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SPECIES DIFFERENCES OF MIXED-FUNCTION OXIDASE INDUCTION BETWEEN RABBITS AND RATS AFTER PRETREATMENT WITH POLYCHLORINATED BIPHENYLS (PCB's)

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Abstract—The inducing effect of PCB on mixed-function oxidase was investigated in rabbits. No induction of microsomal aminopyrine- and *p*-nitroanisole-demethylation as well as of aniline- and 4-chlorobiphenyl-*p*-hydroxylation was found, whereas the cytochrome P-450 level was increased by 150–300 percent normal after Clophen A 50 administration. In rat liver microsomes corresponding enzyme activities and P-450 levels were increased 2–5-fold after identical pretreatment. Similarly, hexobarbital half life remained unchanged in rabbits when hexobarbital sleeping time was reduced by more than 50 percent after the same dose of Clophen A 50 in the rat. Pretreatment of rabbits with different acute doses or with low chronic doses of Clophen A 50 increased P-450-content but confirmed the noninducibility of the enzyme activities measured. Evidence is presented that this phenomenon is not caused by inhibitory action of residual PCBs on mixed-function oxidase. The results are discussed with regard to the well known response of mixed-function oxidase in rabbit liver to 3-methylcholanthrene (3-MC)-treatment.

Studies on the biological effects of polychlorinated biphenyls (PCBs) showed a strong and long lasting induction of the hepatic enzyme system which oxidizes many drugs and lipophilic foreign compounds. Proliferation of smooth endoplasmic reticulum [1–3], increase of the microsomal hemoprotein, cytochrome P-450, and enhancement of drug oxidation *in vitro* and *in vivo* have been demonstrated with PCB-mixtures [4–6], and pure chlorinated biphenyls [7]. Beyond these quantitative effects there are qualitative alterations: the cytochrome P-450 of rats after treatment differs in its spectral properties from that present before treatment and resembles the cytochrome P-448 which is induced by the polycyclic hydrocarbon 3-MC [8]. The marked increase in benzo(a)pyrene-hydroxylase activity after PCB-as well as after 3-MC-administration further indicates similar inducing effects of the two chemicals. In contrast, PCBs induce hydroxylation of hexobarbital and *N*-demethylation of ethylmorphine which are not enhanced by 3-MC but by PB-treatment of rats.

Rabbits are known to differ from rats in their response to polycyclic hydrocarbon inducers [9, 10]. Thus, the rabbit might offer an alternative model to study the induction pattern of mixed-function oxidase by PCBs and to establish their characteristics as inducers. Little is known on the PCB-mediated induction pattern in rabbits. Two communications report on increased aminopyrine demethylation *in vitro* and hexobarbital metabolism *in vivo* [11, 12]. In that, induction by PCBs appeared to be similar to that by phenobarbital (PB). In contrast, we have recently reported some evidence for a 3-MC-type of PCB-induction in rabbits. With respect to the difference spectrum of the reduced P-450-CO-complex and the microsomal metabolism of two substrates, 4-chlorobiphenyl and *p*-nitroanisole, the effects of PCBs were similar to those of 3-MC, but completely different from those of PB [33].

To clarify whether the PCB-mediated induction pattern in rabbit liver can be assigned to one of the two types of induction produced by PB or 3-MC, or has properties of both types, we have investigated the effects of the technical PCB-mixture Clophen A 50, a potent inducer in rats [13] on several types of mixed-function oxidase activities *in vitro* and *in vivo* in male rabbits. Parallel studies were undertaken with rats after identical pretreatment.

MATERIALS AND METHODS

Chemicals

Enzymes and coenzymes were purchased from Boehringer/Mannheim, Germany. Clophen A 50, a mixture of chlorinated biphenyls with an average chlorine content of 5 atoms per biphenyl molecule was obtained from Bayer AG/Leverkusen, Germany. "Bayer Evipan-Na" was a generous gift of Dr. Meyer-Uhl. 4-Chlorobiphenyl was obtained from Riedel-de-Haen, 3016 Seelze, Germany. All other chemicals were of analytical grade and were bought from Merck AG, Darmstadt, Germany. The hydrochlorides of aniline and *p*-aminophenol were prepared by bubbling hydrogen chloride into an ethereous solution of these chemicals.

Animals

Male rabbits of the "White Russian" strain were obtained from Fa. Gassner, Sulzfeld/Germany, or from our own animal breeding station. Rabbits of 1400–1800 g corresponding to an age of 2–3 months and SPF Wistar rats, weighing between 100–200 g were utilized. All animals received water and a nor-

mal labotatory diet (Altromin^R, Lage/Lippe, Germany) *ad lib.* Clophen A 50 was administered in a 5% (w/v) solution in olive oil. For feeding experiments rabbits received daily portions of 200 g Altromin^R containing 100 ppm Clophen A 50.

Preparation of microsomes

At 8 a.m. rabbits were killed by a blow on the neck and exsanguination. Rats were sacrificed by decapitation. Livers were perfused with ice cold NaCl (0.9%)-solution through the vena cava. After storage on ice, approximately 50 g of rabbit liver or the entire rat liver were minced with scissors and further disrupted by a tissue press [14]. The disrupted tissue was homogenized in a cooled Potter-Elvehjem homogenizer (400 rotations/min) in a 4-fold vol. of 0.25 M sucrose, centrifugation was performed as described [15]. The pellets of the last centrifugation were resuspended and recentrifuged in 0.15 M KCL. The final pellets were suspended in sufficient volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose to obtain a protein concentration of 10–20 mg/ml. The suspensions were frozen in liquid nitrogen and stored at $-22°$.

Protein content of microsomes

Protein concentration was determined by the biuret-method described by Szarkowska and Klingenberg [16], with dried bovine serum albumin (Behring-Werke, Marburg/Lahn) as standard. The turbidity arising from insoluble material is substrated from the initial extinction at 546 nm after decolorization with KCN. Microsomal protein content per g fresh liver was calculated by multiplying the microsomal protein content per g perfused liver with a factor of 1.34 obtained from dry weight determinations of fresh and perfused liver tissue respectively.

In vitro *assays of drug metabolism*

Demethylation of *p*-nitroanisole was determined with freshly prepared microsomes. All other *in vitro* assays were performed with microsomes stored for 1 or 2 days. No significant activity changes compared to fresh microsomes were observed.

The activity of p-*nitroanisole-O-demethylase* was determined by a modified procedure of the method described by Netter and Seidel [17]. The assay mixture contained 1 mg microsomal protein, 1 mM *p*-nitroanisole, 10 mM isocitrate, 0.4 units isocitrate-dehydrogenase, 5 mM $MgCl_2$, in a total vol. of 1 ml 0.1 M potassium phosphate buffer, pH 7.85. After temperature equilibration the reaction was started by addition of a mixture of 0.13 μmole of NADP/NADPH. Initial velocities were recorded at 420 nm and 33°. Activity was proportional to protein concentrations of 0.5 and 1.0 mg/ml.

p-*Hydroxylation of* 4-*chlorobiphenyl*. A microcristalline suspension of 4-chlorobiphenyl was prepared by sonification, since organic solvents or detergents like Tween 80 inhibit microsomal hydroxylase activity [18]. Approximately 20 mg of 4-chlorobiphenyl were dissolved in 50 μl of chloroform in a test tube and 5 ml water added in drops. The chloroform solution at the bottom was sonified for 10–20 sec by the microtip of a Branson-sonifier at 50 W output. After evaporating the chloroform and filtering, a suspension being stable for at least 15 min was obtained. Concentration was determined by the UV-absorption in methanol.

Two μmoles of 4-chlorobiphenyl were preincubated with 5 mg microsomal protein for 5 min. The reaction was started by adding this mixture to a medium containing 0.05 M Tris-HCl buffer, pH 8.6, 10 mM glucose-6-phosphate (G6P), 1 mM NADP, 0.5 units/ml glucose-6-phosphate-dehydrogenase (EC 1.1.1.49) (G6PDH) and 4 mM $MgSO_4$ in a total vol. of 10 ml. The mixture was incubated for 4 min at 33°. Extraction with ethylacetate terminated the reaction and the metabolite 4-chloro-4′-hydroxybiphenyl was purified on thin layer plates of Kieselgel G (Merck, Darmstadt) with chloroform as solvent ($R_F = 0.2$–0.3). The absorption difference between 266 and 320 nm of the solution in methanol was taken for quantitative determination. The authentic sample had a molar extinction coefficient of 22 000 litre: $mol^{-1} \cdot cm^{-1}$. The overall yield starting with the ethylacetate extraction was 70 per cent. Under these conditions extinction of 0.2 was equal to an enzyme activity of 1.30 nmoles metabolite·mg protein^{-1}·min^{-1}. Enzyme activity was linear for 5 min with 0.5 and 1.0 mg of protein/ml. The K_M with microsomes from phenobarbital-treated rabbits was $2 \cdot 10^{-4}$ M. With rabbit microsomes no further phenolic metabolite could be detected when the TLC-plates were stained with $FeCl_3$/$K_3(Fe(CN)_6)$ reagent.

Aniline-p-hydroxylase. Determination by the indophenol-method was performed according to Imai *et al.* [19], as described by Mazel [20]. Optimal conditions were found with fivefold higher concentrations of substrate and NADPH-regenerating system. Enzyme activity in rabbit liver microsomes was found to be linear with respect to protein concentration at 0.5 and 1.0 mg/ml within 30 min. Incubation time was 20 min at 0.5 mg protein/ml. Microsomes from rat liver gave similar results within 15 min. Incubation time here was 10 min at 0.5 mg protein/ml.

Aminopyrine-N-*demethylase*. The metabolite aminoantipyrine was determined by diazotation and coupling with α-naphtol [21]. Substrate and cofactor concentrations were the same as described by Mazel [20], except that nicotinamid was omitted. Incubation procedures and linearity of enzyme activity were the same as described for aniline-*p*-hydroxylase.

Cytochrome P-450 *content of microsomes*. Determined according to Omura and Sato [22], using a molar extinction coefficient of $91 \cdot mM^{-1} \cdot cm^{-1}$, in a Beckman-Spectrophotometer, model Acta C-III. Wavelength accuracy was checked by the absorption lines at 418.5 and 453.4 nm of holmium-oxide and the D β-line of the deuterium lamp at 486.0 nm. For accurate determination of maximal wavelength difference spectra were recorded with a chart expansion of 2 nm/inch and evaluated graphically according to R. B. Mailman *et al.* [23].

In vivo *assays*

Half life of hexobarbital in rabbits. Four untreated animals received 40 mg sodium salt of hexobarbital/kg body wt in 2 ml of water by cautious injection into the ear vein. Samples of arterial blood were collected from the other ear into heparinized vessels 5 times within 1.5 hr. Plasma concentration of non-

metabolized hexobarbital was determined according to Cooper and Brodie [24]. Blanks were obtained from two blood samples taken immediately before hexobarbital-injection. Clophen A 50 dissolved in olive oil (5% w/v) was given to the same animals by stomach tube.

Sleeping time of rats. Groups of 10 Wistar rats were starved for 16 hr and then received a 5% (w/v) solution of Clophen A 50 in olive oil by stomach tube. Control groups only received olive oil. After i.p. injection of the sodium salt of hexobarbital (1.5% in 0.9% NaCl) the time until the righting reflexes reappeared (from the side position) was determined.

Gaschromatographic determination of PCBs in tissues

For estimation of PCB-content 1 ml microsomes were extracted by shaking with 5–10 ml hexane. The organic phase was concentrated to 1 ml vol. and chromatographed on a 1 × 10 cm column of Al_2O_3 (Woelm neutral, 5.5% H_2O content) with 15 ml *n*-hexane. Gas liquid chromatography was performed on a Carlo Erba gas chromatograph, model Fractovap 2300, equipped with a 63 Ni-electron-capture detector. A glass column of 6 feet length was packed with 5% SE 30 on Chromosorb W/AW/DMCS, 80/100 mesh. The column temperature was 210°. Argon/methane (90/10) was used as carriergas. Automatic intergration of the gaschromatogram was taken for quantitative estimation.

RESULTS

Rabbit and rat liver drug metabolism in vitro *after 5 × 50 mg/kg of Clophen A 50*

When rabbits and rats were pretreated daily with 50 mg/kg Clophen A 50 for 5 consecutive days, only liver weight and cytochrome P-450 level were markedly increased in rabbits, whereas the rates of *p*-hydroxylation of 4-chlorobiphenyl and aniline as well as of the oxidative demethylation of aminopyrine and *p*-nitroanisole remained unchanged. No augmentation of microsomal protein was observed (Fig. 1).

In contrast, all parameters determined with Clophen-treated rats were significantly increased, in agreement with the results obtained by other authors [4–7].

Two parameters were similarly increased in both species: the relative liver weight by 50% and the cytochrome P-450 level by 140%. The maximal wavelength of the CO-difference spectrum of the rabbit cytochrome was 448.4 ± 0.2 nm. It differs significantly from the maximum of 449.8 ± 0.2 nm after phenobarbital ($P < 0.01$) and from the maximum of 447.8 ± 0.2 nm after 3-MC-pretreatment ($P < 0.001$). In rats, the maximal wavelength of 448.6 ± 0.2 nm after Clophen A 50 treatment was significantly different ($P < 0.001$) from 449.9 ± 0.2 nm, the maximal wavelength of controls.

Hexobarbital half life in rabbits and hexobarbital narcosis in rats after a single oral dose of 50 mg/kg Clophen A 50

The differences of inducibility between the two species observed *in vitro* were further confirmed by the determination of hexobarbital metabolism *in vivo*. In untreated rabbits hexobarbital half life was 28 ± 6 min. Two days later, a similar half life was found in the same animals. This indicates that repeated hexobarbital administration does not alter its own metabolism. When these rabbits were treated with a single oral dose of 50 mg/kg Clophen A 50, a dose which induced P-450-content 2 days later by 60%, hexobarbital half life remained unaffected.

Rats after identical treatment with Clophen A 50 showed a markedly reduced hexobarbital sleeping time (Table 1) 8 hr after PCB-administration. This reduction was not significantly different between both sexes. One, resp. three days later, a reduction to 35% resp. 24% of control values was observed in female rats. With male rats, a similar reduction of sleeping time was found by other authors [5].

Microsomal enzyme activities of rabbits at different times after 5 × 50 mg/kg Clophen A 50

The high P-450 level observed within the first week after 5 × 50 mg/kg Clophen A 50 decreased slowly during the following week whereas microsomal protein content and oxidation of 4-chlorobiphenyl were not significantly altered (Fig. 2).

Demethylation rate of *p*-nitroanisole after 2 wk was the same as 3 days after the last PCB-administration. Liver wt was maximally increased while cytochrome level decreased, indicating that these two responses of the liver cell to PCB-uptake may be not correlated to each other.

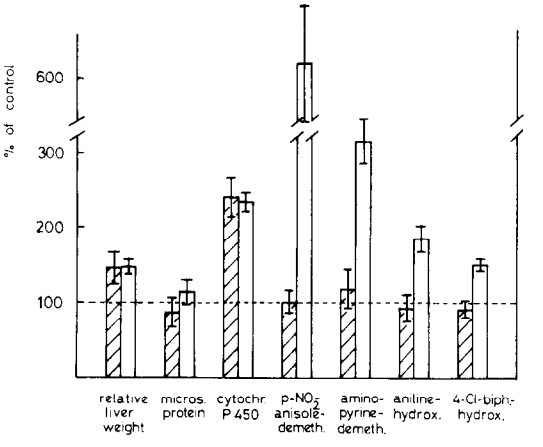

Fig. 1. Induction parameters after i.p.-administration of 50 mg/kg Clophen A 50 given daily on 5 consecutive days to male rabbits and rats. Microsomes were prepared 3 days after the end of treatment. For experimental details see "materials and methods". The columns represent mean values ± S.D. of rabbits ■ and rats □ relative to control. Control values for rabbits and rats were: Relative liver weight: 3.9 ± 0.4 ($n = 22$) and 5.5 ± 0.4 g/100 g body wt, microsomal protein: 8.8 ± 1.5 ($n = 21$) and 18.3 ± 1.1 mg protein/g fresh liver, cytochrome P-450: 1.94 ± 0.25 ($n = 18$) and 1.01 ± 0.20 nmoles/mg protein, *p*-nitroanisole-demethylation: 1.8 ± 0.2 ($n = 12$) nmoles and 0.9 ± 0.2 nmole *p*-nitrophenol/mg protein/min, aminopyrine-demethylation: 0.40 ± 0.05 ($n = 11$) and 0.25 ± 0.09 nmole amino-antipyrinc/mg protein/min, aniline-*p*-hydroxylation: 0.83 ± 0.12 ($n = 12$) and 1.72 ± 0.22 nmole *p*-aminophenol/mg protein/min, 4-chlorobiphenyl-*p*-hydroxylation: 1.2 ± 0.1 ($n = 9$) and 2.6 ± 0.5 nmole 4-chloro-4′-hydroxy-biphenyl/mg protein/min. Number of treated rabbits is the same as indicated for controls. For determination of drug metabolism in rats, groups of 8 animals each weighing 150 ± 20 g were used. Livers of two animals were pooled.

Table 1. Effect of a single oral dose of 50 mg/kg Clophen A 50 on hexobarbital narcosis in rats

Sex	Time after PCB-dosage (hr)	Dose of hexobarbital (mg/kg)	Sleeping time (min) Control	50 mg kg Clophen A 50	% Decrease
male	8	150	42 ± 6 (6)	32 ± 3 (8)	23
female	8	150	113 ± 13 (7)	72 ± 17 (7)	36
female	24	120	92 ± 7 (9)	32 ± 8 (10)	65
female	72	150	97 ± 21 (5)	23 ± 8 (7)	76

Male rats weighing 165 ± 10 g and female rats weighing 120 ± 20 g were used. Data are mean values ± S.D. Groups of 10 animals were treated. *n*. given in brackets was < 10, when animals died or did not sleep.

Effect of feeding 100 ppm Clophen A 50 on microsomal drug metabolism in rabbits

To find out whether chronic low dosage has an effect similar to acute high dosage, rabbits were fed with a diet containing 100 mg/kg Clophen A 50, which corresponds to an approximate daily uptake of 10 mg/kg. After 6 wk, no changes in drug metabolizing enzymes could be observed, whereas cytochrome P-450 content was doubled.

DISCUSSION

PCBs are known inducers of P-450 dependent microsomal drug metabolism in rats [4–6] and mice [25] and of steroid metabolism in birds [26–28]. There are similarities and some striking differences between rabbits and rats in the response to PCB-treatment (Fig. 1). Some parameters, relative liver wt, cytochrome P-450 content and the wavelength shift of the reduced CO-difference spectrum were similarly altered in rabbits and rats. However, two demethylation reactions, the *N*-demethylation of aminopyrine and the *O*-demethylation of *p*-nitroanisole as well as the aromatic *p*-hydroxylation of aniline and 4-chlorobiphenyl, type II and type I compounds respectively [30,*], were not enhanced in microsomes of Clophen A 50-treated rabbits but in microsomes of rats. 4-Chlorobiphenyl was chosen as substrate instead of the more generally used biphenyl [29] in order to test whether PCBs can stimulate their own metabolism.

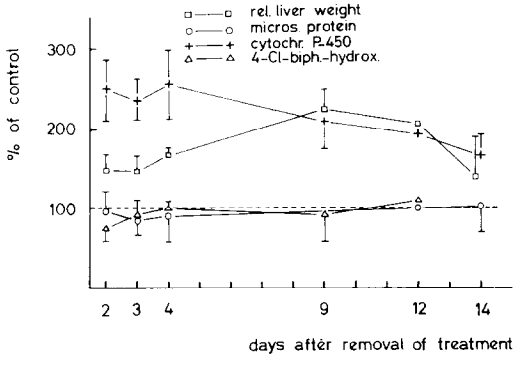

Fig. 2. Induction parameters at different time intervals after administration of 50 mg/kg Clophen A 50 given daily on 5 consecutive days. Each point represents a mean value ± S.D. relative to control of 3 animals 2, 4, 9 and 14 days after treatment and of 2 animals 12 days after treatment. *n* for day 3 after treatment is given under Fig. 1. For experimental details see "materials and methods". Control values see legend of Fig. 1.

Similarly to the *in vitro* activities, *in vivo* hexobarbital half life remained unchanged in Clophen A 50-treated rabbits, whereas in rats shortening of hexobarbital sleeping time soon after uptake of Clophen A 50 indicated accelerated barbiturate metabolism.

These findings are in disagreement with reports of a 3-fold increase in aminopyrine-demethylation [11], or shortened hexobarbital sleeping time [12] after administration of a low dose of Aroclor 1254 to rabbits. Differences in rabbit strains which were made responsible for large differences in hexobarbital metabolism rate after phenobarbital treatment [32] may account for the conflicting results. However, the rabbit strain we used does not generally behave as a non- or weakly inducible strain, because after pretreatment with phenobarbital we observed a marked induction of all enzyme parameters measured [33].

Considering the missing induction of several P-450 dependent reactions, it is possible that residual PCBs remaining in the microsomes during isolation inhibit the *in vitro* metabolism sufficiently to mask induced mixed-function oxidase activity. This appears unlikely, since gaschromatographic determination showed a microsomal PCB-concentration not more than 0.5–2 nmol/mg protein (Table 2). A 10 per cent inhibition of *p*-nitroanisole-*O*-demethylation *in vitro*

Table 2. Determination of PCB-residues in liver microsomes

Dose (i.p.) (mg/kg)	Animal	Time after removal of administration (days)	Content (μg/g protein)
1 × 50	rabbit	3	95*
5 × 50	rabbit	3	430 ± 55 (4)
5 × 50	rat	3	100 ± 20 (4)

* Pooled livers of 4 animals.

Data are mean values ± S.D. Numbers of individual microsomal preparations are given in brackets.

* S. Hesse, unpublished result.

required the presence of 25 nmol of 2,4,6,2',4',6'-hexachloro- or 40 nmol of 2,5,2',5'-tetrachlorobiphenyl/mg protein, i.e. 10–50 times the residual PCB-concentration. Aniline-hydroxylase was even found to be stimulated when 100 nmol/mg protein were added *in vitro* (results not shown).

Likewise it is unlikely that induction occurs later than 3–4 days after the last dosage, the time chosen for the preparation of microsomes and enzyme assay, since *p*-hydroxylation activity was not found to be increased within two wk after the last application of 5 × 50 mg/kg Clophen A 50 (Fig. 2).

It has been established that 3-MC increases hepatic cytochrome level and shifts the CO-difference spectrum to 448 nm, but does not change the microsomal protein content or the rate of several hydroxylation and demethylation reactions in the rabbit [9, 10]. Recent observations indicate that the 3-MC-induced cytochrome may have specific catalytic properties. Acetanilide-*p*-hydroxylation and N-2-acetylaminofluorene-*N*-hydroxylation were markedly stimulated after treatment [35]. Preliminary results showed that Clophen, like 3-MC causes a 2- and 5–10-fold increase, respectively, in these two microsomal enzymes in rabbits*.

Thus, our results suggest that PCBs induce only one type of mixed-function oxidase in the rabbit liver. The substrate specificity of the induced cytochrome totally differs from the specificity of the PB-type, which is characterized by a general increase in mixed-function oxidase activities [33, 34] and is closely similar to the activity pattern caused by 3-MC-treatment. We are aware of the fact, that the similarity in the enzyme activity pattern after PCB- as well as after 3-MC-treatment does not prove the identity of the two cytochromes.

The failing stimulation of 4-chlorobiphenyl-*p*-hydroxylase in the rabbit liver after PCB-treatment indicates that PCBs do not stimulate their own metabolism in this animal. In the rat, PCBs are enhancing the metabolism of 4-chlorobiphenyl after PCB-administration (Fig. 1). This species difference would explain why the microsomal PCB-content of the rabbit liver is much higher than in rat liver after identical treatment (Table 2). Thus, if PCB-metabolism is merely a detoxification mechanism, PCBs might be more toxic to the rabbit than to the rat.

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* Th. Wolff, in preparation.