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IMMUNOLOGICAL AND ENZYMATIC COMPARISON OF HEPATIC CYTOCHROME P-450 FRACTIONS FROM PHENOBARBITAL-, 3-METHYLCHOLANTHRENE-, β-NAPHTOFLAVONE- AND 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-TREATED RATS

E. LE PROVOST*, T. CRESTEIL, S. COLUMELLI and J. P. LEROUX

Laboratoire de Biochimie (INSERM U 75), Faculté de Médecine Necker-Enfants Malades, 156 rue de Vaugirard, 75730 Paris Cédex 15, France

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Abstract—A comparison of the cytochrome P-450 forms induced in rat liver microsomes by phenobarbital on the one hand, and 3-methylcholanthrene, β-naphtoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the other hand, was performed using specific antibodies: anti-P-450 B_2 PB IG (against the phenobarbital-induced cytochrome P-450) and anti-P-450 B_2 BNF IG (against the β-naphtoflavone-induced cytochrome P-450). On DEAE-cellulose chromatography, four cytochrome P-450 fractions were separated, called P-450 A (non-adsorbed), P-450 Ba, P-450 Bb and P-450 Bc, from control, phenobarbital-, 3-methylcholanthrene, β-naphtoflavone- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated rats. Cytochrome P-450 A fractions appeared to be unmodified by the inducers, whereas the specifically induced cytochrome P-450 forms were always recovered in Bb fractions. The P-450 Bb fractions induced by 3-methylcholanthrene, β-naphtoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exhibited common antigenic determinants, comparable catalytic activities (benzphetamine *N*-demethylase, benzo[*a*]pyrene hydroxylase) and similar mol. wts. Moreover, the inhibition patterns by the two antibodies of benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase activities catalysed by 3-methylcholanthrene, β-naphtoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin microsomes or by the corresponding P-450 Bb fractions in a reconstituted system were quite identical. By these different criteria, β-naphtoflavone, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin seem to induce a common cytochrome P-450 species in rat liver.

Compounds such as 3-MC,† TCDD and BNF are potent inducers of hepatic cytochrome P-450. It is well known that PAH (such as 3-MC) and PB are two distinct classes of inducers, leading to *de novo* synthesis of different cytochrome P-450 species distinguished by their mol. wts, peptide maps, substrate specificities and immunological characteristics, in the rat [1–10], rabbit [11–14] and mouse [15, 16]. Multiple forms of cytochrome P-450 have been extensively studied in the liver of the PB- and PAH-treated rabbits and mice. It is well established that TCDD, BNF and 3-MC induce a similar form of cytochrome P-450 (P-450 LM_4) in rabbit liver [11–13, 17]. In mouse liver, those compounds appear to induce cytochromes P-450 exhibiting the same mobility in polyacrylamide gel electrophoresis and identical patterns of BP metabolites [15, 16]. On the other hand, 3-MC and TCDD are also known to induce two distinct forms of cytochrome P-450, called P-448 and P_1-450, more closely associated with acetanilide hydrolase and BP hydroxylase activities respectively in mouse liver [18, 19], as well as in rabbit liver [20, 21].

The purpose of this study was to determine if TCDD, 3-MC and BNF induce similar cytochrome P-450 species in rat liver. To this end, cytochromes P-450 from liver microsomes of PB-, 3-MC-, BNF- and TCDD-treated rats were separated by DEAE-cellulose chromatography. Microsomes and the specifically induced separated cytochrome P-450 fractions were compared by SDS electrophoresis, and a study of their substrate specificities and immunological characteristics.

MATERIALS AND METHODS

Treatment of rats. Male Sprague–Dawley rats weighing 150–200 g were used. PB dissolved in 0.9% NaCl (w/v) was administered by an intraperitoneal route at a dose of 80 mg/kg body wt, once a day for 3 days. 3-MC and BNF dissolved in sunflower oil were injected at doses of 20 and 80 mg/kg respectively, once a day for 2 days. TCDD (250 μg/ml dioxan) dissolved in sunflower oil was administered at a single dose of 10 μg/kg. PB-, 3-MC-, BNF- and TCDD-treated rats were killed 24, 48, 48 and 96 hr respectively after the last injection. Control rats received no treatment, since it had been shown that treatment by vehicle only did not modify monooxygenase activities.

* To whom correspondence should be addressed.

† Abbreviations: PB, phenobarbital; 3-MC, 3-methylcholanthrene; BNF, β-naphtoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH, polycyclic aromatic hydrocarbons; BP, benzo[*a*]pyrene; NI, non-induced; SDS, sodium dodecyl sulfate.

Liver microsomes were prepared as previously described [22], resuspended in 100 mM sodium phosphate buffer (pH 7.4) containing 20% (w/v) glycerol, and stored at −80°. They will be called NI-, PB-, 3-MC-, BNF- and TCDD-microsomes respectively according to whether they were prepared from NI, PB-, 3-MC-, BNF- or TCDD-treated rats. Cytochrome P-450 was determined according to Omura and Sato [23] and protein according to Lowry *et al.* [24].

Separation of cytochrome P-450 fractions on DEAE-cellulose. Microsomal proteins were solubilized as previously described [22] and applied on a DEAE-cellulose column. The chromatography was developed at room temp according to Warner *et al.* [25], and four fractions containing cytochrome P-450 were collected: fraction A non-adsorbed on DEAE-cellulose, and fractions Ba, Bb and Bc eluted with 0.03, 0.08 and 0.15 M NaCl in the buffered detergent solution. Cytochrome P-450 was estimated and pooled fractions were concentrated by ultrafiltration on Amicon PM 30. Emulgen was eliminated by calcium phosphate treatment until absorbance at 280 nm became lower than 0.040 [26]. Only fractions A and Bb, the latter containing specifically-induced cytochrome P-450 fractions, were utilized for enzymatic and immunological studies. The use of this single DEAE-cellulose chromatography allowed us to separate different fractions of P-450, but not to purify them extensively, as judged by the P-450 sp. acts [3–6 nmoles P-450 × (mg protein)$^{-1}$] and by SDS–polyacrylamide gel electrophoresis.

Purification procedure of cytochromes P-450. In order to prepare antibodies, some forms of cytochrome P-450 were extensively purified from A and Bb fractions previously separated by DEAE-cellulose chromatography. Cytochromes P-450 A_2 PB, P-450 A_2 TCDD and P-450 A_2 NI were respectively purified from P-450 A fractions of PB-, TCDD- and NI-microsomes. The three specifically-induced forms, P-450 B_2 PB, P-450 B_2 TCDD and P-450 B_2 BNF were respectively prepared from Bb fractions of PB-, TCDD- and BNF-microsomes. The purification procedure, already described [22, 27], involved successive chromatographic separations on octyl-Sepharose (Pharmacia), DEAE-cellulose (DE 52 Whatman) and CM cellulose (CM 52 Whatman). A further purification step on hydroxylapatite [6] was added for cytochrome P-450 B_2 BNF. These procedures led to the preparation of purified cytochrome P-450, as judged by SDS–polyacrylamide gel electrophoresis. NADPH–cytochrome *c* reductase fractions eluted from the octyl-Sepharose column were further purified on 2′,5′-ADP-Sepharose [28].

Electrophoresis was performed as described by Laemmli [29] on polyacrylamide gels (1 mm thin, 15 cm length) in the presence of SDS.

Preparation of the antibodies. Antibodies have been raised in female New Zealand rabbits against the highly purified PB-induced cytochrome P-450 (P-450 B_2 PB) and against the cytochromes P-450 A_2 from non-treated and PB-treated rats (P-450 A_2 NI and P-450 A_2 PB respectively) as previously described [27]. A similar procedure was utilized to obtain antibodies against the purified BNF-induced cytochrome P-450 (P-450 B_2 BNF). The immunoglobulin fractions were purified by ammonium sulfate precipitation and further by DEAE-cellulose chromatography [27]. Control IG was isolated in an identical manner from control rabbit sera.

Ouchterlony double-diffusion analysis. The immunodiffusion plates were prepared with 0.9% agarose, 80 mM NaCl, 15 mM sodium azide, 0.2% Emulgen 911 dissolved in 1 M glycine buffer (pH 7.4) as previously described [27]. Twenty-microlitre wells contained 0.04 nmoles of cytochrome P-450 and 0.1 mg purified immunoglobulins. The immunoprecipitin bands were stained for proteins with 0.1% naphtol blue black (Amido Schwarz) in 5% acetic acid for 3 hr, then destained in 5% acetic acid [30].

Quantitative immunoprecipitation. Twenty micrograms highly purified cytochrome P-450 (P-450 B_2 PB or P-450 B_2 BNF) were incubated with gradual amounts of specific immunoglobulins (from 0.6 to 3 mg protein) in 10 mM potassium phosphate buffer (pH 7.5) containing 0.9% NaCl, 0.25% sodium cholate and 0.25% Emulgen 911 (final vol. 1.5 ml) [4]. The mixture was incubated at 37° for 30 min, then at 4° for 24 hr. The immunoprecipitates were collected by centrifugation at 3000 *g* for 15 min and washed 3 times with 10 mM potassium phosphate buffer (pH 7.5) containing 0.9% NaCl. Protein determinations in the immunoprecipitates were carried out after solubilization with 1 M NaOH [24]. Immunoreaction mixtures without antibodies and with control immunoglobulins were performed in the same conditions and subtracted as backgrounds. In other experiments, we have performed the immunoprecipitation of increasing amounts of cytochrome P-450 (from 5 to 50 μg protein) by a fixed amount of the homologous antibody (0.5 mg anti-P-450 B_2 BNF IG or 0.75 mg anti-P-450 B_2 PB IG) in the absence or presence of the heterologous antigen (10 μg).

Enzymatic assays. The activities of benzphetamine *N*-demethylase and BP hydroxylase were assayed on microsomes or in reconstituted systems by described methods: for benzphetamine *N*-demethylase, formaldehyde produced was measured according to Nash [31], and the overall activity of BP hydroxylase was measured using [^{3}H] BP as substrate [32].

Reconstitution experiments for benzphetamine *N*-demethylase were performed as described previously [27] using 0.4 nmoles cytochrome P-450. Reconstitution experiments for BP hydroxylase were carried out with 0.2 nmoles cytochrome P-450, 100 μg sodium cholate, 50 μg dilauroylphosphatidylcholine, NADPH–cytochrome P-450 reductase (100 nmoles cytochrome *c* reduced in 1 min), and 40 nmoles [^{3}H]BP, allowed to reassociate for 15 min at 37°. The vol. was then adjusted to 0.5 ml with Tris–HCl buffer (pH 7.6) to a final concn of 50 mM containing 5 mM $MgCl_2$, 0.45 mM NAD, 0.4 mg bovine serum albumin and a NADPH-generating system, and the reaction was started by addition of glucose-6-phosphate dehydrogenase.

Inhibition of enzymatic activities by antibodies. Inhibitions of benzphetamine *N*-demethylase and BP hydroxylase activities were performed on both microsomes and reconstituted systems.

For benzphetamine *N*-demethylase, microsomes (0.5 nmoles cytochrome P-450) or P-450 fractions

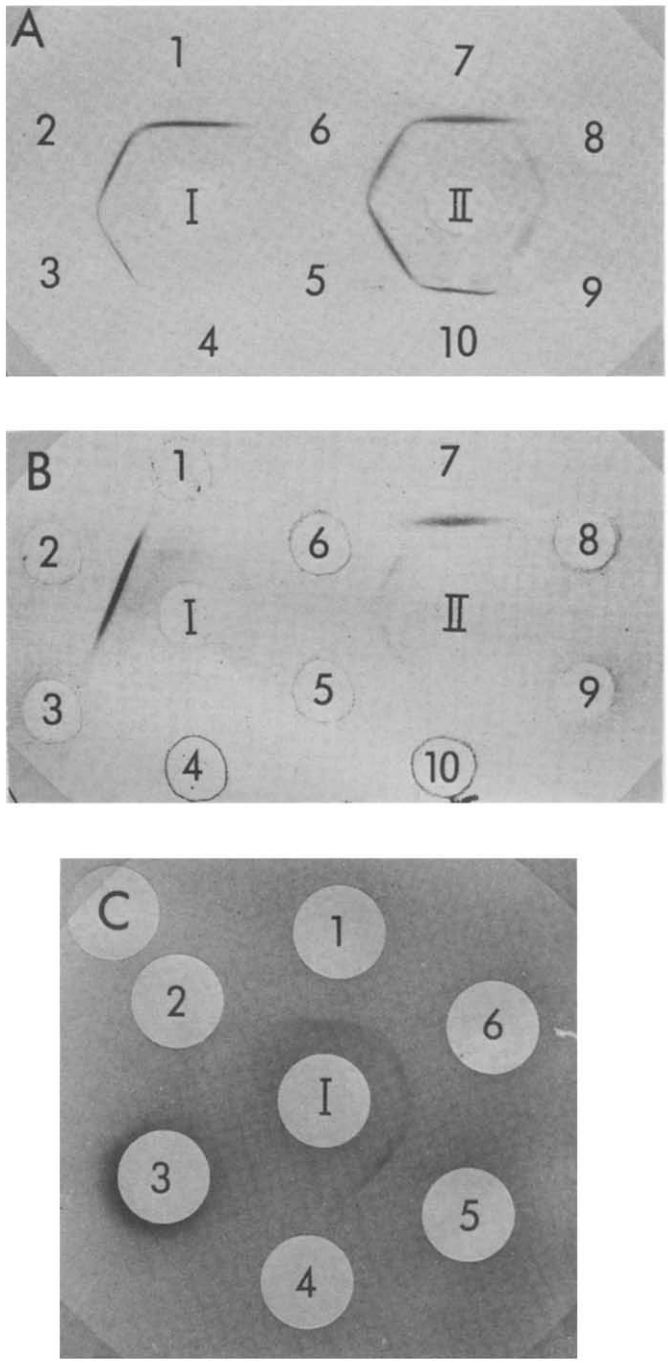

Fig. 1. Ouchterlony double-immunodiffusion. (A) Wells contained 0.04 nmoles of cytochrome P-450. 1 and 8: Bb PB; 2: B_2 PB; 3 and 9: Bb NI; 4 and 10: Bb 3-MC; 5: B_2 TCDD; 6: Bb BNF; 7: B_2 BNF. I: 0.1 mg of anti-P-450 B_2 PB IG; II: 0.1 mg of anti-P-450 B_2 BNF IG. (B) Wells contained 0.04 nmoles of cytochrome P-450. 1 and 8: A PB; 2: Bb PB; 3 and 9: A NI; 4 and 10: A 3-MC; 5: A_2 TCDD; 6: A BNF; 7: Bb BNF. I: 0.1 mg of anti-P-450 B_2 PB IG; II: 0.1 mg of anti-P-450 B_2 BNF IG. (C) Wells contained 0.05 nmoles of cytochrome P-450. 1: A PB; 2: Bb PB; 3: Bb NI; 4: adult rat hemoglobin; 5: A 3-MC; 6: A NI. I: 0.1 mg anti-P-450 A_2 NI IG.

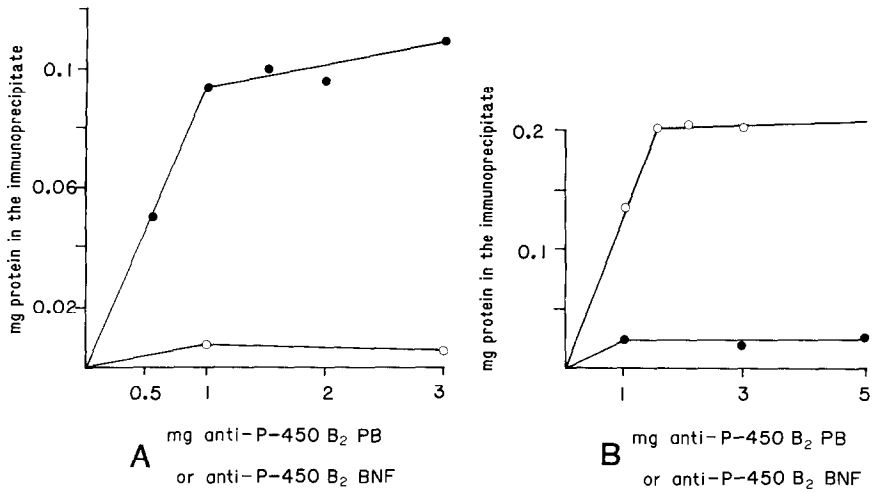

Fig. 2. Quantitative immunoprecipitation of cytochromes P-450 B_2 PB and P-450 B_2 BNF by anti-P-450 B_2 PB IG and anti-P-450 B_2 BNF IG. (A) Immunoprecipitation of P-450 B_2 BNF (20 μg) by increasing amounts of anti-P-450 B_2 BNF IG (●—●) or anti-P-450 B_2 PB IG (○—○); (B) immunoprecipitation of P-450 B_2 PB (20 μg) by increasing amounts of anti-P-450 B_2 PB IG (○—○) or anti-P-450 B_2 BNF IG (●—●). Results of a typical experiment. Another experiment gave similar results.

(0.4 nmoles cytochrome P-450) were preincubated with increasing amounts of specific or control immunoglobulins at 23° for 10 min in 100 mM sodium phosphate buffer (pH 7.4) (final vol. 0.37 ml) before addition of the remaining incubation components.

For BP hydroxylase, microsomes (0.1 nmoles cytochrome P-450) or P-450 fractions (0.2 nmoles cytochrome P-450) were preincubated with increasing amounts of specific or control immunoglobulins at 23° for 10 min in 100 mM Tris–HCl buffer (pH 7.6) before addition of the remaining incubation components (final vol. 0.22 ml).

The inhibitory effect of the antibodies was calculated using the catalytic activities in the presence of the same amount of control immunoglobulins as 100%.

RESULTS

Specificity of the antibodies

The different fractions of cytochrome P-450 separated on DEAE-cellulose and two highly purified cytochromes P-450 (P-450 B_2 PB and P-450 B_2 BNF) were tested by Ouchterlony double-diffusion against anti-P-450 B_2 PB IG and anti-P-450 B_2 BNF IG. In Fig. 1A, the anti-P-450 B_2 PB IG formed a single intense precipitin line with highly purified cytochrome P-450 B_2 PB and P-450 Bb PB fractions, but only a faint band with the P-450 Bb NI fraction. No crossreaction occurred with cytochromes P-450 Bb BNF, P-450 B_2 TCDD and P-450 Bb 3-MC. The anti-P-450 B_2 BNF IG gave a precipitin line of total identity with P-450 B_2 BNF, P-450 Bb BNF, P-450

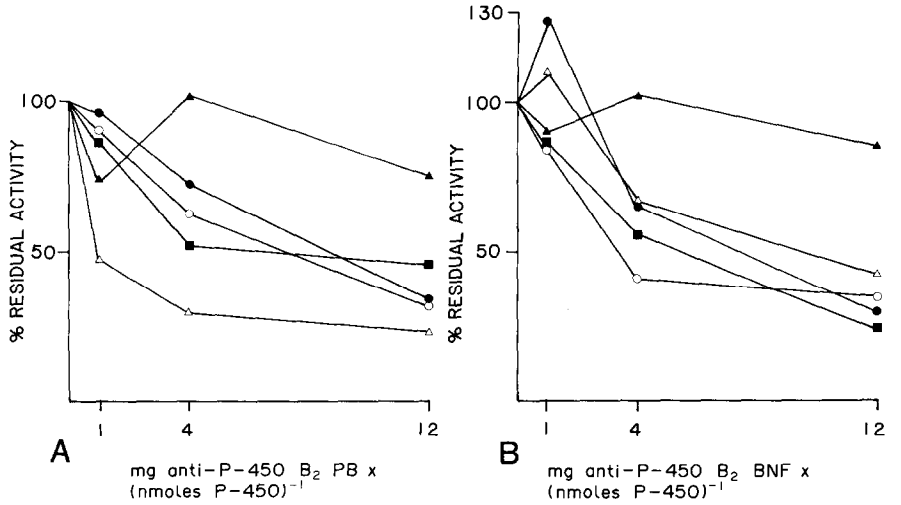

Fig. 3. Effect of anti-P-450 B_2 PB IG (A) and anti-P-450 B_2 BNF IG (B) on benzphetamine *N*-demethylase activity catalyzed by microsomes from control (▲), PB-(△), 3-MC-(●), BNF-(○) and TCDD (■)-treated rats. 0.5 nmoles of microsomal cytochrome P-450 were used for the enzymatic assay. Results of one experiment.

Table 1. Molecular activities of benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase, and cytochrome P-450 content of microsomes from control (NI), PB-, 3-MC-, BNF- and TCDD-treated rats

	NI-microsomes	PB-microsomes	3-MC-microsomes	BNF-microsomes	TCDD-microsomes
Benzphetamine *N*-demethylase*	2.4	10.3	2.2	3	2.4
Benzo[*a*]pyrene hydroxylase*	2.7	1.7	6.2	5.5	5.1
Cytochrome P-450 content†	1.2	2.5	2.1	1.7	2.5

Results are expressed as *nmoles of product formed $\times$ min^{-1} $\times$ (nmoles of cytochrome P-450)$^{-1}$ and †nmoles of cytochrome P-450 $\times$ (mg of microsomal protein)$^{-1}$.

Results of a typical experiment.

B_2 TCDD and P-450 Bb 3-MC (Fig. 1A); a very faint reaction was observed with P-450 Bb NI and P-450 Bb PB but this precipitin line was not correlated to the line occurring between homologous antigen and antibody. Consequently, P-450 Bb Ni and P-450 Bb PB fractions were not contaminated by P-450 Bb BNF fractions.

The cytochrome P-450 A fractions were not at all or very slightly recognized by both antibodies (Fig. 1B).

In Fig. 1C, the antibody against the cytochrome P-450 A_2 from control rats (anti-P-450 A_2 NI IG) was tested against P-450 A and P-450 Bb fractions. A fractions from NI, PB- and 3-MC-treated rats were recognized in a single line of total identity by the antibody, whereas P-450 Bb fractions did not react. Similar results were obtained with the antibody against the cytochrome P-450 A_2 from PB-treated rats (data not shown). In other experiments, A fractions from control, PB- and 3-MC-treated neonatal and fetal rats were tested against the two anti-P-450 A_2 antibodies. We observed one single line of total identity between P-450 A fractions from adult, fetal and neonatal rats irrespective of the induction, indicating that P-450 A fractions were not modified qualitatively by inducers during ontogenesis (data not shown).

The specificity of the antibodies was also clearly demonstrated by immunoprecipitation of purified cytochromes P-450 B_2 PB and P-450 B_2 BNF by their homologous antibodies. Fig. 2A shows that increasing amounts of anti-P-450 B_2 BNF IG were able to precipitate cytochrome P-450 B_2 BNF whereas anti-P-450 B_2 PB IG was not. Conversely, increasing amounts of anti-P-450 B_2 PB IG precipitated cytochrome P-450 B_2 PB whereas anti-P-450 B_2 BNF IG did not (Fig. 2B).

With a fixed amount of anti-P-450 B_2 BNF IG (0.5 mg), the presence of 10 μg of P-450 B_2 PB did not appreciably affect the immunoprecipitation of cytochrome P-450 B_2 BNF. Similarly, addition of 10 μg of P-450 B_2 BNF did not significantly modify the immunoprecipitation of cytochrome P-450 B_2 PB by 0.75 mg of anti-P-450 B_2 PB IG (results not shown).

Inhibition of enzymatic activities by antibodies in microsomes

As expected, benzphetamine *N*-demethylase exhibited a higher activity in microsomes from PB-treated rats than in microsomes from control, 3-MC-, BNF- and TCDD-treated rats (Table 1).

Benzphetamine *N*-demethylase activity was strongly inhibited by anti-P-450 B_2 PB IG and by P-450 B_2 BNF IG in microsomes of all treated animals, whereas it was nearly uninfluenced in control microsomes (Fig. 3). However, the extent of the inhibition was larger in PB-microsomes by anti-P-450 B_2 PB IG (Fig. 3A), and in BNF-, TCDD- and 3-MC-microsomes by anti-P-450 B_2 BNF IG (Fig. 3B). Moreover, the extent of inhibition by anti-P-450 B_2 PB IG on the one hand, and by anti-P-450 B_2 BNF IG on the other hand, was quite similar in 3-MC-, BNF- and TCDD-treated animals.

BP hydroxylase exhibited high and similar molecular activities in microsomes from 3-MC-, BNF-

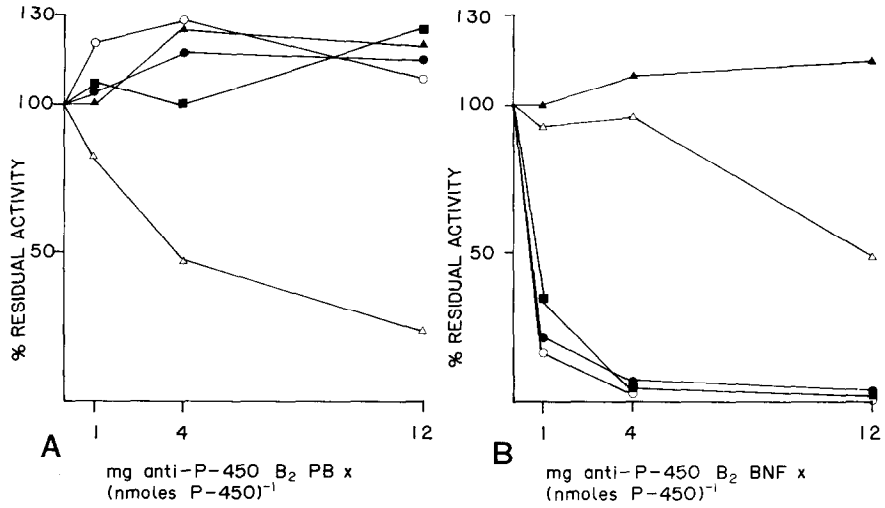

Fig. 4. Effect of anti-P-450 B_2 PB IG (A) and anti-P-450 B_2 BNF IG (B) on benzo[*a*]pyrene hydroxylase activity catalyzed by microsomes from control (▲), PB-(△), 3-MC-(●), BNF-(○) and TCDD (■)-treated rats. 0.1 nmoles of microsomal cytochrome P-450 were used for the enzymatic assay. Results of one experiment.

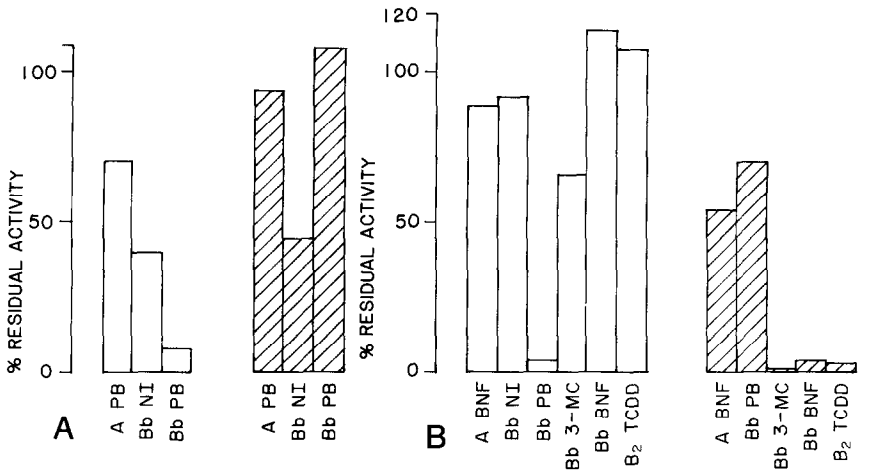

Fig. 5. (A) Effect of 4 mg of anti-P-450 B_2 PB IG (□) and 4 mg of anti-P-450 B_2 BNF IG (▨) on benzphetamine *N*-demethylase activity catalyzed by the reconstituted system containing 0.4 nmoles of cytochrome P-450 (A PB, Bb PB and Bb NI fractions separated on DEAE-cellulose). (B) Effect of 4 mg of anti-P-450 B_2 PB IG (□) and 4 mg of anti-P-450 B_2 BNF IG (▨) on benzo[*a*]pyrene hydroxylase activity catalyzed by the reconstituted system containing 0.2 nmoles of cytochrome P-450 (A, Bb BNF, Bb NI, Bb PB and Bb 3-MC fractions separated on DEAE-cellulose, and highly purified P-450 B_2 TCDD). Results of one experiment.

Table 2. Molecular activities of benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase in P-450 A and P-450 Bb fractions from control, PB-, 3-MC-, BNF- and TCDD-treated rats

	Control		PB		3-MC		BNF		TCDD	
	A	Bb	A	Bb	A	Bb	A	Bb	A_2	B_2
Benzphetamine *N*-demethylase	0.53	2.2	0.79	7.6	0.77	<0.2	0.38	<0.2	0.66	0.41
Benzo[*a*]pyrene hydroxylase	0.11	0.47	0.08	0.11	0.14	2	0.09	2.5	0.07	2.7

Results are expressed in nmoles of product formed × min^{-1} × (nmoles of cytochrome P-450)$^{-1}$.
Results of a typical experiment.

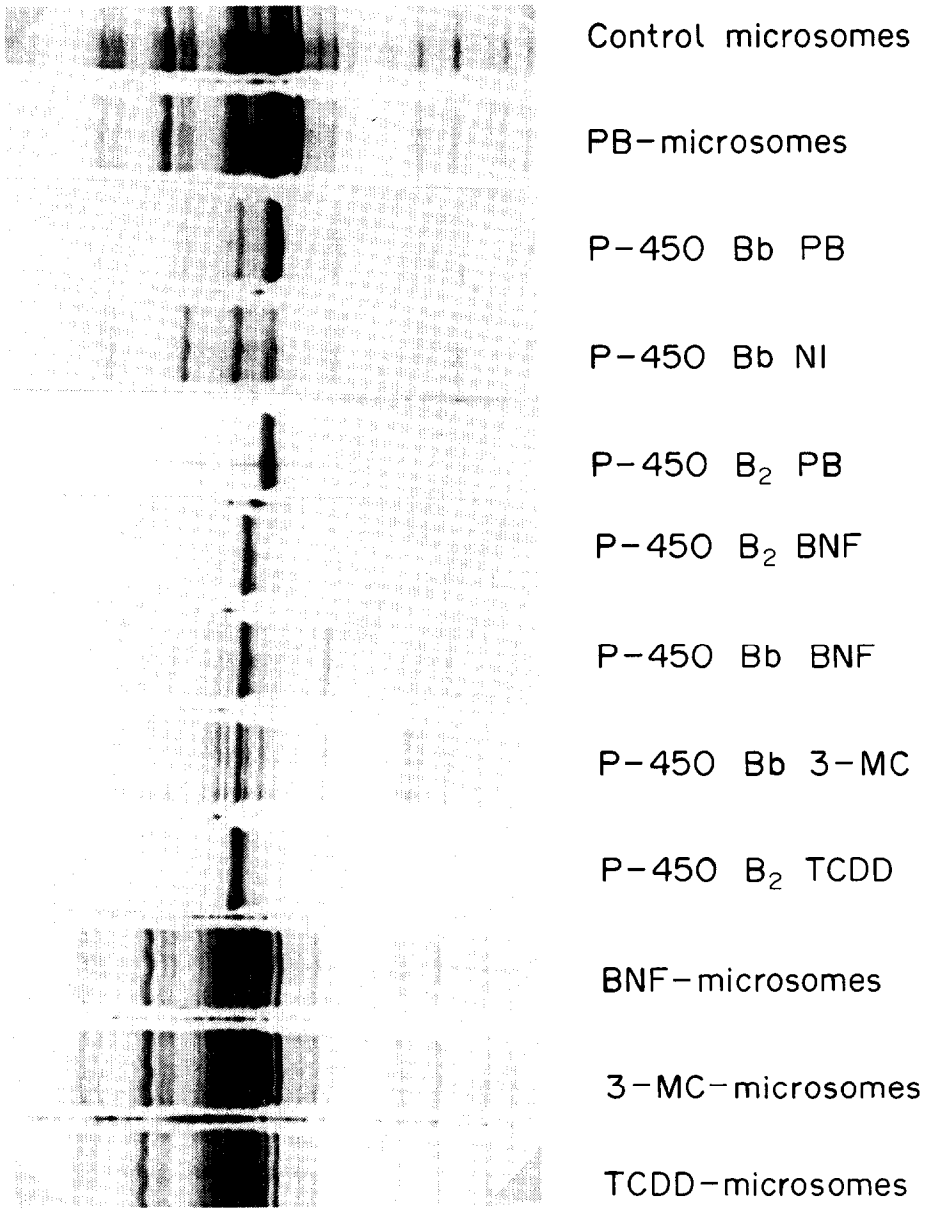

Fig. 6. SDS–polyacrylamide gel electrophoresis of microsomes (13 μg of protein), highly purified (B_2) cytochromes P-450 (3 μg) and partially resolved P-450 Bb fractions (5 μg) from control, PB- and PAH-treated rats.

and TCDD-treated rats whereas these activities were much lower in microsomes from control and PB-treated rats (Table 1).

BP hydroxylation catalyzed by PB-microsomes was inhibited by 80% by the homologous antibody (anti-P-450 B_2 PB IG) whereas there was little or no inhibition in control, BNF-, 3-MC- and TCDD-microsomes (Fig. 4A).

The anti-P-450 B_2 BNF completely inhibited the reaction catalyzed by BNF-, TCDD- and 3-MC-microsomes. No inhibition was observed in control microsomes. In PB-microsomes, there was no inhibition at 4 mg of antibody × (nmoles of P-450)$^{-1}$ but a 50% inhibition at 12 mg of antibody × (nmoles of P-450)$^{-1}$ (Fig. 4B).

Inhibition of enzymatic activities by antibodies in a reconstituted system

Benzphetamine *N*-demethylase activities were reconstituted with the fractions of cytochrome P-450 called P-450 A and P-450 Bb obtained by DEAE-cellulose chromatography from the different types of microsomes. Only fraction P-450 Bb PB presented a high benzphetamine *N*-demethylase activity. This activity was very low in P-450 Bb 3-MC, P-450 Bb BNF and P-450 B_2 TCDD and always lower than the

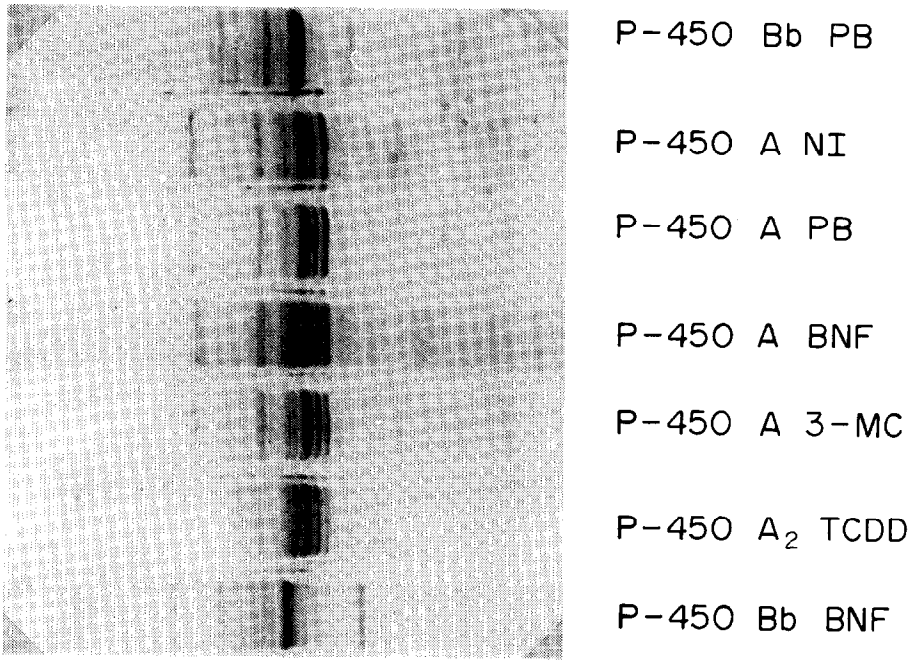

Fig. 7. SDS–polyacrylamide gel electrophoresis of partially resolved P-450 A and Bb fractions (5 μg) from control, PB- and PAH-treated rats.

one measured in P-450 Bb NI. Irrespective of the induction, benzphetamine *N*-demethylation remained low and roughly constant in P-450 A fractions (Table 2). Reconstituted BP hydroxylase activities were high and quite similar in P-450 Bb fractions from 3-MC-, BNF- and TCDD-treated rats, but low in Bb fractions from control and PB-treated rats. In P-450 A fractions, enzymatic activities were always low and nearly constant, irrespective of the induction (Table 2).

Preliminary experiments had shown that, in reconstituted systems, 4 mg of anti-P-450 B_2 PB IG × nmoles P-450)$^{-1}$ inhibited by 85–90% the benzphetamine *N*-demethylation supported by the P-450 Bb PB fraction, whereas the same quantity of the heterologous antibody (anti-P-450 B_2 BNF IG) had no effect on the enzyme activity. Similarly, at the same amount of 4 mg × (nmole of P-450)$^{-1}$, anti-P-450 B_2 BNF IG maximally inhibited the BP hydroxylase activity supported by P-450 Bb BNF fractions, whereas the heterologous antibody (anti-P-450 B_2 PB IG) had no effect on the enzyme activity. Thus, the amount of 4 mg of antibody × (nmoles of P-450)$^{-1}$ was used in subsequent experiments.

Fig. 5A shows that the benzphetamine *N*-demethylase activities of P-450 Bb PB and P-450 A PB were not modified by anti-P-450 B_2 BNF IG. Anti-P-450 B_2 PB IG inhibited the activity of P-450 Bb PB by more than 90%, and that of P-450 A PB by about 30%. The activity of P-450 Bb NI was similarly inhibited by both antibodies (about 60%).

In Fig. 5B, the results of inhibition experiments on BP hydroxylation are shown. Anti-P-450 B_2 BNF IG completely inhibited the enzyme activities of fractions Bb 3-MC, Bb BNF and B_2 TCDD, whereas it exerted a moderate effect on Bb PB (30% inhibition) and A BNF (50% inhibition). Anti-P-450 B_2 PB IG completely inhibited the enzyme activity of P-450 Bb PB, and had no effect on all the other tested fractions (A BNF, Bb NI, Bb BNF and B_2 TCDD) except for a slight inhibition of Bb 3-MC.

SDS–polyacrylamide gel electrophoresis

The purified forms (P-450 B_2 PB, P-450 B_2 BNF and P-450 B_2 TCDD) migrated as the major bands present in Bb fractions from which they were prepared. Moreover, P-450 B_2 BNF and B_2 TCDD migrated identically, and exhibited the same mol. wt as the major band present in P-450 Bb 3-MC (Fig. 6). Specifically PB-induced P-450 exhibited a lower mol. wt than the 3-MC-, BNF- and TCDD-induced forms. On the other hand, all the cytochrome P-450 A fractions migrated identically, irrespective of the induction, and exhibited mol. wts different from those of specifically PB-induced as well as 3-MC-, BNF- and TCDD-induced forms (Fig. 7).

DISCUSSION

In order to compare the inducing effects of 3-MC, BNF and TCDD on the monooxygenase system of rat liver, enzymatic and immunological studies were performed on both microsomal preparations and partially resolved cytochrome P-450 fractions.

This study is mainly based on the use of specific antibodies. Anti-P-450 B_2 PB IG has been shown by Ouchterlony double-immunodiffusion and quantitative immunoprecipitation to recognize only its homologous antigen (B_2 PB-induced P-450). Anti-P-450 B_2 BNF IG gives a faint cross-reaction with

P-450 Bb PB fractions, but this cross-reactivity does not interfere with experimental results, since the immunoprecipitation of cytochrome P-450 B_2 PB is negligible, and anti-P-450 B_2 BNF IG has no inhibitory effect on the benzphetamine *N*-demethylase activity catalysed by P-450 A and P-450 Bb PB fractions.

On DEAE-cellulose chromatography, we observed the same P-450 elution pattern from 3-MC-, BNF- and TCDD-microsomes (data not shown). These induced cytochromes P-450 (as PB-induced P-450) are eluted, at the same ionic strength, in one peak called the P-450 Bb fraction [10, 33].

On Ouchterlony double-immunodiffusion, P-450 Bb fractions from 3-MC-, BNF- and TCDD-microsomes show a single line of total identity with anti-P-450 B_2 BNF IG, indicating common antigenic determinants, and no cross-reaction with anti-P-450 B_2 PB IG. Moreover, in polyacrylamide gel electrophoresis, the main form of 3-MC-, BNF- and TCDD-microsomes or corresponding P-450 Bb fractions exhibit a similar mol. wt. On the other hand, cytochromes P-450 from A fractions are not recognized by both antibodies, but form a continuous precipitin line with anti-P-450 A_2 NI IG and anti-P-450 A_2 PB IG, whatever the induction may have been, and migrate with the same mobility in electrophoresis. Thus P-450 A fractions do not seem to be qualitatively modified by the different inducers in adult rats as well as in fetal and neonatal rats.

It is well established that BP hydroxylase activity is mainly associated with cytochrome(s) P-450 (P_1-450, P-448) induced by PAH, whereas PB-induced cytochrome P-450 is responsible for most of the benzphetamine *N*-demethylase activity. Measurements of molecular activities performed either on microsomes (Table 1) or on separated P-450 fractions (Table 2) largely support that statement. Moreover, the molecular activities of benzphetamine *N*-demethylase on the one hand and BP hydroxylase on the other hand are quite similar in microsomes or P-450 Bb fractions from 3-MC-, BNF- and TCDD-treated animals.

In order to confirm the apparent identity of cytochrome P-450 forms induced by the three tested PAH-like inducers, the pattern of enzyme inhibition by antibodies was determined on microsomes and separated P-450 fractions.

The *N*-demethylation of benzphetamine catalyzed by 3-MC-, BNF- and TCDD-microsomes is strongly inhibited by anti-P-450 B_2 BNF IG as well as by anti-P-450 B_2 PB IG. Similarly, this activity is inhibited in PB-microsomes by anti-P-450 B_2 BNF IG (60%), and by anti-P-450 B_2 PB IG (80%). Thus benzphetamine *N*-demethylase seems to be supported by different P-450 forms, mainly by PB-microsomes, but also by 3-MC-, BNF- and TCDD-microsomes: these forms are present in variable amounts in microsomes from variously treated animals, but also in low amounts in microsomes from control animals. This result is in agreement with previous reports which quantified PB and 3-MC isoenzymes in microsomes [4, 5, 34, 35].

A different picture is observed for the inhibition of BP hydroxylase activity: the complete inhibition of 3-MC-, BNF- and TCDD-induced BP hydroxylase by anti-P-450 B_2 BNF IG and the lack of effect of anti-P-450 B_2 PB IG on these microsomes indicate that BP activity is mainly supported by induced cytochrome P-450 forms. Comparable results are obtained in PB-microsomes: BP hydroxylase is inhibited only by anti-P-450 B_2 PB IG, and thus seems to be supported only by induced P-450. Similar results were reported previously with anti-P-450 PB and anti-P-450 3-MC [3–5, 36]. The absence of inhibition in control microsomes might indicate that BP hydroxylase is supported by other cytochrome P-450 forms unresponsive to anti-P-450 B_2 BNF and anti-P-450 B_2 PB IG.

The inhibition of enzymatic activities in reconstituted systems completely agrees with the data reported above: A fractions show no or little inhibition of both BP hydroxylase and benzphetamine *N*-demethylase activities by anti-P-450 B_2 PB IG and anti-P-450 B_2 BNF IG, and Bb fractions are significantly inhibited only by homologous antibodies. A discrepancy is only observed between the inhibitory effect of anti-P-450 B_2 BNF IG on benzphetamine *N*-demethylase activity catalysed by PB-microsomes and the separated P-450 A and P-450 Bb PB fractions. The enzymatic activity is inhibited by the antibody in PB-microsomes whereas there is no inhibition in the corresponding separated P-450 fractions. This result may indicate that benzphetamine *N*-demethylase activity is supported in PB-microsomes by other P-450 forms than the A and Bb PB fractions, perhaps by a P-450 Bb BNF present at a low amount in these microsomes.

Irrespective of antibodies used and enzymatic activities studied, an identical pattern is always observed in 3-MC-, BNF- and TCDD-microsomes, and in fractions Bb 3-MC, Bb BNF and B_2 TCDD. Thus, in spite of different approaches by electrophoresis, double-immunodiffusion, enzymatic characterization, inhibition by antibodies, and chromatographic separation on DEAE-cellulose, we are unable to distinguish P-450 forms induced by 3-MC, BNF and TCDD. Using other criteria, Madhukar *et al.* [37] have shown that TCDD and 3-MC have a similar inducing effect in the rat. In the mouse and rabbit, many reports conclude in favour of identical effects of different PAH-like inducers [11, 13, 15–17] by the use of several tests.

Nevertheless, some previous reports conflict with our results. Saito and Strobel [38] purified a BNF-induced cytochrome P-446, different from the 3-MC-induced cytochrome P-448 in the rat. Comparison of the inducing effect of 3-MC and TCDD in the rat suggested different molecular mechanisms of induction [37, 39].

According to our results, 3-MC, BNF and TCDD might induce a single form of cytochrome P-450. Moreover, Lau and Strobel [40] have recently purified from liver microsomes of BNF-treated rats five cytochrome P-450 forms which appear to be distinct from one another by all criteria examined. Only one form (called form 5) is associated with PAH-inducible enzymatic activities.

Thus PAH-like inducers might have different effects in the rabbit and mouse on the one hand and in the rat on the other hand. In the adult rabbit, two different forms of hepatic cytochrome P-450 (LM_4

and LM_6) have been purified after TCDD treatment [21]. In the same way, 3-MC and TCDD induce in mouse liver a cytochrome P_1-450 (a 56,000 mol. wt moiety) more closely related to aryl hydrocarbon hydroxylase activity and a cytochrome P-448 (a 55,000 mol. wt moiety) more closely related to acetanilide 4-hydroxylase activity [18, 19]. Recently Chen *et al.* [41] have demonstrated that 3-MC induces two different cytochromes P-450 (P_1-450 and P-448) in the rat, as in the mouse, using antibodies against mouse P_1-450 and P-448. However, these two forms were hardly separated by chromatography, whereas differences between rabbit P-450 LM_4 and P-450 LM_6 are more evident [21]. A common extracellular receptor for PAH-like inducers [42–45] is involved in the molecular events triggering cytochrome P-450 synthesis: TCDD, BNF and 3-MC compete for the binding to this receptor and probably set off a common series of induction processes, leading to the activation of cytochrome P-450 synthesis.

In conclusion, three compounds (3-MC, BNF and TCDD) seem to induce the same (or very closely related) cytochrome P-450 species in rat hepatic microsomes, in regard to chromatographic, electrophoretic, enzymatic and immunological properties.

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