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Bilirubin and uroporphyrinogen oxidation by induced cytochrome P4501A and cytochrome P4502B
Role of polyhalogenated biphenyls of different configuration

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Abstract

In previous work it was shown that hepatic microsomes from rats treated with 3-methylcholanthrene and similar inducers had increased bilirubin-degrading activity. The activity was further stimulated by addition of 3,4-tetrachlorobiphenyl (TCB), a response specifically dependent on CYP1A1. Here, we compared the effect of adding PCBs of either planar or non-planar configuration on rate of bilirubin degradation, monooxygenase activity and NADPH/O_2 consumption by liver microsomes from animals treated with either phenobarbital or 3-methylcholanthrene/β-naphthoflavone. We also examined the oxidation of uroporphyrinogen (hexahydro-uroporphyrin) (URO'gen) under these conditions. Polychlorinated biphenyl (PCBs) stimulated the rate of bilirubin and URO'gen oxidation with microsomes expressing high levels of either CYP2B or CYP1A, inhibiting at the same time their monooxygenase activities (PROD and EROD, respectively); however, non-planar di-*ortho*-substituted PCBs were preferentially active with phenobarbitone-induced microsomes, in contrast to those active with 3-methylcholanthrene/β-naphthoflavone microsomes, where a planar configuration was required for activity. An antibody raised against CYP2B1 markedly inhibited the PCB-dependent bilirubin degradation and PROD activities of phenobarbital-induced microsomes with similar dose–response curves for the two effects. Increased microsomal utilizations of NADPH and O_2 were also caused by PCBs with both types of induced microsomes and here again PCBs of different configuration were preferentially active. It is concluded that PCBs of the appropriate configuration may interact with either CYP1A1 or CYP2B1, increase production of oxidative species by an uncoupling mechanism, and lead to oxidation of target molecules in the cell, among these uroporphyrinogen and bilirubin.

Keywords: CYP1A1; CYP2B1; Bilirubin degradation; Uroporphyrinogen oxidation; Congenital jaundice; Polyhalogenated biphenyls

1. Introduction

In previous work [1,2] a bilirubin-degrading system has been described in the microsomal fraction of rat liver, which can be induced by treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in vivo*. Hepatic microsomes from rats treated with this inducer displayed an increased rate of NADPH-dependent bilirubin degradation *in vitro* and this rate could be further doubled by addition to the microsomal incubation of a planar polyhalogenated biphenyl, 3,4,3′,4′-tetrachlorobiphenyl (3,4-TCB). This bilirubin-degrading system, which could also be induced to high activities by treatment with either β-naphthoflavone (BNF) or 3-methylcholanthrene (3-MC), operated both in non-jaundiced Wistar rats and in congenitally jaundiced Gunn rats, where induction with these agents was accompanied by a significant alleviation of jaundice [1,3,4]. More recent work [5], where mice of different strains were used, including *cyp1a2* null mutant mice, implicated both CYP1A1 and CYP1A2 in the bilirubin-degrading activity of the induced liver microsomes and suggested that manipulation of the CYP1A system may be of therapeutic benefit in patients with congenital jaundice due to defects of bilirubin conjugation. CYP1A2 apparently possesses a

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Abbreviations: BNF, β-naphthoflavone; CYP, cytochrome P450; ECOD, 3-cyano-7-ethoxycoumarin-*O*-deethylase; EROD, 7-ethoxyresorufin-*O*-deethylase; HCB, hexachlorobiphenyl; 3-MC, 3-methyl cholanthrene; PCB, polychlorinated biphenyl; PROD, 7-propoxyresorufin-*O*-depropylase; TCB, tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO'gen, uroporphyrinogen (hexahydro-uroporphyrin).

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high bilirubin-degrading activity, intrinsically; whereas CYP1A1 only acquires it when challenged with a planar PCB, such as 3,4-TCB. Addition of this compound was also found to cause a marked inhibition of the 7-ethoxyresorufin-*O*-deethylase (EROD) activity of the microsomal fractions from induced animals [1,2], suggesting that the planar PCB interacted with the active site of CYP1A1, leading to inhibition of its monooxygenase activity and to increased production of oxidizing species by an uncoupling mechanism.

The purpose of this present work has been 2-fold. First, since treatment of jaundiced patients with phenobarbital has been reported to be beneficial in reducing plasma bilirubin levels [6], we have examined the possibility that phenobarbital-inducible CYP2B1 may also promote bilirubin degradation. We now report that when animals were treated with phenobarbital, their liver microsomes exhibited an increased rate of bilirubin degradation *in vitro* and, also in this case, the addition of PCB stimulated the rate of bilirubin degradation, while inhibiting the monooxygenase activity (7-propoxyresorufin-*O*-depropylase, PROD [7]) of the induced CYP2B1 enzyme. However, in contrast to the planar PCBs which were active with CYP1A1, non-planar PCBs were preferentially active with phenobarbital-induced CYP2B enzymes.

The second main point we have addressed is whether, under conditions where CYP-dependent bilirubin degradation is seen, oxidation of URO'gen can also be demonstrated. CYP1A2 has been clearly implicated in the oxidation of URO'gen [8,9], thus playing a critical role in the mechanism of induction of hepatic uroporphyria [10]. However, there is still uncertainty as to whether CYP1A1 and CYP2B may also be involved in URO'gen oxidation and if so whether oxidation of this substrate also results from increased production of oxidizing species by an uncoupling mechanism. We now present evidence that both CYP1A1 and CYP2B could, on interaction with PCBs of the appropriate configuration, catalyze increased oxidation of URO'gen *in vitro*. Increased utilization of NADPH and oxygen was also caused by the PCBs with both types of induced microsomes and the stimulation of NADPH oxidation, as well as the oxygen uptake and the bilirubin degradation caused by a PCB, all exceeded on a molar basis the amount of PCB added.

The present findings help substantiate the hypothesis that interaction of PCBs with the active sites of CYP enzymes may lead to increased production of reactive oxygen species by an uncoupling mechanism, thus resulting in increased oxidation of cellular molecules, among these URO'gen and bilirubin. The extension of these effects to liver microsomes induced by phenobarbital suggests that polyhalogenated chemicals, which are inducers of CYP2B, rather than CYP1A enzymes, may also initiate toxicity through an oxidative stress mechanism. In addition, these results raise the possibility that increased oxidation of bilirubin may contribute to the jaundice-lowering effect of phenobarbital in people with high plasma levels of bilirubin. A preliminary report of some of these findings has been given in an abstract form [11].

2. Materials and methods

2.1. Animals

Wistar male rats (body weight 155–185 g) and New Zealand White male rabbits were allowed food and water *ad libitum* until they were killed. Treatment with inducers involved a single intraperitoneal injection of TCDD (300 μg/kg body weight) or of 3-MC (80 mg/kg), both in corn oil, and the animals were killed 72 and 63 hr after injection, respectively. Rats were given BNF as two intraperitoneal injections of 80 mg/kg, 48 and 24 hr before killing. Phenobarbital (Na salt) was given in drinking water to rats (1 mg/mL) or rabbits (0.5 mg/mL) for 7 days and the animals were then allowed ordinary water for 24 hr before being killed. Chicken embryos, 16 days of age, of the Rhode Island × White Rock strain, were given BNF (3 mg per embryo) by injection through the air sac, 24 hr before killing. Liver microsomal fractions from chick embryos and rats were isolated, washed and stored frozen as described in [1]; rabbit microsomes as described in [5].

All procedures involving animal care or treatments were in accordance with institutional guidelines in compliance with national and international laws and policies.

2.2. Analytical techniques

Protein was measured by the method of Lowry *et al.* [12], using crystalline bovine serum albumin as a standard, and cytochrome P450 (CYP) content by the method of Omura and Sato [13].

2.3. Source of special chemicals and reagents

NADPH (type III), control rabbit serum and bilirubin were obtained from Sigma Chemical Company and uroporphyrin I from Porphyrin Products; 7-ethoxyresorufin and 7-propoxyresorufin were from Boehringer Mannheim GmbH; 3,4-TCB was from Ultra Scientific; 2,4,2′,4′-tetrachlorobiphenyl (2,4-TCB) was from Analabs Inc; 2,4,5,2′,4′,5′-hexachlorobiphenyl (2,4,5-HCB) and 3,4,5,3′,4′,5′-hexachlorobiphenyl (3,4,5-HCB) were from Greyhound Chromatographic and Allied Chemicals; β-naphthoflavone was from Aldrich Chemical Company and TCDD was a gift from J.B. Greig. A rabbit immune serum raised against rat CYP2B1, but recognizing on immunoblotting both CYP2B1 and CYP2B2 [14], was a gift from A.J. Paine. Its IgG fraction (and the IgG fraction from control rabbit serum) were purified by precipitation with polyethylene glycol [15], redissolved in a volume of phosphate-buffered saline (0.14 M NaCl/8 mM sodium phosphate buffer, pH

7.3) equal to the volume of the original serum, and used in the microsomal inhibition studies.

2.4. Experiments with isolated microsomal fractions

The rate of metabolism of bilirubin and URO'gen I by isolated microsomal fraction was studied in the incubation mixture as previously described [1], using EDTA (final concentration 2 mM) in all experiments, final concentrations of bilirubin and URO'gen of 10 and 2.8 μM, respectively, and liver microsomes corresponding to a concentration of total cytochrome P450 of 65–130 pmol/mL of incubation mixture. The concentrations of bilirubin and URO'gen used here were those employed in previous work [1,16]. Uroporphyrin I (free carboxylate) was fully reduced to the corresponding porphyrinogen, URO'-gen, by treatment with sodium amalgam under N_2. The oxidation of URO'gen was followed in plastic disposable cuvettes (incubated in the presence of air at 28°) by monitoring the increase in absorbance of the Soret maximum and expressed as pico moles of URO'gen oxidized per minute, using an εmM = 260, obtained experimentally under the conditions of the assay. The degradation of bilirubin was followed under identical conditions, by monitoring the loss of absorbance at 450 nm and using an εmM = 55.8.

When the inhibitory effect of the polyclonal antibody on the bilirubin-degrading activity of phenobarbital-induced rat liver microsomes was studied, the final concentration of cytochrome P450 was 32.5 pmol/mL. After incubating the microsomes with increasing concentrations of the IgGs for 5 min, 2,4-TCB in dimethylsulfoxide (DMSO) or DMSO alone was added and, 5 min later, the reaction was started by addition of NADPH and bilirubin. The final concentration of DMSO in both control and 2,4-TCB-treated incubations was 2.8 μL/mL.

The EROD and PROD activities of liver microsomes were measured by a modification [1] of the method of Burke and Mayer [17], using final concentrations of 7-ethoxyresorufin and 7-propoxyresorufin of 2 and 10 μM, respectively, and microsomes delivering 8–40 pmol cytochrome P450/mL of incubation. In one experiment the 3-cyano-7-ethoxycoumarin-*O*-deethylase (ECOD) activity of 3-MC-induced microsomes was also studied, as described by White [18], using a final concentration of substrate of 40 μM and the same concentration of cytochrome P450 as in the 7-alkoxyresorufin assays. The inhibitory effect of PCBs on the monooxygenase activity of microsomes was studied by adding a PCB in DMSO (or DMSO alone) to the incubation mixture containing the dilute suspension of microsomes followed, 1 min later, by the 7-alkoxyresorufins or 3-cyano-7-ethoxycoumarin, and, after 1 min, by NADPH. The inhibitory effect of the antibody raised against CYP2B1 on the PROD activity of phenobarbital-induced rat liver microsomes was also studied, employing IgG/cytochrome P450 ratios similar to those used to investigate the effect of the same antibody on the rate of bilirubin degradation.

The effect of 2,4-TCB and 3,4-TCB on the rate of oxygen uptake and NADPH oxidation was studied in an incubation mixture containing the following components, with final concentrations in parentheses; Tris–HCl buffer, pH 8.2 (100 mM); KCl (19.5 mM); $MgCl_2$ (10 mM); EDTA (2 mM); NaN_3 (0.2 mM), NADPH (83 mM) and microsomal fraction corresponding to a concentration of total cytochrome P450 of 518 pmol/mL. DMSO was present in the incubation mixture at the final concentration of 1.4 μL/mL. NADPH oxidation was followed by monitoring the absorbance at 340 nm (εmM = 6.2 [19]) in the thermostatically controlled, 28°, compartment of a recording spectrophotometer; while the oxygen uptake was measured in the closed cell of a Hanstech oxygen electrode apparatus maintained at 28° [20], calculating the results on the basis of a starting concentration of oxygen of 450 ng atom/mL [21].

3. Results

3.1. Stimulation of bilirubin degradation and inhibition of monooxygenase activity caused by PCBs in microsomal incubations

Treatment of rats with phenobarbital and 3-MC stimulated markedly the rate of microsomal 7-alkoxyresorufin dealkylases (PROD and EROD, respectively), as expected. The rate of basal bilirubin degradation (the rate, that is, observed in presence of NADPH, but without adding a PCB) was also increased in comparison to that shown by microsomes from control rats, but the effect was only modest, roughly in-line with the increase in total CYP concentration observed upon induction (see Table 1). However, induced microsomes responded to the addition of PCBs with a marked increase in rate of bilirubin degradation (Table 1).

The structures of the PCBs tested are shown in Fig. 1. Microsomes obtained from the liver of rats induced with phenobarbital responded to the addition of both di-*ortho*-substituted PCBs (2,4-TCB and 2,4,5-HCB) with a marked stimulation of the rate of bilirubin degradation, whereas both planar congeners (3,4-TCB and 3,4,5-HCB) were only moderately active. In contrast, microsomes obtained from the liver of rats induced with 3-MC responded to the addition of both planar PCBs with a marked stimulation of the rate of bilirubin degradation, whereas the di-*ortho*-substituted PCBs were virtually inactive. Liver microsomes from TCDD and BNF-treated rats gave a pattern of stimulation by PCBs similar to that given in Table 1 for 3-MC-treated rats (results not shown). The microsomes from phenobarbital-induced rabbits and BNF-induced chick embryos were also tested for the response of their bilirubin-degrading activity to addition of various PCBs *in vitro* (Table 1), as these two species have been reported to

Table 1
Effect of PCBs on the rate of bilirubin degradation by liver microsomal fractions obtained from rats, rabbits and chick embryos treated with inducers of CYP1A or CYP2B enzymes

Species	Treatment *in vivo*	Percentage stimulation of bilirubin degradation			
		2,4-TCB	2,4,5-HCB	3,4-TCB	3,4,5-HCB
Rat	None	4 ± 11 (3)	n.d.	2.9 ± 2.6 (3)	n.d.
	Phenobarbital	214 ± 15 (10)[a]	113 ± 3 (4)[b]	33 ± 5.5 (4)	12.5 ± 2 (5)
	3-MC	11.2 ± 5.4 (4)[a]	−7.7 ± 2.5 (4)[b]	145.8 ± 10 (4)	269 ± 16 (4)
Rabbit	Phenobarbital	306 ± 11 (4)[a]	257 ± 6 (4)[b]	18 ± 2 (4)	21 ± 5 (4)
Chick embryo	BNF	4 ± 7 (5)[a]	−7 ± 4 (5)[b]	603 ± 52 (5)	440 ± 25 (5)

Washed microsomes were incubated with bilirubin and NADPH in the presence of one of the biphenyls listed (added as a solution in DMSO) and the stimulation of the rate of bilirubin degradation was calculated from the basal rate obtained with the same microsomes incubated in presence of the vehicle alone. The amount of biphenyl added was 1 μg/3.5 mL total incubation with microsomes from rats and rabbits and 300 ng/3.5 mL with microsomes from chick embryos, as preliminary experiments showed the latter to be much more responsive. Results are given as averages ± SEM of the number of observations in parentheses. n.d. = not done. Basal, unstimulated rates (pmoles/min/mg protein) were as follows: rats, control uninduced, 320 ± 8 (3); phenobarbital-induced, 880 ± 270 (5); 3-MC-induced, 440 ± 20 (5); phenobarbital-induced rabbits, 1070 ± 30 (4); BNF-induced chicken, 161 ± 15 (5).

[a] $P < 0.001$, when compared with corresponding values obtained with 3,4-TCB.

[b] $P < 0.001$, when compared with corresponding values obtained with 3,4,5-HCB.

be resistant to induction of lipid peroxidation [22,23]: they were found to respond to the addition of PCBs with an even greater stimulation of bilirubin degradation than shown by similarly treated rats, maintaining however the same selectivity of response (with regard, that is, to the planar/non-planar PCB configuration). Liver microsomes obtained from rabbits induced with 3-methylcholanthrene also responded to addition of the PCB of the appropriate configuration *in vitro* as microsomes from induced rats, though less markedly (results not shown).

The addition of superoxide dismutase (1000 unit/mL) or catalase (1000 or 2000 unit/mL) to incubations mixtures containing 2,4-TCB-stimulated phenobarbital microsomes from rat liver did not reduce significantly the rate of bilirubin degradation: catalase was completely ineffective and superoxide dismutase may have produced a small reduction in rate, but this was no more than 6% of the rate without the addition (results not shown).

PCBs also inhibited the monooxygenase activity of the induced microsomes and here again non-planar isomers were more active with phenobarbital-induced rat and rabbit microsomes (PROD activity); while planar congeners were more effective in inhibiting the EROD and ECOD activity of 3-MC-induced microsomes (Table 2). Dose–response experiments (Fig. 2) confirmed that non-planar compounds were considerably more potent than planar isomers with microsomes from phenobarbital-induced rats and this applied both to the stimulation of bilirubin degradation and to the inhibition of the appropriate dealkylase (PROD). In contrast, in similar log dose–response experiments, with 3-MC (or TCDD) microsomes, planar isomers exhibited greater potency (results not shown and see also [1]).

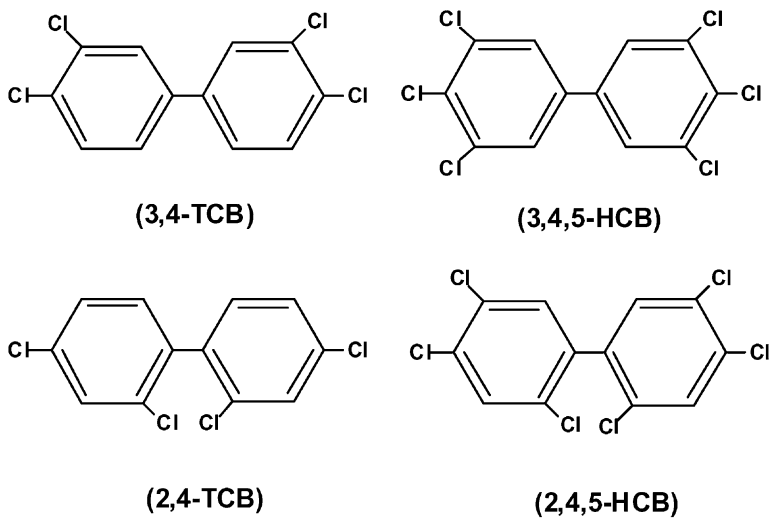

Fig. 1. The structure of the four PCBs discussed in this work. Note that 3,4,3′,4′-tetrachlorobiphenyl and 3,4,5,3′,4′,5′-hexachlorobiphenyl can both take up a planar configuration, resembling that exhibited, typically, by TCDD. For this reason they are referred to as planar. In contrast, 2,4,2′,4′-tetrachlorobiphenyl and 2,4,5,2′,4′,5′-hexachlorobiphenyl are di-*ortho*-substituted biphenyls, where the co-planar arrangement of the two rings is sterically hindered, and are therefore referred to as non-planar.

Table 2
Effect of PCBs, added *in vitro* to microsomal incubations, on the PROD activity of phenobarbital-induced rats and rabbits and on the EROD and the ECOD activities of 3-MC-induced rats

Treatment *in vivo*	Species	Enzyme activity tested	Percentage inhibition of appropriate 7-alkoxy-*O*-dealkylase activity			
			2,4-TCB	2,4,5-HCB	3,4-TCB	3,4,5-HCB
Phenobarbital	Rat	PROD	61.4 ± 0.6 (3)[a]	42.9 ± 3 (4)[c]	31 ± 2.9 (3)	19.3 ± 1.1 (3)
	Rabbit	PROD	65 ± 1 (4)[a]	50 ± 1 (4)[d]	14 ± 2 (4)	3 ± 0.8 (4)
3-MC	Rat	EROD	13.1 ± 11 (3)[b]	9.2 ± 12 (3)[d]	74 ± 5.6 (3)	48.9 ± 2.8 (3)
	Rat	ECOD	23 (33, 14)	14 (14, 14)	86 (87, 85)	n.d.

Washed liver microsomes were incubated for 1 min with one of the biphenyls listed, added in DMSO (200 ng/2.25 mL total incubation). The appropriate resorufin (or coumarin) substrate was then added and, 1 min later, the reaction was started by adding NADPH. Results were calculated as percentage inhibition of dealkylase activity [compared to controls receiving solvent (DMSO) alone] and are averages ± SEM of the observations in parentheses or averages with individual observations in parentheses. n.d. = not done. Control, uninhibited rates (pmol/min/mg protein) were as follows: PROD-rat, 1360 ± 390 (6); PROD-rabbit, 1166 ± 34 (4); EROD, 5590 ± 530 (11); ECOD, 3620 ± 120 (3).

[a] $P < 0.001$, compared to corresponding values obtained with 3,4-TCB.

[b] $P < 0.01$, compared to corresponding values obtained with 3,4-TCB.

[c] $P < 0.05$, compared to corresponding values obtained with 3,4,5-HCB.

[d] $P < 0.01$, compared to corresponding values obtained with 3,4,5-HCB.

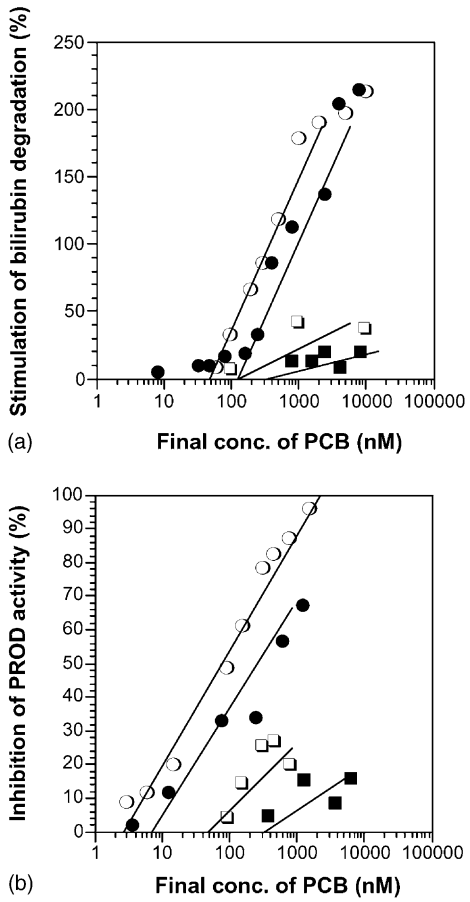

Fig. 2. Log dose–response for the effect of various PCBs on bilirubin degradation and PROD activity of phenobarbital-induced rat liver microsomes. (a) Percentage stimulation of bilirubin degradation caused by the PCBs; (b) percentage inhibition of PROD activity. The following PCBs were added in DMSO to the final concentration indicated: 2,4-TCB (○); 2,4,5-HCB (●); 3,4-TCB (❑); 3,4,5-HCB (■). All data are averages of at least two experiments and are expressed as percentages of the corresponding control activities obtained with microsomal samples receiving solvent (DMSO) alone. Note that the amount of liver microsomes taken for bilirubin degradation and PROD activity corresponded, respectively, to a concentration of total cytochrome P450 of 65 and 8 pmol/mL of incubation.

3.2. Effect of an antibody directed against the phenobarbital-inducible cytochrome(s) P450

The stimulation of bilirubin degradation caused by 3,4-TCB in microsomes from TCDD-induced rats required the activity of CYP1A1, as shown by the inhibitory effect of an antibody raised against this enzyme [2]. Similar results are now presented to implicate CYP2B in the bilirubin-degrading activity of phenobarbital-induced rat liver microsomes. An antibody directed against CYP2B1 (which also recognizes CYP2B2 [14]) inhibited both the basal NADPH-dependent rate of bilirubin degradation of the microsomes from phenobarbital-induced rats and the subsequent stimulation of this rate which was due to 2,4-TCB. The inhibition applied preferentially to the PCB-stimulated rate (Fig. 3a). The antibody was also strongly inhibitory on the PROD activity of the same microsomes, and when the effects of the antibody on the PROD activity and on the bilirubin degradation due to 2,4-TCB addition (calculated by difference from the corresponding unstimulated rates) were compared, the two dose–inhibition curves were found to be very similar, though not exactly superimposable (Fig. 3b).

3.3. Stimulation of microsomal URO'gen oxidation caused by PCBs and correlation with bilirubin degradation

PCBs could also stimulate the microsomal rate of oxidation of URO'gen (Table 3); however the stimulation was considerably lower than observed under identical conditions for bilirubin degradation and in the case of rats, more variable. Thus, phenobarbital-induced rabbits and BNF-induced chick embryos showed a similar pattern of stimulation as shown in Table 1 for bilirubin, again with non-planar PCBs exhibiting preferential activity after phenobarbital induction and planar compounds after BNF.

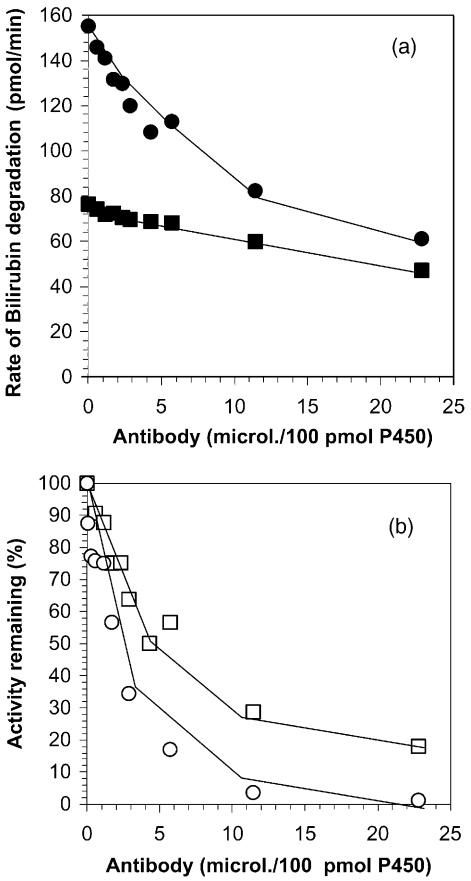

Fig. 3. Effect of an antibody raised against CYP2B1 on the rates of bilirubin degradation and PROD activity of liver microsomes from phenobarbital-induced rats. The two activities were assayed as described in Section 2.4, in the presence of increasing amounts of the antibody, so as to achieve the ratios of antibody to total CYP, which are indicated. (a) The effect of the antibody on the basal rate of bilirubin degradation (■) and on the rate stimulated by 2,4-TCB (1 μg per incubation (●)). (b) The inhibitory effect of the antibody on PROD activity (○) and the 2,4-TCB-dependent bilirubin degradation (□) are compared; the latter rate was calculated as the difference between the bilirubin degradation seen in presence of 2,4-TCB and the corresponding values obtained in its absence. Note that, in contrast to the immune IgG, control IgG only exhibited a slight inhibitory effect on the 2,4-TCB-stimulated rate of bilirubin degradation (8% inhibition vs. 60%, at 11 μL/100 pmol CYP) and caused a slight stimulation of PROD activity (17% stimulation vs. 100% inhibition at 22 mL/100 pmol CYP).

The rate of URO'gen oxidation of microsomes from 3-MC-induced rats was stimulated by 3,4-TCB in most cases (Table 3), a stimulatory effect shared by 3,4,5-HCB, with 2,4-TCB and 2,4,5-HCB showing no stimulation. However, in the case of 4 out of a total 11 rats, 3,4-TCB failed to stimulate the oxidation of URO'gen, even though it could still stimulate the rate of bilirubin degradation, albeit to a lower extent than found with the "responsive" microsomes shown in Table 3. As previously reported for bilirubin degradation [2], the stimulation of URO'gen oxidation by 3,4-TCB could be demonstrated in the kidneys of rats given 3-MC (where induction of CYP1A1, but not CYP1A2, has been reported) and showed a good correlation with CYP1A1 after treatment of the 3-MC-induced rats with selective suicide substrates of CYP1A enzymes (results not shown). Liver microsomes from rats induced with BNF also responded to 3,4-TCB, 1 μg per incubation, with a stimulation of URO'gen oxidation: activities of URO'gen oxidation were (pmol/min/mg protein, averages ± SEM of four observations) basal oxidation rate, 27 ± 0.95; 3,4-TCB-stimulated rate, 45.2 ± 0.5, $P < 0.001$.

Further similarities between bilirubin degradation and URO'gen oxidation were obtained with microsomes from BNF-induced chick embryos, by comparing metabolism of the two substrates after adding increasing amounts of 3,4-TCB (Fig. 4a) or after challenge with a standard dose of different PCBs. A very good correlation between bilirubin degradation and oxidation of URO'gen was found in both cases (Fig. 4b), with all experimental points falling within the same close relationship.

3.4. Effect of PCBs of different configuration on the utilization of oxygen and NADPH by induced liver microsomes and attempted calculation of stoichiometry for the PCB-induced responses

Table 4 shows that both 2,4-TCB and 3,4-TCB could stimulate the oxygen uptake and NADPH oxidation of induced rat liver microsomes and also in this case 2,4-TCB

Table 3
Effect of PCBs on the rate of URO'gen oxidation by liver microsomal fractions obtained from animals treated with inducers of CYP1A or CYP2B enzymes

Species	Treatment *in vivo*	Percentage stimulation of URO'gen oxidation			
		2,4-TCB	2,4,5-HCB	3,4-TCB	3,4,5-HCB
Rat	3-MC	−6 ± 4 (4)[a]	−4 ± 5 (3)[b]	69 ± 7 (7)	72 ± 16 (5)
Rabbit	Phenobarbital	28.6 ± 7 (4)[a]	24.8 ± 3 (4)[c]	−1 ± 2.4 (4)	−2.3 (2.4, −4.7)
Chick embryo	BNF	−2 ± 4 (5)[a]	12 ± 5 (5)[c]	241 ± 20 (5)	171 ± 18 (5)

Washed microsomes were incubated with uroporphyrinogen I and NADPH in the presence of one of the biphenyls listed (added as a solution in DMSO) and the stimulation of the rate of URO'gen oxidation was calculated from the basal rate obtained with the same microsomes incubated in presence of the vehicle alone. The amount of biphenyl added was 1 μg/3.5 mL total incubation, with microsomes from rats and rabbits and 300 ng/3.5 mL with microsomes from chick embryos. Results are given as averages ± SEM of the number of observations in parentheses, or as averages with individual observations in parenthesis. Basal, unstimulated rates of URO'gen oxidation (pmoles/min/mg protein) were as follows: 3-MC-induced rats, 32 ± 5 (7); phenobarbitone-induced rabbits, 77 ± 8 (4); BNF-induced chick embryos, 15 ± 1.6 (5).

[a] $P < 0.001$, when compared with corresponding values obtained with 3,4-TCB.
[b] $P < 0.05$, when compared with corresponding values obtained with 3,4,5-HCB.
[c] $P < 0.001$, when compared with corresponding values obtained with 3,4,5-HCB.

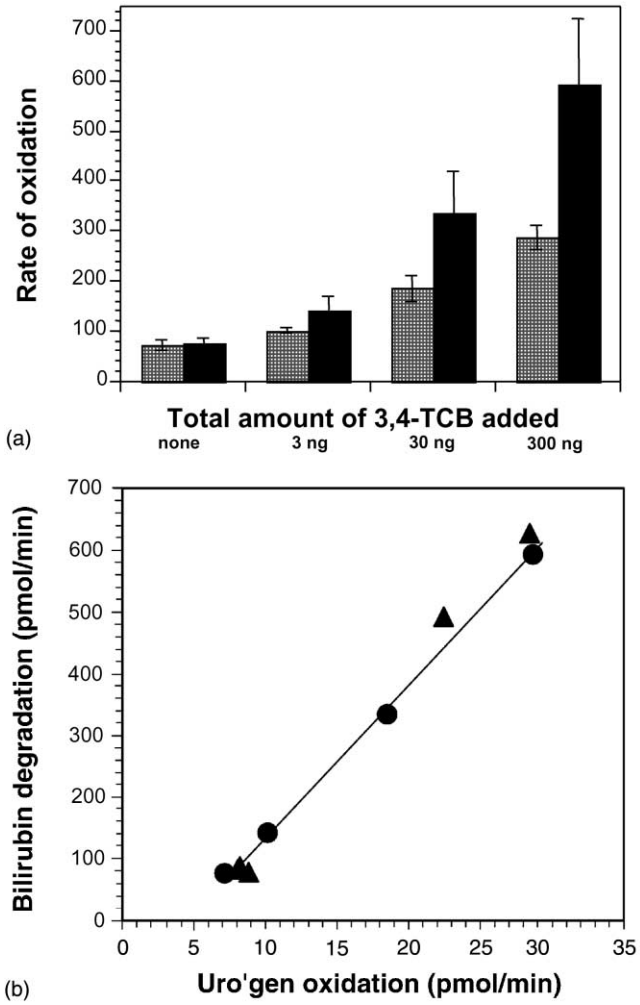

Fig. 4. Effect of 3,4-TCB on BNF-induced chick embryo liver microsomes. Comparison of the stimulation of bilirubin degradation with that of URO'gen oxidation. (a) The rates of bilirubin degradation (pmol/min, black columns) and those of URO'gen oxidation (pmol/10 min, shaded columns) in presence of increasing amounts of 3,4-TCB or in its absence. The results (averages ± SEM of four observations) are expressed as total amount of bilirubin degraded or URO'gen oxidized per 3.5 mL incubation containing 227 pmol total CYP. The TCB-stimulated rates were significantly different from corresponding control rates ($P < 0.05$), when the dose of TCB was either 30 or 300 ng/3.5 mL. (b) A comparison between average rates of bilirubin degradation and corresponding rates of URO'gen oxidation of BNF-induced chicken microsomes incubated under identical conditions with either substrate: (●) data taken from the dose–response experiment; (▲) results obtained with microsomes challenged with a standard dose of different PCBs (see Tables 1 and 3). Note a close correlation ($R^2 = 0.99$) between the two rates in both experiments.

exhibited greater activity after induction with phenobarbital, while 3,4-TCB was more effective with 3-MC-induced microsomes. In these experiments higher concentrations of CYP and TCBs, than in the bilirubin and URO'gen oxidation experiments, were found to be necessary in order to achieve acceptable rates of NADPH/O_2 consumption, particularly with 3-MC microsomes, which were less active. Although preferential stimulation by the appropriate TCB of both NADPH utilization and oxygen uptake was repeatedly demonstrated with both types of microsomes, a NADPH/O_2 stoichiometry proved difficult to measure, particularly with 3-MC-induced microsomes where the results were less reproducible. In four experiments with phenobarbital-induced microsomes, the apparent NADPH/O_2 ratios obtained for the effect of 2,4-TCB were as follows (concentration of TCB given in parenthesis): (2.9 μg/mL) 1.86, 1.82; (0.29 μg/mL) 2.11, 1.73. This may indicate that most of the oxygen taken up at an increased rate under the influence of 2,4-TCB is fully reduced to water (see later); however, in view of the complexity of the system investigated and the small amount of data so far obtained on this point, the results can only be considered as preliminary and will require further investigation.

The fate of the reducing equivalents and oxygen which are utilized at an increased rate under the influence of the TCBs is not known. Both 2,4-TCB and 3,4-TCB are potential substrates for metabolism by CYP enzymes. However, it could be calculated that the increased utilization of reducing equivalents and oxygen caused by either 2,4-TCB (with phenobarbital microsomes) or by 3,4-TCB (with 3-MC microsomes) exceeded in molar terms the amount of TCB added. For example, the increased NADPH utilization caused by adding 3.4 nmol (1 μg) 2,4-TCB to phenobarbital-induced microsomes already exceeded 15 nmol by 5 min incubation, at a time when both the unstimulated and TCB-dependent rates were still linear. A similar excess could be documented for the oxygen uptake by phenobarbital microsomes and for the NADPH utilization by 3,4-TCB-stimulated 3-MC microsomes. The ability to utilize NADPH at a stimulated rate was maintained, after a single challenge with TCB, for incubation times as long as 15 min, provided that the NADPH supply was maintained, making the molar excess of NADPH utilized (compared to the TCB added) even greater, when calculated over incubation periods longer than 5 min.

Similar calculations were applied to the rate of bilirubin degradation over the period of time in which both the basal unstimulated rate and that seen in the presence of a biphenyl were found to be linear. When the effect of the biphenyl, calculated by difference between the two rates, was compared to the amount of biphenyl added, the biphenyl-dependent bilirubin degradation was again found to be in excess compared to the amount of biphenyl added, especially at low doses of biphenyl and with chick embryo microsomes, which were by far the most active. For example, in chick embryo microsomes challenged with 30 ng (100 pmol) 3,4-TCB, see Fig. 4a, the amount of bilirubin degraded due to TCB over a 10 min linear period, calculated by difference from the corresponding unstimulated rate, was $3300 - 760 = 2540$ pmol, a 25-fold excess over the amount of TCB added. These findings therefore indicate that the increased rate of utilization of reducing equivalents and oxygen (involved, presumably, in generating the bilirubin- and URO'gen oxidizing species), and also the increased degradation of bilirubin, one of the target

Table 4
Effect of PCBs added *in vitro* to microsomes from induced rats on the rates of microsomal NADPH oxidation and oxygen uptake

Treatment of rats *in vivo*	Biphenyl added (μg/mL of incubation)	NADPH oxidation (nmol/min/mg protein)	Oxygen uptake (nmol/min/mg protein)
Phenobarbital	None	10.1 ± 0.36 (3)	12.15 ± 0.65 (4)
	2,4-TCB (2.86)	27.5 ± 0.76 (3)[a,d]	21.5 ± 1.95 (4)[a]
	3,4-TCB (2.86)	14.0 ± 0.34 (3)	17.8 ± 0.5 (4)
3-MC	None	5.66 ± 0.36 (4)	4 ± 0.35 (4)
	2,4-TCB (2.86)	6.83 ± 0.5 (4)	5.75 ± 0.6 (4)[c]
	3,4-TCB (2.86)	8.39 ± 0.57 (4)[b]	7.33 ± 0.42 (4)[a]

Rat liver microsomes, corresponding to a concentration of cytochrome P450 of 518 pmol/mL of incubation mixture, were incubated in the presence of a biphenyl or in its absence and the rates of NADPH oxidation and oxygen uptake were measured as indicated in Section 2.4. Results are averages ± SEM of the number of observations in parentheses.

[a] $P < 0.001$, when compared to corresponding values obtained in presence of 3,4-TCB.

[b] $P < 0.01$, when compared to corresponding values obtained in presence of 3,4-TCB.

[c] $P < 0.05$, when compared to corresponding values obtained in absence of a biphenyl.

[d] $P < 0.01$ when compared to corresponding values obtained in the absence of a biphenyl.

molecule studied, were all influenced by the configuration of the TCB and all exceeded in molar terms the amount of TCB added.

4. Discussion

This present work has extended the findings previously reported after induction with TCDD and similar inducers to microsomes obtained from phenobarbital-induced animals and here again PCBs are shown to inhibit cytochrome P450-dependent monooxygenation activity, while stimulating the rate of bilirubin degradation. However, biphenyls with different steric configuration were active with the phenobarbital-induced microsomes, suggesting that a molecular recognition of the effective biphenyls by the active sites of the induced cytochromes (CYP2B1/2B2 and CYP1A1, respectively) operated in these effects. In the case of BNF-induced chick embryos, CYP1A5 [24] instead of CYP1A1 may be the enzyme recognized by planar PCBs, with similar effects. This conclusion is supported by the finding [25] that biphenyls possessing similar steric configuration could also serve as selective substrates of the corresponding cytochromes, provided that the pattern of halogen substitution was compatible with metabolism. It will be noted, however, that both inhibition of monooxygenase activity and stimulation of bilirubin degradation were also caused by 3,4,5-HCB and by 2,4,5-HCB, biphenyls which are metabolized very tittle if at all [26,27], lending support to the concept [1,2] that binding of a (relatively) poor substrate to the CYP active site is the essential feature of the interaction.

We have now found that not only bilirubin, but also URO'gen was oxidized at a faster rate as a result of the interaction of a PCB with CYP2B, as well as CYP1A, and here again PCBs of different configuration were active with the two enzymes. A very strong correlation was found between bilirubin degradation and URO'gen oxidation under conditions where different doses of a stimulatory PCB were tested (or a standard dose of PCBs with different structures), suggesting the activation, under these conditions, of a CYP-dependent reaction, which may oxidize other molecules in the cell, in addition to bilirubin, with potential toxicological significance. For example, since URO'gen oxidation appears to be important in the induction of hepatic uroporphyria [8–10], the present findings that both CYP1A1 and CYP2B could oxidize URO'gen at a faster rate, when challenged with the appropriate PCBs, may have implications for the induction of this disorder, an aspect which deserves further investigation.

We have also shown in this paper that TCBs increased the rate of NADPH oxidation and of O_2 utilization by induced microsomes and also in this case the steric configuration of the TCB afforded a degree of selectivity. We have no information on the fate of the reducing equivalents and oxygen in TCB-stimulated microsomes but we have shown that the effects of the appropriate TCB on these parameters and on rate of bilirubin degradation all exceeded in molar terms the amount of TCB added. So, a catalytic process, rather than a process stoichiometric with respect to the TCB added, appears to be involved. Lipid peroxidation is known to propagate itself in an autocatalytic fashion [28] and the work of Billing and coworkers [29] has shown lipid peroxides to be able to degrade bilirubin. We cannot completely rule out a contributing role of lipid peroxides in the bilirubin-degrading activity of the liver microsomes in our experiments. However, the following findings do not support a major role of lipid peroxides in our experiments. (a) Our incubations were all carried out in the presence of 2 mM EDTA, a powerful inhibitor of microsomal lipid peroxidation. (b) PCBs stimulated bilirubin and URO'gen oxidation in microsomes from species—like rabbits and chick embryos—which are resistant [22,23] to induction of lipid peroxidation. (c) The process of bilirubin degradation due to 2,4-TCB required the catalytic activity of CYP2B1, as shown by its sensitivity to a step-wise inhibition by an antibody raised against this enzyme. (d) Finally, the addition of PCB

was shown to produce stimulation of bilirubin degradation and inhibition of 7-alkyloxy-resorufin dealkylase activity with similar log dose–responses for the two effects (Fig. 2 and see also [1]), suggesting that the PCB-dependent oxidative process may be related to PCB occupancy of the active site of the CYP enzyme. Taking into account all these points, the interpretation we favor is that the PCBs may, by binding the corresponding CYP, stimulate reduction of O_2 and produce an oxidizing species, probably by an uncoupling mechanism.

A similar interpretation has been previously put forward for both the degradation of bilirubin (and alleviation of jaundice [2]) and the synergistic increases caused by iron of PCB-induced uroporphyria, genotoxicity and cancer [30,31], toxic manifestations all thought to arise by an oxidative mechanism [32–34]. Previous work with model enzymic systems [35], including the xanthine oxidase system [36], or with model chemical systems [1], has indicated that, in presence of a heme compound or of non-heme iron, H_2O_2 is very effective at promoting bilirubin degradation, so the proximal oxidant may be a species closely related to H_2O_2, possibly, an iron bound form. O_2^- and H_2O_2 can both be produced by microsomes on binding of substrates and are thought to arise from abortive breakdown of the oxygenated complexes of the CYP catalytic cycle [37]. Schlezinger *et al*. [38] have also proposed that 3,4-TCB, on binding to CYP1A1, stimulates production of reactive oxygen species and have reported a time-dependent inactivation of CYP1A1 by 3,4-TCB, which they attributed to increased, TCB-dependent production of O_2^- and H_2O_2. 3,4-TCB-dependent inactivation of CYP1A could be demonstrated in several animal species and correlated inversely with rates of 3,4-TCB oxidation [39], supporting the role of the 3,4-TCB as a potentially stable substrate, acting as an uncoupler. Some of the O_2^- and H_2O_2 produced in excess would be responsible for CYP1A inactivation, some would escape from the enzyme and be available, presumably, for oxidation of other molecules in the cell. These findings are compatible with the originally proposed mechanism of increased released of reactive oxygen species as the mechanism leading to oxidation of bilirubin and URO'gen. However, remains to be explained why, as we have reported in this paper, the bilirubin-degrading process due to CYP/PCB interaction is insensitive to catalase, in contrast to the bilirubin oxidation caused by xanthine oxidase [36] or purely chemical system [1], where catalase afforded protection. Another source of uncertainty relates to the reported ability of polyhalogenated chemicals to act as uncouplers of the CYP catalytic cycle, inducing, however, full reduction of O_2 to H_2O. This property has been documented for perfluorohexane [40] and other non-metabolisable halogenated chemicals [41], including 2,4,6,2′,4′,6′-hexachlorobiphenyl [42], all of which stimulated NADPH and oxygen utilization by microsomes from phenobarbital-induced rabbits with a molar stoichiometry ratio of NADPH:O_2 consumption of 2:1. The microsomal production of O_2^- and H_2O_2 was also measured directly after addition of some of these uncouplers and found not to be increased [40,41], in agreement with them stimulating full reduction of O_2 to H_2O. This may apply to the conditions of some of our experiments, for example to those with HCBs, which like perfluorohexane are not easily metabolisable (or even to 2,4-TCB, as suggested by the preliminary results we have obtained). On this basis, it is more difficult to visualize an active diffusable bilirubin-degrading (and URO'gen-oxidizing) species, but the oxidation of these molecules may instead take place in close proximity to the active site of the enzyme, by the intermediary oxygen species (presumably the ferryl-oxo complex [43,44]) being intercepted before it has undergone full reduction to water.

In conclusion, although there is still uncertainty on the precise role of cytochrome P450 in bilirubin and URO'gen oxidation and on the nature of the oxidizing species involved, the present findings help substantiate the hypothesis that interaction of polyhalogenated chemicals with the active sites of CYP enzymes may lead to increased production of reactive oxygen species by an uncoupling mechanism. The extension of these effects to liver microsomes induced by phenobarbital suggests that polyhalogenated chemicals, which are inducers of CYP2B, rather than CYP1A, enzymes, may also be potentially toxic by an oxidative stress mechanism. This may apply to pure non-planar PCB isomers, as well as to the complex mixtures containing planar and non-planar congeners [45]. In addition, the present findings raise the possibility that increased oxidation of bilirubin may contribute to the jaundice-lowering effect of phenobarbital in people with high plasma levels of bilirubin.

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