Biochemical Pharmacology, Vol. 32, No. 14, pp. 2269–2279, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

INDUCTION OF RAT HEPATIC MICROSOMAL CYTOCHROME P-450 BY 2,3′,4,4′,5,5′-HEXACHLOROBIPHENYL

ANDREW PARKINSON, LARRY W. ROBERTSON and STEPHEN H. SAFE*
Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843, U.S.A.

(*Received* 13 *September* 1982; *accepted* 28 *December* 1982)

Abstract—The following evidence suggests that 2,3′,4,4′,5,5′-hexachlorobiphenyl resembles isosafrole as an inducer of hepatic microsomal cytochrome P-450d in the immature male Wistar rat. First, the major hepatic microsomal polypeptide (M_r = 52,000), intensified after treatment of rats with 2,3′,4,4′,5,5′-hexachlorobiphenyl, comigrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with cytochrome P-450d (i.e. the major isosafrole-inducible polypeptide) but had an electrophoretic mobility intermediate between cytochrome P-450b ($M_r \approx$ 51,500) and cytochrome P-450c (M_r = 56,000) (i.e. the major phenobarbital- and 3-methylcholanthrene-inducible polypeptides respectively). Second, when pairs of various xenobiotics were coadministered to rats at doses effecting maximal induction of hepatic microsomal cytochrome P-450, the inductive effects of 2,3′,4,4′,5,5′-hexachlorobiphenyl were additive with those of phenobarbital, 3-methylcholanthrene and pregnenolone-16α-carbonitrile but not with those of isosafrole. The inductive effects of phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile and isosafrole were all expressed additively with each other. Third, in contrast to phenobarbital and pregnenolone-16α-carbonitrile treatment, treatment of rats with 2,3′,4,4′,5,5′-hexachlorobiphenyl, isosafrole or 3-methylcholanthrene failed to increase markedly the proportion of total cytochrome P-450 capable of forming a 446 nm-absorbing complex with metyrapone. Fourth, the *in vitro* metabolism of isosafrole, catalyzed by hepatic microsomes from rats treated with 2,3′,4,4′,5,5′-hexachlorobiphenyl, isosafrole or 3-methylcholanthrene, produced complexes between ferrous cytochrome P-450 and a methylenedioxyphenyl metabolite, the spectra of which were between 400 and 500 nm and were similar to each other but which were readily distinguishable from the spectra of the product adducts formed during the metabolism of isosafrole by hepatic microsomes from rats treated with corn oil (control), phenobarbital, or pregnenolone-16α-carbonitrile.

Phenobarbital (PB) and 3-methylcholanthrene (MC) are prototypes of two classes of inducers of hepatic microsomal cytochrome P-450 [1]. The major PB-inducible form of cytochrome P-450, designated cytochrome P-450b†, and the major MC-inducible form, designated cytochrome P-450c, have been purified to apparent homogeneity from rat liver microsomes and shown to be true isozymes with differing catalytic, spectral, electrophoretic and immunologic properties [2–5]. A third isozyme, designated cytochrome P-450a, is modestly inducible by both PB and MC [2, 3, 6].

Not all xenobiotics that induce hepatic microsomal cytochrome P-450 can be categorized as PB- or MC-type inducers. Two notable exceptions are pregnenolone-16α-carbonitrile (PCN) [6–9] and isosafrole [10–13]. The major isosafrole-inducible form of cytochrome P-450, designated cytochrome P-450d [11], and the major PCN-inducible form, designated cytochrome P-450$_{PCN}$ [8, 9], have been purified to electrophoretic homogeneity from rat liver microsomes. These hemoproteins have been shown by several criteria to be distinct from the major PB- and MC-inducible forms of cytochrome P-450.

The commercial polychlorinated biphenyl (PCB) mixture, Aroclor 1254, simultaneously induces cytochromes P-450a, P-450b and P-450c and is classified, therefore, as a mixed (PB + MC)-type inducer [2, 6, 14, 15]. The large number of individual PCB congeners that have been shown to induce rat liver microsomal cytochrome P-450 have been categorized exclusively into PB-, MC- or mixed-type inducers [16–21]. We have reported previously that, when administered to immature male Wistar rats, 2,3′,4,4′,5,5′-hexachlorobiphenyl (HCBP) resembles a PB-type inducer of hepatic microsomal cytochrome P-450 [19]. However, this finding was unexpected for two reasons. First, the bromo analogue of HCBP, namely 2,3′,4,4′,5,5′-hexabromobiphenyl, has been reported to be a mixed-type inducer [22]. Second, only four PCB congeners with strictly MC-type inducing characteristics have been identified (namely 3,4,4′,5-tetra-, 3,3′,4,4′-tetra-, 3,3′,4,4′,5-penta- and 3,3′,4,4′,5,5′-hexachlorobiphenyls), none of which is chlorinated in the *ortho* (2, 2′, 6 or 6′) positions [16–20]. Of the eight possible mono-*ortho* derivatives of these four MC-type PCBs, all were categorized as mixed-type inducers of cytochromes P-450 with the exception of HCBP [19, 20, 23]. The available evidence suggests, therefore, that HCBP lacks the MC-type inducing charac-

* Send correspondence to: Dr. Stephen H. Safe, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843, U.S.A.

† When applicable, the nomenclature of Ryan and her coworkers is used.

teristics exhibited by both its bromo analogue and by various structurally related PCBs.

In view of its unexpected properties, we have further studied the induction of rat hepatic microsomal cytochrome P-450 by HCBP.* We report here the unexpected finding that HCBP resembles isosafrole as an inducer of cytochrome P-450d in the immature male Wistar rat.

MATERIALS AND METHODS

Chemicals. PCN and 2-*n*-heptylbenzimidazole were gifts from G. D. Searle & Co. and Dr. S. R. Challand of the Wellcome Research Laboratories, Beckenham, England, respectively. Isosafrole (1,2-[methylenedioxy]-4-propenyl benzene) was purchased from Eastman Organic Chemicals, Rochester, NY. Metyrapone (2-methyl-1,2-di[3-pyridyl]-1-propanone) and *trans*-stilbene oxide were obtained from the Aldrich Chemical Co., Milwaukee, WI.

Tritiated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (sp. act. 50–52 Ci nmole) was purchased from KOR Isotopes, Cambridge, MA. The radiolabeled TCDD contained approximately 80% of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin congener, as judged by the manufacturer and confirmed in our laboratory by gas–liquid chromatography. For safety reasons, no attempt was made to purify further the [^{3}H]TCDD. The synthesis and purification (>99%) of HCBP [19] and 2,2′,4,4′,5,5′-hexachlorobiphenyl [21] and the sources of the other reagents used in this study have been described elsewhere [19–21].

Animal treatment. One-month-old male Wistar rats (Woodlyn Laboratories, Guelph, Ontario, Canada) were housed in wire cages and allowed free access to water and certified Purina Rodent Chow, No. 5002. With the exception of PB, which was dissolved in isotonic saline, the inducers were dissolved or suspended in corn oil and administered by intraperitoneal injection to rats between 9.00 and 10.00 a.m. (N, the number of rats per group, is given in Results). Rats were killed by cervical dislocation 24 hr after three consecutive daily injections of PB (400 μmoles/kg), MC (100 μmoles/kg), isosafrole (1 mmole/kg) or PCN (150 μmoles/kg) or after five consecutive daily injections of *trans*-stilbene oxide (2 mmoles/kg), at the doses indicated. HCBP was administered to rats on days 1 and 3 at doses of 150 or 300 μmoles/kg, and the rats were killed on day 4, 6, 8, 11 or 15. When the inducers were coadministered in various combinations, HCBP and 2,2′,4,4′,5,5′-hexachlorobiphenyl were injected at the 150 μmoles/kg dose on days 1 and 3 and the rats killed on day 5 while PB, MC, isosafrole and PCN were administered at the above doses for three consecutive days commencing on day 3.

Animals injected with corn oil (5 ml/kg) served as controls. All rats were fasted over the last 24 hr to lower liver glycogen levels.

Preparation of liver microsomes. The rat livers were cleared of blood by perfusing via the hepatic-portal vein with ice-cold isotonic saline containing 100 μM EDTA. The blanched livers were transferred to pre-weighed, ice-cold solutions of 0.25 M sucrose containing 100 μM EDTA, and the liver weights were determined. Microsomal fractions were prepared from a 10,000 *g* supernatant fraction of whole liver homogenate by ultracentrifugation (100,000 *g* for 60 min at 4°).

Assays. The concentrations of protein [24], cytochrome P-450 [25] and cytochrome b_5 [26] and the activities of aminopyrine *N*-demethylase [27], benzo[*a*]pyrene hydroxylase [28, 29] and 4-chlorobiphenyl hydroxylase [30] were determined as described. Minor procedural modifications and the conditions for determining the ethylisocyanide-difference spectrum are described elsewhere [19].

The binding of metyrapone (final concentration, 1 mM) to ferrous cytochrome P-450 was determined from the metyrapone-difference spectrum of dithionite-reduced microsomes between 400 and 500 nm. The concentration of ferrous cytochrome P-450:metyropone complex was estimated from the increase in absorbance at 446 nm (A_{446}-A_{490}) based on a millimolar extinction coefficient of 52 $mM^{-1} \cdot cm^{-1}$ [31].

The *in vitro* metabolism of isosafrole, with the subsequent formation of a 427/425 nm-absorbing complex between a methylenedioxyphenyl metabolite of isosafrole and ferrous cytochrome P-450, was studied essentially as described [32, 33]. Microsomal suspensions of 1 mg protein/ml potassium phosphate buffer (100 mM, pH 7.4) containing $MgCl_2$ (5 mM) and NADPH (200 μM) were divided equally between two matched cuvettes, and a baseline of equal absorbance was recorded. Isosafrole, dissolved in phosphate buffer–DMSO (9:1, v/v), was added to the sample cuvette (final concentration, 200 μM), and a corresponding volume of solvent was added to the reference cuvette. Product adduct formation was monitored by scanning between 400 and 500 nm at 1- or 2-min intervals for up to 20 min of incubation. Spectra were recorded with a Cary 118C spectrophotometer equipped with a repetitive scan accessory.

Displacement of the isosafrole metabolite from cytochrome P-450. To assess more accurately the ligand-binding properties of hepatic microsomes isolated from rats pretreated with isosafrole, the isosafrole metabolite formed *in vivo* [11, 32–34] was displaced from cytochrome P-450 *in vitro* by 2-*n*-heptylbenzimidazole as described [34]. Briefly, 2-*n*-heptylbenzimidazole (final concentration, 200 μM) was added in 10 μl of *N,N*-dimethylformamide to 10 ml of microsomal suspension (1 mg protein/ml) that had been preincubated at 37° for 5 min. Incubation at 37° was continued for 20 min after which the carbon monoxide- and ethylisocyanide-difference spectra were recorded as described above.

Cytosolic receptor binding studies. The sucrose-density gradient centrifugation method of Okey *et al.* [35] was used with minor modifications [36] to determine the concentration (EC_{50}) of isosafrole effecting displacement of 50% of [^{3}H]TCDD specifically bound to rat hepatic cytosolic receptor protein. The final concentrations of [^{3}H]TCDD and cytosolic protein were 1.0 nM and 5–6 mg protein/ml, respectively, and the various concentrations of isosafrole

* The term induction is used in the general sense described by Conney [1] and refers to an increase in specific content or activity.

(10^{-6} to 10^{-2} M) were added in dimethyl sulfoxide (DMSO).

Electrophoresis of microsomal proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [37] using a slab gel apparatus from Hoefer Scientific Instruments. The separating gel contained 7.5% acrylamide and was 0.75 mm thick and 10 cm long. After electrophoresis, proteins were fixed for 1 hr in a solution of 25% 2-propanol and 10% acetic acid, stained for 1 hr in the same solution containing 0.05% Coomassie Brilliant Blue R-250, and destained overnight in 10% 2-propanol and 10% acetic acid.

Microsomal polypeptides corresponding to epoxide hydrolase or electrophorectically distinct forms of cytochrome P-450 were tentatively identified by comparison with the following enzymes purified and provided by D. Ryan and W. Levin of the Department of Biochemistry and Drug Metabolism, Hoffmann–La Roche Inc., Nutley, NJ: epoxide hydrolase (M_r = 49,000), cytochrome P-450b-LE (M_r = 52,000) and cytochrome P-450c (M_r = 56,000) purified from Long Evans rats treated with Aroclor 1254 and cytochrome P-450b-H (M_r = 51,500–52,000) purified from Aroclor 1254-treated Holtzman rats.

Statistical analysis. The statistical significance of differences between the means of control and treated groups for each variable was analyzed by Student's *t*-test and the method of Dunnett for multiple comparisons with a control [38].

RESULTS AND DISCUSSION

Time course of induction by HCBP. A series of experiments was designed to address the possibility that during our previous study on the induction of rat hepatic microsomal cytochrome P-450 by HCBP we failed to detect the MC-type inducing characteristics exhibited by the bromo-analogue of HCBP [22] and by various PCBs that are structurally related to HCBP [19, 23]. A time–course study, in which rats were injected with HCBP (150 μmoles/kg) on days 1 and 3 and killed on day 4, 6, 8, 11 or 15, revealed that the level of hepatic microsomal cytochrome P-450 and the rate of aminopyrine *N*-demethylation were maximally induced (~2.5-fold) by day 6 and remained in steady state until day 15 (Fig. 1). Although the level of total cytochrome P-450 remained essentially constant during the time–course study, the absorbance maximum of the ferrous cytochrome P-450:carbon monoxide complex changed by 1 nm from 450 nm on day 4 to 449 nm on day 15. This progressive hypsochromic shift was accompanied by a time-dependent increase in the rate of hydroxylation of benzo[*a*]pyrene and 4-chlorobiphenyl. Treatment of rats with PB or the PB-type inducer, 2,2′,4,4′,5,5′-hexachlorobiphenyl, also caused a ~2.5-fold increase in cytochrome P-450 content and aminopyrine *N*-demethylase activity but failed to cause (a) a hypsochromic shift in the carbon monoxide-difference spectrum of dithionite-reduced hepatic microsomes, (b) an increase in the rate of 4-chlorobiphenyl hydroxylation, or (c) more than a 2- to 3-fold increase in benzo[*a*]pyrene hydroxylase

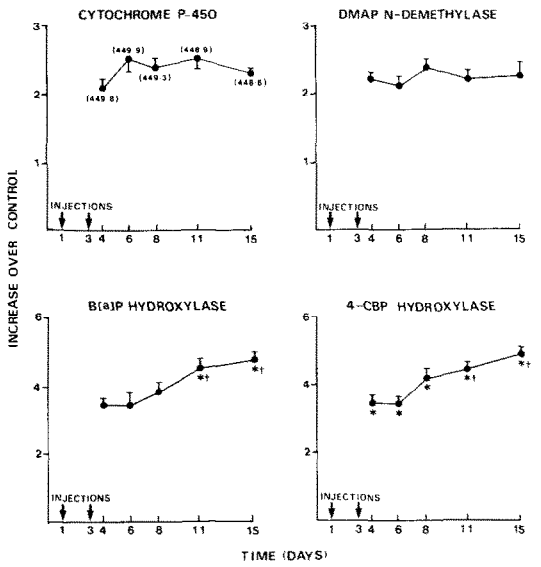

Fig. 1. Time course of induction of rat hepatic microsomal cytochrome P-450 and associated catalytic activities by 2,3′,4,4′,5,5′-hexachlorobiphenyl. Rats (three per group) were injected with HCBP (150 μmoles/kg) on days 1 and 3 and killed on day 4, 6, 8, 11 or 15. For cytochrome P-450, λ_{max} values (nm) are given in parentheses. The substrates DMAP, B[*a*]P and 4-CBP correspond to aminopyrine, benzo[*a*]pyrene and 4-chlorobiphenyl respectively. Typical control values obtained throughout the time–course study for cytochrome P-450 (0.680 ± 0.023 nmole/mg protein), aminopyrine *N*-demethylase [3.65 ± 0.25 nmoles HCHO formed·min^{-1}·(mg protein)$^{-1}$], benzo[*a*]pyrene hydroxylase [175 ± 23 pmoles metabolized·min^{-1}·mg $protein^{-1}$] and 4-chlorobiphenyl hydroxylase [124 ± 15 pmoles metabolized·min^{-1}·(mg protein)$^{-1}$] were as indicated. Each bar represents the standard deviation of the mean. All values were increased significantly (α = 0.01) over values for PB-treated rats (*) or HCBP-treated rats killed on day 6 (†) are labeled as shown.

activity. In contrast, treatment of rats with MC failed to increase aminopyrine *N*-demethylase activity but caused a 2-fold increase in hepatic microsomal cytochrome P-450 content which was accompanied by a 2 nm hypsochromic shift from 450 to 448 nm and a ~15-fold increase in the rate of hydroxylation of both benzo[*a*]pyrene and 4-chlorobiphenyl. The results of the time–course study confirmed that, when administered to rats on days 1 and 3 and the rats killed on day 6, HCBP closely resembles a PB-type inducer of cytochrome P-450 and further demonstrate that, when the duration of induction of cytochrome P-450 by HCBP is prolonged, HCBP apparently exhibits weak MC-type inducing characteristics.

Electrophoresis of microsomal proteins. The major PB- and MC-inducible forms of cytochrome P-450, namely cytochromes P-450b (M_r = 52,000) and P-450c (M_r = 56,000), are readily resolved by SDS-PAGE [2]. Therefore, in an attempt to confirm that HCBP is a PB-type inducer with weak MC-type properties, hepatic microsomes from rats treated with HCBP were subjected to electrophoresis (Fig. 2). Polypeptides corresponding to the major PB- and MC-inducible forms of cytochrome P-450 and to

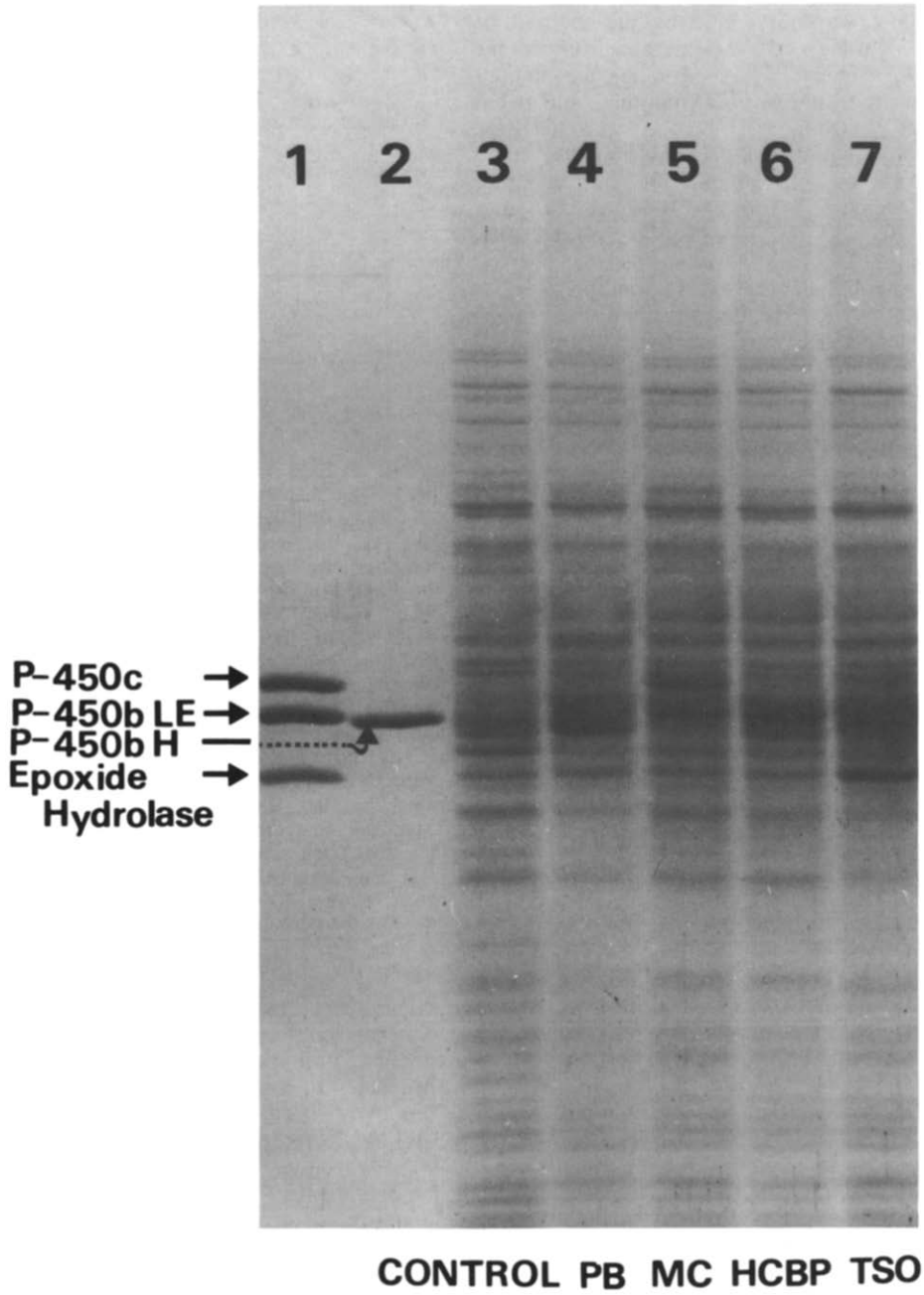

Fig. 2. Electrophoresis of hepatic microsomes from immature male Wistar rats treated with corn oil (control), phenobarbital (PB), 3-methylcholanethrene (MC), 2,3′,4,4′,5,5′-hexachlorobiphenyl (HCBP) or *trans*-stilbene oxide (TSO). Microsomal proteins (6 μg) from control rats (well 3) and from rats treated with PB (well 4), MC (well 5), HCBP (well 6) or TSO (well 7) were subjected to SDS-PAGE according to the method of Laemmli [37]. Rats treated with HCBP on days 1 and 3 were killed on day 6. Well 1 contains a mixture of 0.3 μg epoxide hydrolase (M_r = 49,000), cytochrome P-450b-LE (M_r = 52,000) and cytochrome P-450c (M_r = 56,000), purified from Long Evans rats. Well 2 contains 0.3 μg cytochrome P-450b-H (M_r = 51,500–52,000) purified from Holtzman rats.

epoxide hydrolase were identified by comparisons with hepatic microsomes from rats treated with corn oil (control), PB, MC or *trans*-stilbene oxide and by comparisons with authentic standards supplied by D. Ryan and W. Levin.

Recently, structural differences were detected in cytochrome P-450b purified from different strains of rats [39] for which reason two forms of cytochrome P-450b were used as standards: one was purified from Aroclor 1254-induced Long Evans rats (cytochrome P-450b-LE, $M_r = 52{,}000$) and the other, which has a slightly greater electrophoretic mobility, was purified from Aroclor 1254-induced Holtzman rats (cytochrome P-450b-H, $M_r = 51{,}500$–$52{,}000$). The major hepatic microsomal polypeptide, intensified after treatment of Wistar rats with PB ($M_r = 51{,}500$), was clearly lower in molecular weight than cytochrome P-450b-LE and aligned more closely with cytochrome P-450b-H. This suggests that the strain-dependent difference in cytochromes P-450b that was shown previously to occur between Long Evans and Holtzman rats [39] also occurs between Long Evans and Wistar rats.

In contrast to cytochromes P-450b, cytochromes P-450c from Long Evans and Holtzman rats are indistinguishable by SDS-PAGE [40], for which reason only cytochrome P-450c from Long Evans rats was used as standard. Treatment of Wistar rats with MC resulted in an intensification of two polypeptides of $M_r = 52{,}000$ and 56,000, the larger of which co-migrated with cytochrome P-450c. Although the lower molecular weight protein ($M_r = 52{,}000$) induced by MC co-migrated with cytochrome P-450b-LE, immunochemical analysis indicates that this PB-inducible hemoprotein is not inducible by MC [6]. It has been shown recently that this lower molecular protein in hepatic microsomes from MC-induced Long Evans rats contains cytochrome P-450d [41], a major hemoprotein inducible by isosafrole [11].

The polypeptide ($M_r \approx 51{,}500$) that co-migrated with cytochrome P-450b-H and represented the major hepatic microsomal polypeptide intensified after treatment of Wistar rats with PB was, unexpectedly, only a minor polypeptide in hepatic microsomes from HCBP-treated rats. The major polypeptide intensified by HCBP treatment ($M_r = 52{,}000$) co-migrated with cytochrome P-450b-LE and the lower molecular weight protein induced by MC. The electrophoretic profiles of purified epoxide hydrolase ($M_r = 49{,}000$) and hepatic microsomes from rats treated with the potent epoxide hydrolase inducer, *trans*-stilbene oxide [6, 42–44], clearly indicated that the major HCBP-inducible polypeptide was not epoxide hydrolase. Similarly, the major HCBP-inducible polypeptide cannot correspond to cytochrome P-450a ($M_r = 48{,}000$) because this hemoprotein has an even greater electrophoretic mobility than epoxide hydrolase [2].

Based on its electrophoretic mobility, the major HCBP-inducible polypeptide could be one of the following four previously characterized forms of rat hepatic microsomal cytochrome P-450: (1) cytochrome P-450d ($M_r = 52{,}000$), the major isosafrole-inducible and a minor MC-inducible hemoprotein [11, 41]; (2) cytochrome P-450$_{PCN}$, which was purified from Sprague–Dawley rats and assigned a M_r of 51,000 compared to 50,000 for the major PB-inducible hemoprotein and 54,000 for the major MC-inducible hemoprotein [8]; (3) cytochrome P-450b-LE ($M_r = 52{,}000$) in which case the Wistar rat would have to possess the structural genes for both cytochromes P-450b-H and P-450b-LE, or (4) cytochrome P-450e ($M_r = 52{,}000$–$52{,}500$), a minor PB-inducible hemoprotein recently purified from Aroclor 1254-treated Long Evans rats [45].

The electrophoretic profiles of hepatic microsomes from rats treated with HCBP, PCN or isosafrole are consistent with the first of these possibilities, namely that the major HCBP-inducible polypeptide is cytochrome P-450d, but are inconsistent with the second possibility, namely that the major HCBP-inducible polypeptide is cytochrome P-450$_{PCN}$ (Fig. 3). The major HCBP- and isosafrole-inducible polypeptides ($M_r = 52{,}000$) each had a slightly higher molecular weight than the major PCN-inducible polypeptide ($M_r \approx 51{,}500$) and both co-migrated with cytochrome P-450b-LE, which has been shown to have an identical electrophoretic mobility to cytochrome P-450d [11]. Furthermore, the pattern of polypeptides unique to hepatic microsomes from rats treated with isosafrole or HCBP was also observed upon electrophoresis of hepatic microsomes from rats coadministered isosafrole with HCBP.

Metyrapone binding. The third and fourth possibilities, namely that the major HCBP-inducible polypeptide is either cytochrome P-450b-LE or cytochrome P-450e, are incompatible with the results of the metyrapone-binding study shown in Table 1. The addition of metyrapone (1 mM) to dithionite-reduced microsomes from rat liver resulted in the formation of a stable ferrocytochrome P-450:metyrapone complex which absorbed maximally at 446 nm. The concentration of metyrapone complex increased by 4.3-, 2.2- and 3.0-fold following treatment of rats with PB, MC or HCBP, respectively, whereas the concentration of cytochrome P-450 (carbon monoxide complex) increased 2.5-, 2.1 and 2.5-fold respectively. The proportion of the total cytochrome P-450 that bound metyrapone increased 1.8-fold from 44 (control) to 78% following treatment of rats with PB. In contrast to PB treatment, treatment of rats with either MC or HCBP caused little or no increase (<1.2-fold) in the proportion of total cytochrome P-450 that bound metyrapone. Cytochromes P-450b-LE, P-450b-H and P-450e are essentially indistinguishable by their interaction with metyrapone [40, 45]. Therefore, the ability of PB, but not HCBP, to increase the proportion of cytochrome P-450 that bound metyrapone establishes that the major HCBP-inducible polypeptide is neither cytochrome P-450e nor a second form of cytochrome P-450b. We reported recently [46] that treatment of rats with PCN increased the proportion of cytochrome P-450 that bound metyrapone to 80% (i.e. to the same extent as PB treatment), whereas a slight decrease was observed following treatment of rats with isosafrole. Furthermore, Ryan *et al.* [45] have shown that metyrapone does not form a 446 nm-absorbing complex with ferrous cytochrome P-450d. Therefore, the results of the metyrapone-binding study support the hypothesis based on

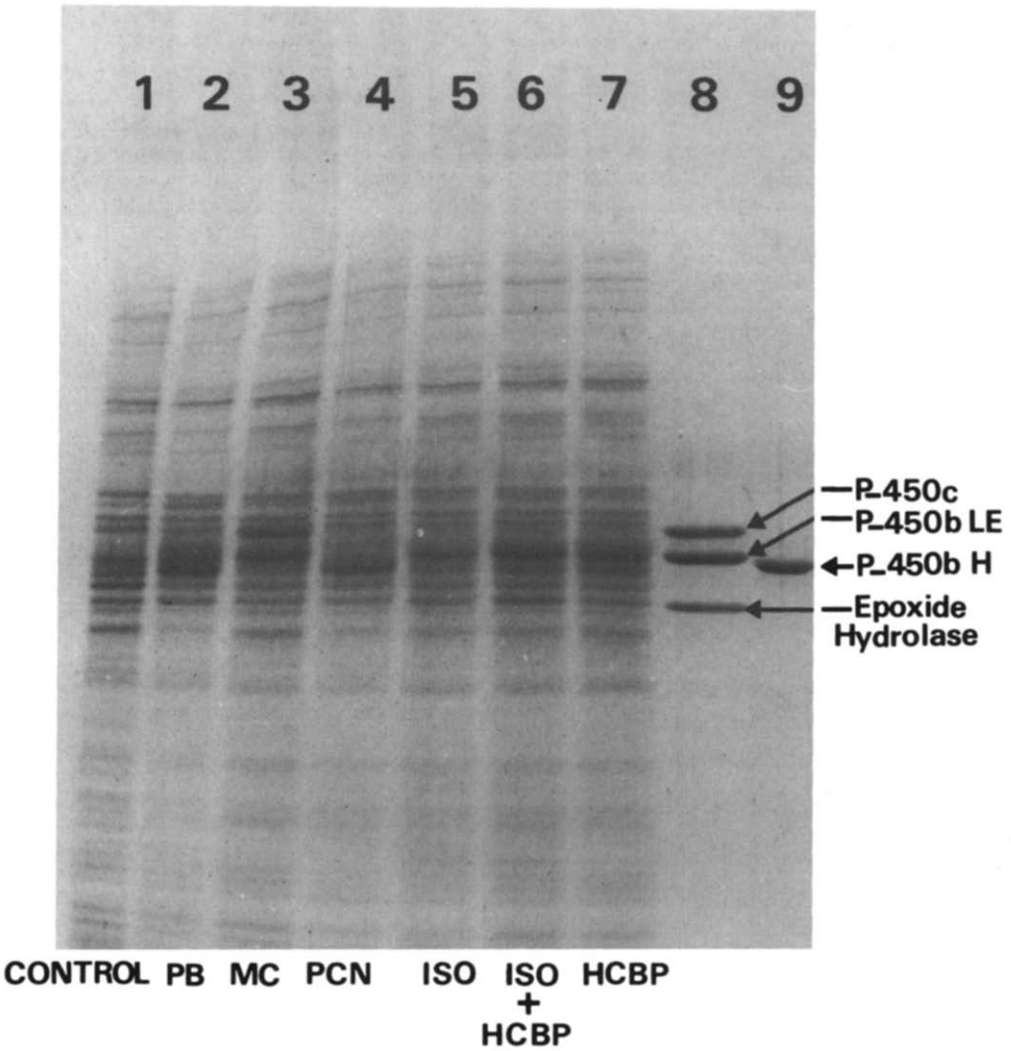

Fig. 3. Electrophoresis of hepatic microsomes from variously treated immature male Wistar rats. Microsomal proteins (6 μg) from rats treated with corn oil (control, well 1), PB (well 2), MC (well 3), PCN (well 4), isosafrole (ISO, well 5), isosafrole plus HCBP (well 6) or HCBP (well 7) were subjected to SDS-PAGE according to the method of Laemmli [37]. Rats treated with HCBP on days 1 and 3 were killed on day 6. Well 8 contains a mixture of 0.3 μg epoxide hydrolase (M_r = 49,000), cytochrome P-450b-LE (M_r = 52,000) and cytochrome P-450c (M_r = 56,000), purified from Long Evans rats. Well 9 contains 0.3 μg cytochrome P-450b-H (M_r = 51,500–52,000) purified from Holtzman rats.

Table 1. Effects of cytochrome P-450 induction on the binding of metyrapone to dithionite-reduced hepatic microsomes*

Treatment (N = 4)	Concentration of metyrapone complex (nmoles/mg protein)	Concentration of carbon monoxide complex (nmoles/mg protein)	% Cytochrome P-450 bound to metyrapone
Corn oil	0.28 ± 0.03	0.63 ± 0.01	44 ± 3
Phenobarbital	1.2 ± 0.1†	1.6 ± 0.1†	78 ± 6†
3-Methylcholanthrene	0.62 ± 0.04†	1.3 ± 0.2†	48 ± 7
2,3′,4,4′,5,5′,Hexachlorobiphenyl	0.85 ± 0.09†	1.6 ± 0.2†	53 ± 4‡

* The metyrapone- and carbon monoxide-difference spectra of dithionite-reduced hepatic microsomes were recorded between 400 and 500 nm. The concentration of metyrapone complex was calculated from the increase in absorbance at 446 nm (A_{446}–A_{490}) based on the millimolar extinction coefficient 52 $mM^{-1} \cdot cm^{-1}$ [31]. The concentration of carbon monoxide complex (i.e. cytochrome P-450) was calculated from the increase in absorbance at 448–450 nm (A_{max}–A_{490}) based on the millimolar extinction coefficient 91 $mM^{-1} \cdot cm^{-1}$ [25]. Values represent the mean ± S.D. of four determinations.

† Values are significantly different from control, $P < 0.01$.

‡ Values are significantly different from control, $P < 0.05$.

SDS-PAGE that the major HCBP-inducible polypeptide corresponds to cytochrome P-450d and not cytochrome P-450$_{PCN}$.

Coadministration studies. It has been unequivocally established [6, 47–49] that the ability of PB and MC to induce two independently regulated forms of cytochrome P-450 explains why their inductive effects are essentially additive [50]. To determine whether the major HCBP-inducible form of cytochrome P-450 was cytochrome P-450b, P-450c, P-450d or P-450$_{PCN}$, HCBP was coadministered to rats with PB, MC, isosafrole or PCN at doses effecting maximal induction of hepatic microsomal cytochrome P-450.

These coadministration studies were complicated by the fact that the full extent of induction of hepatic microsomal cytochrome P-450 by isosafrole can be observed spectrally only after a methylenedioxyphenyl metabolite formed *in vivo* is displaced from the heme of cytochrome P-450 *in vitro* [33, 34]. A variety of structurally diverse displacers has been described [34]. The carbon monoxide-difference spectrum of dithionite-reduced hepatic microsomes isolated from rats treated with isosafrole (either alone or together with other inducers) was determined both before and after the isosafrole metabolite formed *in vivo* was displaced from cytochrome P-450 *in vitro* by 2-*n*-heptylbenzimidazole, the most effective displacer known [34]. Incubation of microsomes from isosafrole-treated rats with 200 μM 2-*n*-heptylbenzimidazole increased the carbon monoxide-binding capacity of cytochrome P-450 by 63% and resulted in a 1.3 nm hypsochromic shift in the absorbance maximum of the ferrous cytochrome P-450:carbon monoxide complex, as shown in Table 2. When MC was coadministered to rats with isosafrole, displacement with 2-*n*-heptylbenzimidazole elicited an even greater increase in carbon monoxide-binding capacity (140 vs 63%). Compared to hepatic microsomes from rats treated with isosafrole alone, microsomes from rats coadministered isosafrole with either PB or PCN displayed little increase in carbon monoxide-binding capacity following incubation with 2-*n*-heptylbenzimidazole. In contrast to MC, PB and PCN, the coadministration of HCBP with isosafrole did not influence the magnitude of the increase in carbon monoxide-binding capacity following displacement of the isosafrole metabolite from cytochrome P-450 (65 vs 63%). A similar study on the effects of coadministering isosafrole with PB or MC gave comparable results to those in Table 2 [51].

The effects of coadministering PB, MC, HCBP, PCN and isosafrole in pairs on hepatic microsomal cytochrome P-450 content are shown in Fig. 4. For each coadministration, the percent increase in cytochrome P-450 observed experimentally is shown alongside a theoretical additive value calculated from the sum of the individual values. The results in Fig. 4 show that the inductive effects of PB, MC, PCN and isosafrole were expressed additively. Similarly, the inductive effects of PB, MC and PCN were essentially additive with those of HCBP. In contrast, the inductive effects of isosafrole were not additive with those of HCBP. Since the xenobiotics were coadministered at maximally effective doses, the lack of additivity between the inductive effects of isosafrole and HCBP suggests that these two xenobiotics may induce the same major form of cytochrome P-450, i.e. cytochrome P-450d.

In view of the reported similarity between HCBP and PB [19], it is noteworthy that the effects of their coadministration to rats were not simply those of a strong PB-type inducer. For example, the ethylisocyanide-difference spectrum of dithionite-reduced microsomes from rats treated with both PB and MC represented qualitatively and quantitatively the sum of the two individual spectra as shown in Fig. 5. In contrast, the ethylisocyanide-difference spectra of dithionite-reduced microsomes from rats treated simultaneously with HCBP and PB or with HCBP and MC were not simply the composite of

Table 2. Effects of the isosafrole metabolite displacer, 2-*n*-heptylbenzimidazole, on the carbon monoxide-binding capacity of cytochrome P-450 in hepatic microsomes from variously treated rats*

Animal treatment (N = 5)	Cytochrome P-450 (nmoles/mg protein) (λ_{max}, nm)		Percent increase following displacement (hypsochromic shift)
	Before displacement	After displacement	
Isosafrole	0.831 ± 0.033 (449.8)	1.35 ± 0.15 (448.5)	63 (1.3 nm)
Isosafrole + phenobarbital	1.70 ± 0.12 (450.0)	1.96 ± 0.21 (448.9)	15 (1.1 nm)
Isosafrole + 3-methylcholanthrene	0.950 ± 0.141 (448.9)	2.28 ± 0.21 (447.6)	140 (1.3 nm)
Isosafrole + 2,3',4,4',5,5'-hexachlorobiphenyl	0.862 ± 0.041 (449.8)	1.42 ± 0.13 (448.4)	65 (1.4 nm)
Isosafrole + pregnenolone-16α-carbonitrile	1.52 ± 0.09 (449.8)	1.87 ± 0.23 (448.8)	23 (1.0 nm)

* The carbon monoxide-difference spectrum of dithionite-reduced hepatic microsomes was recorded both before and after effecting the dissociation of the isosafrole metabolite:cytochrome P-450 complex by incubation of microsomes (1 mg protein/ml) with 200 μM 2-*n*-heptylbenzimidazole. Values represent the mean ± S.D. of five determinations.

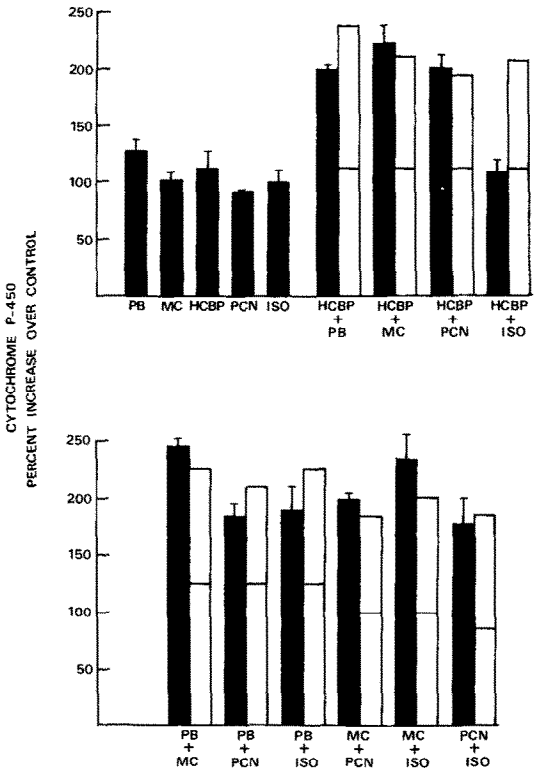

Fig. 4. Effects of coadministering various inducers on rat hepatic microsomal cytochrome P-450. Rats (three to six per group) were treated as described in Materials and Methods. Values are the mean percent increases over controls (0.63 to 0.70 nmole cytochrome P-450/mg protein) and each bar represents the standard deviation. To the right of the appropriate empirical values (black) are theoretical additive values (white) calculated from the sum of the individual values.

the individual spectra. In both cases, the height of the 455 nm peak exceeded that anticipated based on the sum of the individual spectra. Furthermore, when coadministered with PB, the hypsochromic shifts in both the carbon monoxide- and ethylisocyanide-difference spectra elicited by HCBP were augmented.

In contrast to HCBP, no evidence of any additivity between inductive effects was observed when PB was coadministered to five rats with the strictly PB-type congener, 2,2′,4,4′,5,5′-hexachlorobiphenyl (results not shown).

Metabolism of isosafrole to a product adduct. Many substrates for the cytochrome P-450-dependent monooxygenase system are preferentially metabolized by specific forms of cytochrome P-450, enabling the induction of certain cytochrome P-450 isozymes to be measured indirectly by an increase in catalytic activity. For example, cytochromes P-450b and P-450c preferentially catalyze the *N*-demethylation of benzphetamine and the hydroxylation of benzo[*a*]pyrene respectively [2]. Unfortunately, a substrate that is preferentially metabolized by cytochrome P-450d and whose metabolism is diagnostic of cytochrome P-450d induction has not been identified [11, 13].

Cytochromes P-450b, P-450c and P-450d, but not cytochrome P-450a, effectively catalyze the oxidation of isosafrole to a metabolite that forms a complex with the ferrous hemoprotein [11]. The ferrous cytochrome P-450:isosafrole metabolite complex displays absorbance maxima at 430 and 455 nm which, like the two Soret peaks of the ferrous cytochrome P-450:ethylisocyanide complex, are influenced by both pH and the source of cytochrome P-450 [11]. The spectral characteristics of the complex between ferrous cytochrome P-450 and a methylenedioxyphenyl metabolite formed during the *in vitro* metabolism of isosafrole catalyzed by hepatic microsomes

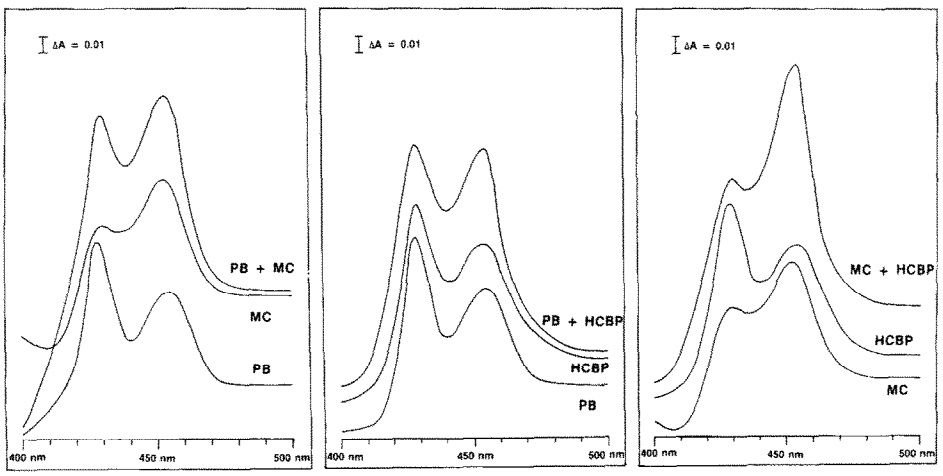

Fig. 5. Ethylisocyanide-difference spectra of dithionite-reduced hepatic microsomes from rats treated with phenobarbital (PB), 3-methylcholanthrene (MC) or 2,3′,4,4′,5,5′-hexachlorobiphenyl (HCBP) either individually or in various combinations. Ethylisocyanide (final concentration, 4.5 mM) was added to one of two balanced cuvettes containing dithionite-reduced microsomes (1 mg protein/ml), and the spectrum was recorded between 400 and 500 nm.

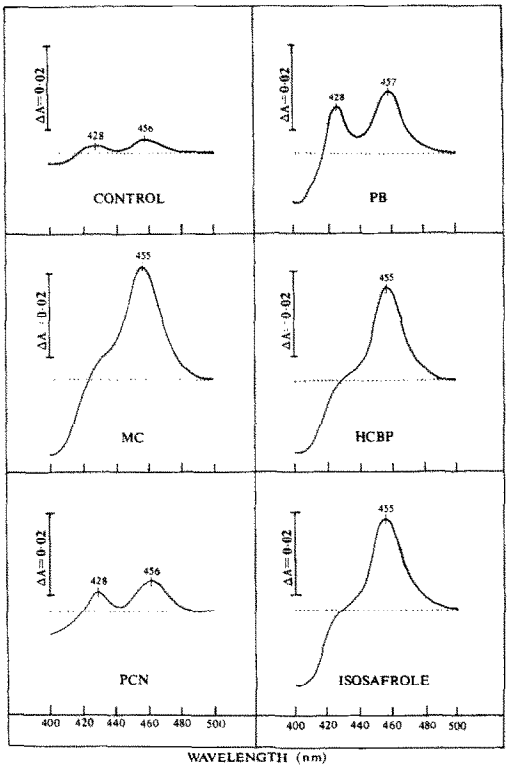

Fig. 6. Spectral properties of the complex formed between ferrous cytochrome P-450 and a methylenedioxyphenyl metabolite formed during the *in vitro* metabolism of isosafrole. Isosafrole was incubated with hepatic microsomes from rats treated with corn oil (control), phenobarbital (PB), 3-methylcholanthrene (MC), 2,3',4,4',5,5'-hexachlorobiphenyl (HCBP), pregnenolone-16α-carbonitrile (PCN) or isosafrole as described in Materials and Methods. The spectra shown were recorded after 5 min of incubation.

from variously treated rats are shown in Fig. 6. The product adducts formed when isosafrole was metabolized for 5 min by hepatic microsomes from rats treated with corn oil (control), PB or PCN displayed absorbance maxima at 428 and ~456 nm. At pH 7.4, these two peaks were of approximately equal height. In contrast, the product adduct formed by hepatic microsomes from rats treated with MC, isosafrole or HCBP displayed a prominent peak at 455 nm with only a shoulder around 430 nm.

The product adducts formed during the metabolism of isosafrole by hepatic microsomes from rats treated with PB, MC or isosafrole displayed spectral properties very similar to those reported for adducts between the isosafrole metabolite and ferrous cytochromes P-450b, P-450c and P-450d respectively [11]. The results in Fig. 6 further distinguish HCBP from PB as an inducer of cytochrome P-450 and lend further support to the possibility that the major HCBP-inducible polypeptide is cytochrome P-450d.

HCBP versus isosafrole. Although cytochrome P-450d is the major isosafrole-inducible hemoprotein in rat liver, isosafrole also induces cytochromes P-450b and P-450c [11]. The results of the previous [19] and present study on HCBP suggest that, like isosafrole, HCBP may also induce cytochromes P-450b and P-450c, albeit to a lesser extent than cytochrome P-450d. Taking advantage of the relatively low catalytic activity of cytochrome P-450d toward several substrates [11], the abilities of HCBP and isosafrole to induce cytochromes P-450b and P-450c were assessed indirectly by the induction of aminopyrine *N*-demethylase and benzo[*a*]pyrene hydroxylase activity respectively. The results shown in Table 3 suggest that HCBP is a better inducer of cytochrome P-450b (aminopyrine *N*-demethylase activity) than isosafrole but that, conversely, isosafrole is the better inducer of cytochrome P-450c (benzo[*a*]pyrene hydroxylase activity). Increasing the dose of HCBP from 150 to 300 μmoles/kg gave no further increases in hepatic microsomal cytochrome P-450 content or associated catalytic activities (results not shown).

Table 3 also compares the potency with which HCBP and isosafrole competitively displaced [^{3}H]TCDD from the rat hepatic cytosolic receptor that apparently regulates the induction of cytochrome P-450c and various other enzymes [35, 52, 53]. The EC_{50} values for HCBP and isosafrole were 16 μM and 1 mM, respectively, indicating that HCBP was bound much more avidly than isosafrole to the cytosolic receptor. Although HCBP was considerably more effective than isosafrole in displacing 50% of the specifically bound [^{3}H]TCDD, HCBP itself is a relatively weak competitor compared to various inducers of cytochrome P-450c including the series of PCBs that are structurally related to HCBP, i.e. the series of mono-*ortho* derivatives of the four MC-type coplanar PCBs [36].

The relatively weak receptor-binding properties of HCBP are consistent with the relatively weak MC-type inducing properties of HCBP. However, the extremely low affinity with which isosafrole bound to the cytosolic receptor is anomalous since isosafrole was a better inducer than HCBP of cytochrome P-450c (benzo[*a*]pyrene hydroxylase activity). One possible explanation for this anomaly is that a metabolite of isosafrole is involved in the induction process as previously proposed by Fennell *et al.* [54–56].

In summary, the results of the present study provide indirect evidence that, in immature male Wistar rats, HCBP induces cytochrome P-450b and, unexpectedly, more cytochrome P-450d than cytochrome P-450c. This profile of hepatic microsomal cytochrome P-450 isozymes is qualitatively similar to that observed following treatment of rats with isosafrole [57]. We have recently completed an immunochemical analysis of the profile of cytochromes P-450a–P450e in hepatic microsomes from immature male Long Evans rats treated with a variety of halogenated biphenyl isomers and congeners [58]. We confirmed by this direct approach that, like isosafrole [57], HCBP induces cytochrome P-450b and induces more cytochrome P-450d than cytochrome P-450c. Although qualitatively similar, HCBP and isosafrole differed quantitatively in the extent to which they induced different cytochrome P-450 isozymes. HCBP was the better inducer of cytochrome P-450b whereas isosafrole was the better inducer of cyto-

Table 3. Comparison of 2,3′,4,4′,5,5′-hexachlorobiphenyl and isosafrole as rat hepatic microsomal enzyme inducers and as competitors with [^{3}H]TCDD for rat hepatic cytosolic receptor binding*

Compound	Microsomal enzyme induction		Receptor binding (EC_{50})
	Aminopyrine *N*-demethylase	Benzo[*a*]pyrene hydroxylase	
2,3′,4,4′,5,5′-Hexachlorobiphenyl	2.1- to 2.4-fold	4- to 5 fold	16 μM
Isosafrole	1.3- to 1.5-fold	8- to 10-fold	1 mM

* The rate of aminopyrine *N*-demethylation and benzo[*a*]pyrene hydroxylation catalyzed by hepatic microsomes from rats treated with HCBP or isosafrole (N = 5) is expressed as an increase over control (typical values for which are given in the legend to Fig. 1). The increases over controls were statistically significant ($\alpha = 0.01$) as were the differences in the rate of metabolism of each substrate catalyzed by microsomes from rats treated with HCBP versus isosafrole. The concentration of isosafrole effective in displacing 50% of the [^{3}H]TCDD specifically bound to rat hepatic cytosol (EC_{50}) was determined as described in Materials and Methods. The EC_{50} value for HCBP is from Ref. 36.

chrome P-450c [58]. These quantitative differences are consistent with differences in the catalytic activity of hepatic microsomes isolated from HCBP- or isosafrole-treated rats (Table 3).

Acknowledgements—The financial assistance of the National Institutes of Environmental Health Sciences (1-RO1-ES02798-01), Natural Sciences and Engineering Research Council of Canada and the Extramural Research Programs, Health and Welfare Canada are gratefully acknowledged. The assistance of S. Bandiera, K. Riley, L. Uhlig and L. Safe is appreciated. We thank D. Ryan and W. Levin for the purified enzymes and free access to unpublished data and Dr. S. R. Challand for the 2-*n*-heptylbenzimidazole.

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