specificities and electrophoretic protein patterns [4].

The hepatic microsomal mono-oxygenases induced after pretreatment with a commercial PCB preparation, such as Aroclor 1254, exhibit properties consistent with a mixed induction pattern produced by the simultaneous administration of both PB and 3-MC. However, it has been shown recently that individual PCB isomers can be categorized on a structural basis into PB-type and 3-MC-type inducers of microsomal enzyme activity [5–7]. The structure–activity relationships indicate that PCB congeners, chlorinated in both the para (4,4′) and ortho (2,2′ or 2,2′,6,6′) positions, are PB-type inducers of cytochrome P-450 activity. Congeners chlorinated in both the para (4,4′) and meta (3,3′; 3,3′,5 or 3,3′,5,5′) positions are 3-MC-type inducers of cytochrome P-448 activity, and any alteration in this substitution pattern results in the loss of this induction activity.

Recent work in our laboratory using a 4-chlorobiphenyl hydroxylase assay system has indicated that some PCB isomers may be mixed inducers [8]. This possibility is reinforced by a report by Dannan *et al.* [9] which describes the simultaneous induction of cytochrome P-450 and cytochrome P-448 activity by 2,3′,4,4′,5,5′-hexabromobiphenyl, isolated as a minor component of the commercial polybrominated biphenyl mixture, Firemaster BP-6. It was proposed that the single isomer possessed the structural requirements for a cytochrome P-448 inducer (3,3′4,4′,5-substitution) and the addition of the single ortho bromo substituent did not eliminate this activity and was also sufficient to impart the capability of microsomal cytochrome P-450 induction. A test for this mixed induction

capability for a PCB isomer was performed using a highly purified (>99.5 per cent) synthetic PCB isomer, 2,3,3',4,4',5,5'-heptachlorobiphenyl (HCBP).

The HCBP congener was prepared by the diazo coupling of 3,4,5-trichloroaniline in excess 1,2,3,4-tetrachlorobenzene, using amyl nitrate [10]. The crude product was purified by a Florisil column chromatographic cleanup and thin layer chromatography (t.l.c.) on silicic acid, using hexane as solvent for both procedures. Crystallization from methanol gave a product which was >99.5 per cent pure as determined by gas chromatography, using a Hewlett–Packard model 5710 chromatograph equipped with a ^{63}Ni electron capture detector and an 0.6 cm × 1.2 m glass column packed with 3 per cent OV 101 on Ultrabonded Carbowax 20M, 80–100 mesh (RFR Corp., Hope, RI). Gas chromatographic analysis of HCBP did not show any minor peaks with retention times corresponding to 3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl. The 220 MHz nuclear magnetic resonance spectrum of the product (in CCl_4) gave singlet resonances at 7.40 (2H) and 7.35 (1H) p.p.m.

Each inducer was administered to five 1-month old male Wistar rats (average weight, 100–120 g). HCBP was administered intraperitoneally at 30 μmoles/kg (low dose) or 150 μmoles/kg (high dose) in corn oil (0.5 ml) on days 1 and 3 and killed by cervical dislocation on day 6. 3-MC (100 μmoles/kg) in corn oil (0.5 ml) and PB (400 μmoles/kg) in isotonic saline solution (0.5 ml) were administered individually and co-administered on days 1 and 2 to the groups of experimental animals which were then killed on day 3 by cervical dislocation. Microsomes from perfused livers were collected as a 100,000 *g* pellet from a 10,000 *g* supernatant fraction as described [11]. For both spectral and enzymatic assays, the final concentration of microsomal protein was 1.0 mg/ml. The carbon monoxide (CO)-difference spectrum was determined as described [12] from the dithionite-reduced microsomes:CO complex minus dithionite-reduced microsomes spectrum. The peak or shoulder at 420 nm was attributed to small amounts of contaminating hemoglobin as identified by the carbon monoxyhemoglobin minus oxyhemoglobin spectrum. The ethylisocyanide (EIC)-difference spectrum was determined in a manner similar to the CO-difference spectrum except that EIC was added to the sample cuvette (final concentration 4.5 mM) instead of carbon monoxide. All spectra were recorded on a Cary 118C spectrophotometer with a repetitive scan accessory. Spectra were calibrated with holmium oxide.

The rate of oxidative *N*-demethylation of 4-dimethylaminoantipyrine (DMAP) was measured by quantifying the production of formaldehyde. The formaldehyde, trapped as the semicarbazone, was developed in double strength Nash reagent [13]. The rate of benzo[*a*]pyrene hydroxylation was measured by a radioassay [14] in which the base-soluble metabolites were quantified following hexane extraction to remove the benzo[*a*]pyrene starting material. The activity of NADPH–cytochrome P-450 reductase was measured by the reduction of cytochrome *c* [15]. The content of cytochrome b_5 was measured spectrophotometrically from the NADH-reduced microsomes minus oxidized microsomes [15] using the corrected extinction coefficient of 185 cm^{-1} mM^{-1} [12]. Residues of HCBP in liver were determined as described [16].

The data summarized in Fig. 1 and Table 1 confirm that the spectral and kinetic properties of rats pretreated with heptachlorobiphenyl at 30 μmoles/kg are similar to those observed for non-induced animals. At higher dose levels (150 μmoles/kg) the results were consistent with a mixed induction pattern. Cytochrome P-450 levels increased and the reduced cytochrome P-450: CO binding spectrum peak shifted 1.5 nm downfield from 450.0 to 448.5 nm. The ethylisocyanide-difference spectrum 455/428 ratio increased from 0.48 to 1.22 with the 455.0 peak shifted downfield to 452.4 nm and the 428.0 nm peak shifted upfield to 429.0 nm. Like the spectral characteristics of microsomes prepared from rats co-administered PB and 3-MC, the spectral characteristics of microsomes harvested from the HCBP-pretreated rats were intermediate to microsomes prepared from the PB-pretreated and 3-MC-pretreated rats. The activities of benzo[*a*]pyrene hydroxylase, 4-dimethylaminoantipyrine *N*-demethylase and NADPH-cytochrome *c* reductase were stimulated over controls by 11.3-, 2.5- and 1.7-fold, respectively. The magnitude of the increases in these microsomal enzyme activities were commensurate with induction by both PB and 3-MC (Table 1). Residue analysis of pooled liver samples identified 5.1 and 59.4 μg

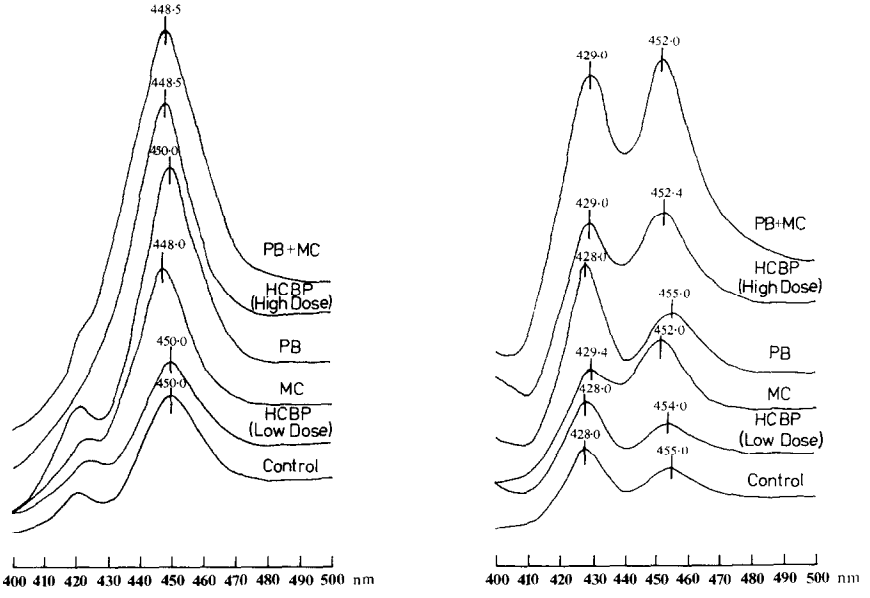

Fig. 1. Carbon monoxide (left panel)- and ethylisocyanide (right panel)-difference spectra of microsomes prepared from control (corn oil-pretreated), phenobarbitone (PB)-, 3-methylcholanthrene (MC)-, phenobarbitone plus 3-methylcholanthrene (PB + MC)- and 2,3,3',4,4',5,5'-heptachlorobiphenyl (HCBP)-pretreated rats.

Table 1. Comparison of the effects of various pretreatments on several variables of hepatic microsomal drug metabolism

Treatment	DMAP *N*-demethylase (nmoles/mg protein-min)	Benzo[*a*]pyrene hydroxylase (nmoles/mg protein-min)	NADPH-cytochrome *c* reductase (nmoles/mg protein-min)	Cytochrome b_5 (nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)	Ethylisocyanide-difference spectra		
						Peak 428 nm (A_{428}-A_{500})	Peak 455 nm (A_{455}-A_{500})	Ratio of 455 nm peak to 428 nm peak
Corn oil	3.47 ± 0.12	0.158 ± 0.021	68.6 ± 10.9	0.19 ± 0.02	0.64 ± 0.07	0.033 ± 0.005	0.016 ± 0.001	0.48 ± 0.07
Phenobarbitone	8.38 ± 0.70	0.539 ± 0.054	182 ± 33	0.28 ± 0.04	1.48 ± 0.15	0.092 ± 0.026	0.053 ± 0.012	0.58 ± 0.07
3-Methylcholanthrene	3.79 ± 0.34	2.41 ± 0.13	79.0 ± 16.1	0.27 ± 0.03	1.22 ± 0.08	0.032 ± 0.001	0.055 ± 0.008	1.72 ± 0.25
Phenobarbitone plus 3-methylcholanthrene	8.70 ± 2.1	2.83 ± 0.16	163 ± 35	0.31 ± 0.09	2.31 ± 0.55	0.119 ± 0.024	0.136 ± 0.027	1.14 ± 0.16
2,3,3',4,4',5,5'-Heptachlorobiphenyl (low dose)*	3.79 ± 0.21	0.243 ± 0.062	77.2 ± 6.7	0.21 ± 0.02	0.70 ± 0.09	0.039 ± 0.004	0.021 ± 0.004	0.54 ± 0.07
2,3,3',4,4',5,5'-Heptachlorobiphenyl (high dose)†	8.57 ± 0.75	1.78 ± 0.13	115 ± 12	0.24 ± 0.03	1.70 ± 0.09	0.049 ± 0.013	0.060 ± 0.016	1.22 ± 0.18

* Low dose = 30 μmoles/kg.
† High dose = 150 μmoles/kg.

HCBP/g of liver in rats treated with the low and high dose, respectively. Although the residue levels were disproportionally lower in the low dose animals, the failure of this dose to induce micosomal enzyme activity cannot be explained by the inability of the chemical to reach the liver.

The synthetic isomer 2,3,3′,4,4′,5,5′-heptachlorobiphenyl was chosen because it contains the structural requirements for a cytochrome P-448 inducer (3,3′,4,4′,5,5′-hexachloro substitution) plus one additional ortho chloro substituent. The HCBP has been tested previously in female rats and shown at low doses (14 μmoles/kg) not to be an inducer of the microsomal mono-oxygenases [6]. The isomer was purified via chromatography and crystallization, and the Florisil chromatographic procedure was used to remove any possible chlorinated dibenzofuran contaminants.

The results demonstrate that a single PCB isomer can induce both cytochrome P-448- and P-450-type spectral properties and enzyme activities. Although previous reports have indicated that a number of hexachlorobiphenyl isomers were mixed inducers [17, 18], it has been suggested that the observed 3-MC-type activity may have been due to highly active impurities [19]. This was illustrated by the identification of 2,3,7,8-tetrachlorodibenzofuran (TCDF) as a contaminant in a 99 per cent pure 2,2′,4,4′,5,5′-hexachlorobiphenyl preparation [19]. The >99.5 per cent pure HCBP used in this study was chromatographed on a Florisil column to remove any possible TCDF and related polar contaminants, and was further cleaned up by t.l.c. This latter step readily separated the less polar HCBP from any possible contamination by the more polar 3-MC-type PCB inducers, 3,3′,4,4′,5,5′-hexachlorobiphenyl and 3,3′,4,4′-tetrachlorobiphenyl. It has been shown that commercial PCBs are also mixed inducers [1]; however, the PCB congeners which induce 3-MC-like activity, 3,3′,4,4′-tetrachlorobiphenyl, 3,3′,4,4′,5,5′-hexachlorobiphenyl and 3,3′,4,4′,5-pentachlorobiphenyl, are only minor constituents of these mixtures [20, 21]. It is conceivable that TCDF, a highly potent inducer of cytochrome P-448 hemoproteins, and the other chlorinated dibenzofuran congeners, which have been detected in trace amounts in commercial PCBs [22, 23], may be partially responsible for the observed mixed induction activity. Although HCBP is a minor component of commercial PCB mixtures, a number of structurally related congeners which contain the 3,3′,4,4′-tetrachloro and the 3,3′,4,4′,5-pentachloro substitution pattern plus one ortho chloro substituent (e.g. 2,3,3′,4,4′,5-hexachlorobiphenyl, 2,3′,4,4′,4,5′-hexachlorobiphenyl, 2,3′,4,4′,5-pentachlorobiphenyl and 2,3,3′,4,4′-pentachlorobiphenyl) have been identified in commercial PCBs [17, 18]. The synthesis of these compounds is currently in progress and investigation of their effects as microsomal enzyme inducers will clarify their role in the commercial PCB mixtures.

It has been suggested that some of the toxic properties of several classes of halogenated aromatics may be related to their induction of microsomal cytochrome P-448 activity [5] and, therefore, the biological effects, environmental occurrence and toxicity of the mixed inducers warrant further investigation.

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