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# Interaction of polyhalogenated compounds of appropriate configuration with mammalian or bacterial CYP enzymes Increased bilirubin and uroporphyrinogen oxidation *in vitro*

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**Abstract**

Polyhalogenated compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, are associated with toxic *Uroporphyria* and cause alleviation of jaundice in the Gunn rat. These effects have been attributed to a microsomal oxidation of uroporphyrinogen and bilirubin for which supportive evidence has been obtained *in vitro*. CYP1A1 required planar polyhalogenated biphenyls for these oxidative reactions, while CYP1A2 was capable of oxidation in their absence.

We have now used rat CYP1A1 and confirmed with the pure enzyme that increased bilirubin oxidation was caused by the addition of 3,4,3′,4′-tetrachlorobiphenyl. CYP1A2 was more active than CYP1A1 at oxidizing bilirubin in presence of NADPH alone and reacted to addition of 3,4,3′,4′-tetrachlorobiphenyl with a depression rather than a stimulation of bilirubin oxidation.

We have also tested a bacterial enzyme, CYP102. Dodecanoic acid and its polyhalogenated analogue (perfluorododecanoic acid) both stimulated NADPH oxidation by CYP102, but only the perfluoro analogue stimulated markedly bilirubin oxidation. The analogue exhibited much greater potency than the normal substrate in stimulating NADPH and bilirubin oxidation and also showed greater affinity for CYP102, as measured by the binding constant, *K*s. The molar stoichiometry ratio between NADPH and $O_2$ consumption was 1 in the case of the substrate, but approximated 2 with the perfluoro analogue. We conclude that halogenated substrate analogues can interact with different CYPs to increase production of oxidative species, probably by an uncoupling mechanism. A role of the ferryl-oxygen intermediate is suggested in the oxidation of biologically important molecules, with possible implications for the therapy of jaundice and for toxic oxidative reactions, such as uroporphyria and cancer.


*Keywords:* CYP1A1; CYP1A2; CYP102; Bilirubin degradation; Uroporphyrinogen oxidation; Polyhalogenated biphenyls; Perfluorododecanoic acid

## 1. Introduction

Previous work has shown that polyhalogenated aromatic compounds are associated in man and in experimental animals with a toxic variety of *Sporadic Uroporphyria*, a disorder of liver heme metabolism characterized by accumulation of uroporphyrin. This is thought to arise through an oxidative stress mechanism, involving oxidation of URO'gen and leading to accumulation of uroporphyrin, the fully oxidized aromatic derivative, which cannot be metabolized further and will therefore accumulate (reviewed in [1]). One such halogenated chemical, TCDD, has also been reported to cause a significant alleviation of jaundice in the Gunn rat [2], a strain suffering from congenital jaundice, and this effect has also been attributed to an increased microsomal oxidative process. In agreement with such an oxidative mechanism, liver microsomes isolated from animals treated with either TCDD, 3-MC or BNF showed an increased rate of NADPH-dependent oxidation of URO'gen or bilirubin, and the rate of oxidation of both substrates was further increased by the *in vitro* addition of PCBs [3–6]. The rate of oxidation of these substrates shown by induced microsomes in presence of

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*Abbreviations:* ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BNF, β-naphthoflavone; CYP, cytochrome P450; 3-MC, 3-methyl cholanthrene; PCBs, polyhalogenated biphenyls; 3,4-TCB, 3,4,3′,4′-tetrachlorobiphenyl; 2,4-TCB, 2,4,2′,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; URO'gen, uroporphyrinogen (hexahydro-uroporphyrin).

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NADPH alone could be attributed to CYP1A2; whereas the stimulation of these rates caused by adding PCBs appeared to depend on CYP1A1 ([3] and references therein). In more recent work [7], liver microsomes from animals induced with phenobarbitone also responded to the *in vitro* addition of PCBs with a stimulation of the rate of URO'gen and bilirubin oxidation. However, non-planar di-*ortho*-substituted PCBs were active with phenobarbital-induced microsomes, in contrast to those active with 3-MC/BNF microsomes, where a planar configuration of the PCBs was required for stimulatory activity. It was concluded that PCBs of the appropriate configuration may interact with either CYP1A1 or CYP2B1, increase production of oxidative species by uncoupling the catalytic cycle of the enzyme and lead to oxidation of target molecules in the cell, among these URO'gen and bilirubin. A role of lipid peroxidation [8] and of other membrane-related effects in the PCB-dependent stimulation of microsomal oxidative reactions appeared less likely, but could not be completely discounted.

The purpose of this present work has been 2-fold. First we wanted to confirm, employing genetically expressed pure rat enzymes, the differential behaviour of CYP1A1 and CYP1A2 with respect to bilirubin oxidation and verify that the stimulation of oxidation due to PCBs is related to PCB occupancy of the active site of CYP1A1. The second aim of this work was to gain more information on the mechanisms, in the absence of lipid peroxidation and other membrane-related effects, by employing a pure soluble bacterial enzyme, CYP102 (BM3).

We have now shown, with genetically overexpressed rat CYP1A1, that the rate of oxidation of bilirubin could be stimulated by a planar PCB, whereas the non-planar congener was not stimulatory. In contrast, CYP1A2 was more active than CYP1A1 in degrading bilirubin in presence of NADPH alone and addition of a planar PCB was inhibitory, rather than stimulatory. These results confirm data [3] previously obtained with induced mice liver microsomes.

We have also shown in this paper that addition of the appropriate polyhalogenated substrate analogue (perfluorododecanoic acid) to the soluble bacterial enzyme, CYP102 (BM3) will stimulate bilirubin and URO'gen oxidation, altering at the same time the NADPH/$O_2$ stoichiometry ratio to values compatible with an uncoupled catalytic cycle. The production of $H_2O_2$ was not increased under these conditions, suggesting that some other oxidizing species produced during uncoupling played a major role in bilirubin oxidation.

Some of these findings have already been given in an abstract form in [9].

## 2. Materials and methods

### 2.1. Source of special chemicals

NADPH (type III), ABTS, diammonium salt (98% pure), horse radish peroxidase (987 U/mg), dodecanoic acid (sodium salt) and bilirubin were obtained from Sigma Chemical Company; perfluorododecanoic acid and $H_2O_2$ (puriss. p.a., 30%) from Fluka; and uroporphyrin I from Porphyrin Products. 3,4-TCB and 2,4-TCB were from Ultra. Sodium mercury amalgam (containing 5% Na) was obtained in beads form from Aldrich Chemical Company. Chelex 100, employed to render iron-free the Tris buffer, was from Bio-Rad.

### 2.2. Analytical techniques

Protein was measured by the method of Lowry *et al.* [10], using bovine serum albumin as a standard, and CYP content by the method of Omura and Sato [11].

### 2.3. Experiments with CYP enzymes

"Supersome preparations", expressing either rat CYP1A1 or rat CYP1A2, together with rat cytochrome P450 reductase were obtained from Gentest Corporation. The bilirubin-degrading activity was measured *in vitro* in presence of NADPH and oxygen, after adding either a planar or a non-planar PCB (or solvent alone), in the incubation mixture previously described [4], total volume 1 mL, using EDTA (final concentration 2 mM) in all experiments, 0.1 M Tris–HCl buffer, pH 8.2 and final concentrations of bilirubin and NADPH of 12 and 68.6 μM, respectively. The concentration of CYP1A1/CYP1A2 was of 32.5 pmol/mL of incubation mixture. *Bacillus megaterium* cytochrome P450 BM3 (CYP102), expressed in *E. coli* and purified to homogeneity, was a generous gift from Prof. Gordon C.K. Roberts of the University of Leicester and was the intact enzyme, containing both heme and reductase domains [12,13]. In the case of CYP102, the effects of adding dodecanoic acid, the normal substrate (0.95–1500 μM), and its polyhalogenated analogue (perfluorododecanoic acid, 0.95–3.5 μM) were compared under identical incubation conditions, using a final concentration of CYP102 of 27 pmol/mL. Dodecanoic acid was usually tested at concentrations 100–400-fold higher than its perfluoro analogue, on account of the considerable difference in potency between the two compounds in stimulating NADPH and bilirubin oxidation. Also, in some experiments, higher concentrations of CYP102 and of NADPH were taken in order to obtain more easily measurable rates of oxygen uptake in the oxygen electrode apparatus.

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 (final concentration 2.8 μM) was followed [6] in plastic disposable cuvettes by monitoring the increase in absorbance of the Soret maximum (at 398 nm) and expressed as pmoles of URO'gen oxidized per minutes, using an $\varepsilon_{mM} = 260$ [7], obtained experimentally under the conditions of the assay. The oxidation of bilirubin was followed under identical conditions, by monitoring the

difference in absorbance between 450 and 500 nm, using an $\varepsilon_{mM}$ value of 47.3 [3]. In this and in additional studies where monitoring of optical absorbance was involved, a recording Beckman DU640 spectrophotometer was used.

When the rates of NADPH oxidation, $O_2$ consumption and hydrogen peroxide production were measured, the incubation mixture (1 mL total volume) contained the following components, with final concentrations in parentheses: Tris–HCl buffer, pH 8.2 (100 mM), KCl (26 mM), EDTA (2 mM), NADPH (120–240 μM), CYP102 (140 pmol/mL), and either dodecanoic acid (150 μM) or perfluorododecanoic acid (1.5 μM). NADPH oxidation was followed by monitoring the absorbance at 340 nm, using an $\varepsilon_{mM} = 6.2$ [14]; and the rate of $O_2$ consumption in the closed cell of a oxygen electrode apparatus (Digital Model 10, Rank Brothers, Ltd.), calculating the results on the basis of a starting concentration of oxygen of 450 ng atom/mL [15].

The amount of $H_2O_2$ produced during the CYP incubation reactions was measured by a horseradish peroxidase/ABTS assay [16], as follows. The same incubation conditions described above for the NADPH oxidation were used, but, since NADPH was found to inhibit the ABTS-derived colour, the reaction was allowed to proceed until the NADPH consumption was complete (usually 2 min), when 100 μL 1 N HCl were added, followed by 60 μL 1 N NaOH and by 100 μL 5 M $K_2HPO_4/NaH_2PO_4$ buffer, so as to bring the pH value of the final mixture to 5.6. Horseradish peroxidase (25 U) and ABTS (final concentration, 1 mM) were then added and the colour development followed by monitoring the absorbance at 405 nm. Appropriate blanks (containing no NADPH) were routinely run and also samples of composition corresponding to the incubation mixtures, but containing known amounts of $H_2O_2$ as internal standards. In separate experiments, it was demonstrated that incubation of the whole system, containing NADPH, with $H_2O_2$ did not lead (when corrected with appropriate blanks) to a demonstrable loss of preformed $H_2O_2$, compared to similar incubations conducted without NADPH. Neither did the presence of $H_2O_2$ in the CYP102 incubation system lead to a faster rate of NADPH oxidation.

All reactions were carried out at the temperature of 28°.

## 3. Results

### 3.1. Experiments with genetically overexpressed rat CYP1A1 and CYP1A2

The structures of the various polyhalogenated compounds examined in this work (PCBs and perfluorododecanoic acid) are shown in Fig. 1. It must be stressed that the halogen substitution we are concerned with here is one where metabolism is hindered, but there is no impairment of ES complex formation. In the first experiment, "supersomes" expressing rat CYP1A1 showed increased bilirubin-oxidizing activity, when challenged with 3,4-TCB; in contrast, 2,4-TCB, the non-planar isomer, was almost inactive (Fig. 2). The "supersomes" containing CYP1A2 were more active than those expressing CYP1A1 at oxidizing bilirubin in presence of NADPH alone, and reacted to addition of 3,4-TCB with a depression rather than a stimulation of bilirubin oxidation (Fig. 2). These results showing a different behaviour of the two rat enzymes with respect to bilirubin degradation are in line with previous findings obtained with intact liver microsomes obtained from BNF-induced mice [3].

### 3.2. Experiments with CYP102

#### 3.2.1. Stimulation of NADPH oxidation caused by dodecanoic acid and perfluoro dodecanoic acid

The effects of either dodecanoic acid or of its perfluoro analogue on the rate of NADPH oxidation by CYP102 was studied. Both compounds stimulated the rate of NADPH oxidation, the perfluoro analogue showing more potency

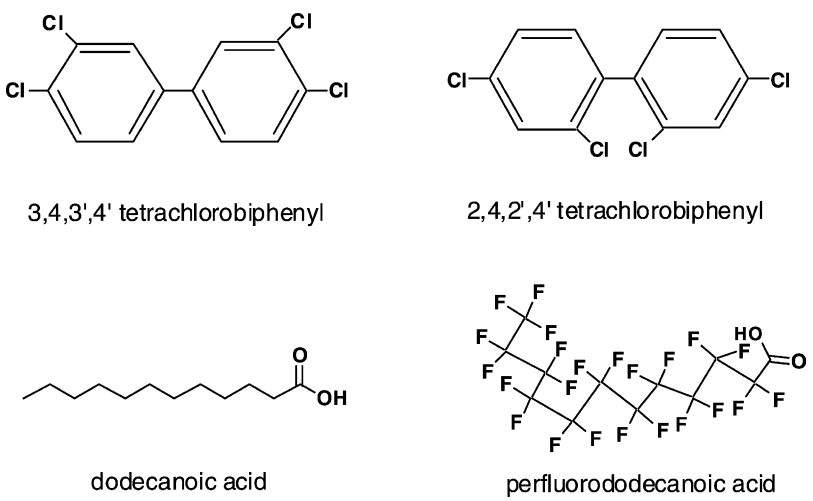


Fig. 1. The structure of polyhalogenated chemicals employed in this work. Dodecanoic acid, the normal substrate of CYP102, is also shown.

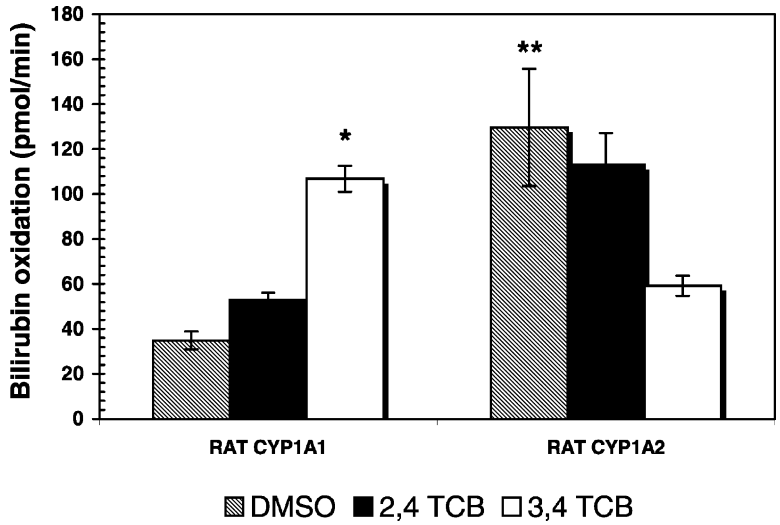


Fig. 2. Effect of either 2,4-TCB or 3,4-TCB on the rate of bilirubin oxidation by "supersome" preparations containing CYP1A1 or CYP1A2. "Supersomes" corresponding to 32.5 pmol cytochrome P450 were incubated, in a final volume of 1 mL, with bilirubin (12 μM) and NADPH (68.6 μM), in the presence of a standard dose (286 ng/mL) of either 2,4-TCB or 3,4-TCB. Control received the same volume of DMSO alone (1.43 μL/mL). $^{*}P < 0.001$, when compared with corresponding values obtained with CYP1A1 after adding either DMSO (control) or 2,4-TCB. $^{**}P < 0.01$, when compared with corresponding DMSO (control) values obtained with CYP1A1.

(at least 100-fold greater) but less efficacy than dodecanoic acid (see Fig. 3, where the log concentration–responses for the effects of the two compounds are shown, either as a percentage of the maximum stimulation achieved (panel a) or as the absolute rates of NADPH oxidation which were observed (panel b)).

### 3.2.2. *Stimulation of bilirubin and URO'gen oxidation caused by perfluorododecanoic acid*

The effects of dodecanoic acid and of its perfluoro analogue on bilirubin and URO'gen oxidation were then examined. Both oxidizing activities were significantly stimulated by perfluorododecanoic acid, whereas the normal substrate (dodecanoic acid) was very much less active. This was true whether the two compounds were tested at the same concentration (0.95 μM) or at higher concentrations shown by the concentration–response curves of Fig. 3 to provide a maximal (or near-maximal) stimulation of NADPH oxidation (Table 1).

The log concentration–responses for the effects of the two compounds on bilirubin oxidation were also examined and are shown in Fig. 4, again as a percentage of the maximum stimulation achieved (panel a) or as the absolute rates of bilirubin degradation which were observed (panel b). As reported in Fig. 3 for NADPH oxidation, the perfluoro analogue showed at least a 100-fold greater potency at increasing bilirubin oxidation than did dodecanoic acid. However, when the actual rates of bilirubin oxidation were compared to the corresponding rates of NADPH oxidation, different NADPH/bilirubin molar consumption ratios were found for the substrate and its analogue (Table 2). With both compounds only a small proportion of the NADPH consumed was utilized for bilirubin oxidation, but the proportion of NADPH so

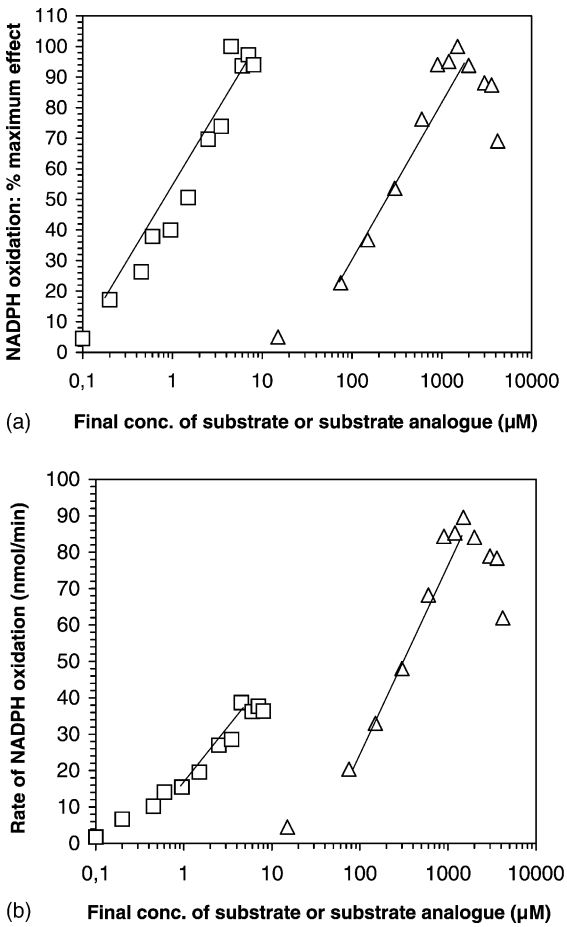


Fig. 3. Log concentration–response curves for NADPH oxidation activity obtained with CYP102 in the presence of various concentrations of either dodecanoic acid (△) or perfluorododecanoic acid (□). Note that data are shown in panel (a) as percent of the maximum stimulated activity; whereas panel (b) shows the rates of NADPH oxidation which were actually observed.

Table 1
Effect of either dodecanoic acid or perfluorododecanoic acid on the rate of oxidation of NADPH, bilirubin and URO'gen by CYP102

| Addition and final concentration | Rate of NADPH oxidation (nmol/min) | Rate of bilirubin oxidation (pmol/min) | Rate of URO'gen oxidation (pmol/min) |
|---|---|---|---|
| DMSO (1.43 μL/mL) | 0.54 ± 0.02 (3) | 48.6 ± 20.2 (3) | 1.67 ± 0.4 (3) |
| Dodecanoic acid (0.95 μM) | 0.76 ± 0.19 (3)[a] | 54.3 ± 6.5 (3)[a] | 1.5 ± 0.4 (3)[a] |
| Perfluorododecanoic acid (0.95 μM) | 17.1 ± 1.2 (3)[b] | 536 ± 55.6 (3)[b] | 3.85 ± 0.4 (3)[c] |
| Dodecanoic acid (1.5 mM) | 62.4 ± 1.9 (3)[c,d] | 260 ± 26 (3)[c,d] | 1.8 ± 0.2 (3) |
| Perfluorododecanoic acid (3.5 μM) | 33.4 ± 0.6 (3)[c] | 872.7 ± 19 (3)[c] | 3.72 ± 0.2 (3)[e] |

CYP102 (27 pmol/mL) was incubated in the presence of dodecanoic acid or of its perfluoro analogue at the concentrations stated, with NADPH (68.6 μM) alone and the rate of NADPH oxidation monitored by loss of absorbance at 340 nm. In order to measure the rate of oxidation of bilirubin or URO'gen, either compound was also added (at a concentration of 12 and 2.8 μM, respectively) together with NADPH and their rates of oxidation monitored as described in Section 2. Results are given as averages ± SEM of the number of observations in parentheses. Note that the stimulatory effects of two substrates were compared both at the same concentration (0.95 μM) and also at concentrations capable of providing a maximal (or near-maximal) stimulation of NADPH and bilirubin oxidation (see dose–response of Figs. 3 and 4).

[a] $P < 0.01$, when compared to corresponding values obtained with perfluorododecanoic acid, 0.95 μM.
[b] $P < 0.01$, when compared to corresponding values obtained with DMSO alone.
[c] $P < 0.001$, when compared to corresponding values obtained with DMSO alone.
[d] $P < 0.001$, when compared to corresponding values obtained with perfluorododecanoic acid, 3.5 μM.
[e] $P < 0.05$, when compared to corresponding values obtained with either DMSO alone or dodecanoic acid, 1.5 mM.

utilised was significantly greater in the case of perfluoro dodecanoic acid.

The rate of bilirubin oxidation was found to be strictly dependent on the addition of *both* NADPH and the halogenated compound, as, even though a very slight stimulation was caused by NADPH alone, a marked effect was only noted when both were present (Table 3).

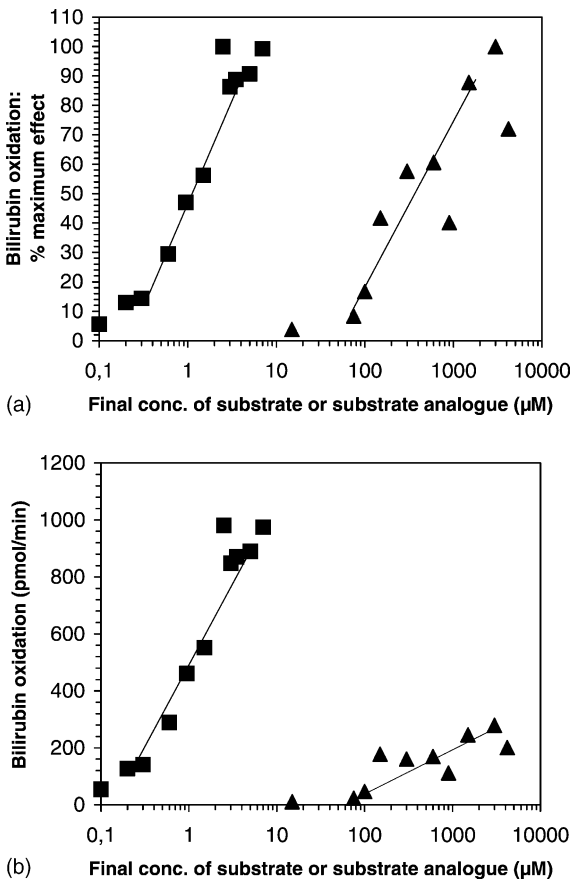


Fig. 4. Log concentration–response curves for bilirubin oxidizing activity obtained with CYP102 in presence of various concentrations of either dodecanoic acid (▲) or perfluorododecanoic acid (■). Note that data are shown in panel (a) as percent of the maximum stimulated activity; whereas panel (b) shows the rates of bilirubin degradation which were actually measured.

We conclude that both the substrate and the halogenated analogue can stimulate the degradation of bilirubin in presence of NADPH. With dodecanoic acid, most of the NADPH oxidized is probably utilized for the monooxygenation of the substrate; whereas, with perfluorododecanoic acid, a compound which—presumably—does not undergo metabolism to a significant extent, an alternative pathway(s) of NADPH utilization may be activated, ultimately leading to full reduction of oxygen to water (see below) and producing one or more bilirubin-oxidizing species.

### 3.2.3. Evidence for binding of dodecanoic acid and perfluoro dodecanoic acid to the active site of CYP102 and attempted calculation of affinity of binding

A type I binding spectrum was produced by adding both compounds to CYP102 (Fig. 5a). Perfluoro dodecanoic acid showed a considerably greater affinity of binding (compared to dodecanoic acid), as measured by *K*s values (Fig. 5b and c), 2.24 and 230 μM, respectively. These values are in fairly close agreement with the $EC_{50}$ values (approx. 1.5 and 300 μM, Figs. 3a and 4a) obtained for the oxidation of NADPH and bilirubin by perfluoro dodecanoic acid and dodecanoic acid, respectively. This suggests that the greater potency of perfluorododecanoic acid in stimulating NADPH and bilirubin oxidation was at least in part related to its greater affinity for CYP102 and supports the view that occupancy of active site is involved in the oxidative effects we have studied.

### 3.2.4. Attempted calculation of the molar NADPH/$O_2$ stoichiometry ratios for the effect of dodecanoic acid and perfluoro dodecanoic acid

The experiment described below indicates that the fate of reducing equivalents from NADPH differed according

Table 2
Comparison between dodecanoic acid and its analogue in their stimulatory activities on NADPH and bilirubin oxidation by CYP102

| Addition and final concentration | Rate of NADPH oxidation (nmol/min) | | Rate of bilirubin oxidation (nmol/min) | | Apparent NADPH/bilirubin molar consumption ratios (A/B) |
|---|---|---|---|---|---|
| | Rate observed | Stimulation due to substrate (A) | Rate observed | Stimulation due to substrate (B) | |
| DMSO (1.43 μL/mL) | 0.54 ± 0.02 (3) | – | 0.049 ± 0.02 (3) | – | – |
| Dodecanoic acid (1.5 mM) | 62.4 ± 1.9 (3) | 61.8 | 0.26 ± 0.026 (3) | 0.21 | 294 |
| Perfluorododecanoic acid (3.5 μM) | 33.4 ± 0.6 (3) | 32.9 | 0.87 ± 0.02 (3) | 0.82 | 40 |

The data of NADPH and bilirubin oxidation are taken from Table 1, where the condition of both assays are described. Note that dodecanoic acid compared to perfluorododecanoic acid was a much weaker stimulant of bilirubin oxidation, but a much stronger stimulant of NADPH oxidation; consequently the corresponding NADPH/bilirubin molar ratios were considerably different. Similar discrepancies in NADPH/bilirubin molar ratios between dodecanoic acid and perfluorododecanoic acid were found at additional concentration of the two substrates (results not shown), with dodecanoic acid showing much higher ratios in all cases. Results are given as averages ± SEM of the number of observations in parentheses.

Table 3
Effect of adding NADPH and perfluorododecanoic acid, either on their own or together, on the rate of bilirubin oxidation by CYP102

| Addition and final concentration | Rate of bilirubin oxidation (pmol/min) | |
|---|---|---|
| | −NADPH | +NADPH |
| DMSO (1.43 μL/mL) | 12 ± 5.3 (3) | 28.2 ± 1.2 (3)[a] |
| Perfluorododecanoic acid (0.95 μM) | 10.6 ± 7.3 (3) | 452.4 ± 14.8 (3)[b,c] |

The conditions of the assay are described in the legend to Table 1.
[a] $P < 0.05$.
[b] $P < 0.001$, when compared to corresponding values obtained in absence of NADPH.
[c] $P < 0.001$, when compared to corresponding values obtained in presence of NADPH alone.

to whether dodecanoic acid or its perfluoro analogue were added to the CYP102 enzyme. Both dodecanoic acid and the perfluoro analogue, added in concentrations previously shown to stimulate markedly NADPH oxidation, increased the rate of oxygen uptake, but the NADPH/$O_2$ consumption ratios were significantly different for the two compounds (Table 4): a molar stoichiometry ratio of NADPH/$O_2$ consumption of approximately 1 was obtained for the effects of dodecanoic acid, compatible with this compound undergoing metabolism by monooxygenation; in contrast, a ratio of approximately 2 was obtained with perfluoro dodecanoic acid, suggesting that, with the halogenated analogue, most of the oxygen was fully reduced to water.

In one experiment, an attempt was made to relate the effect of the perfluoro compound in stimulating bilirubin degradation (calculated by difference between the stimulated rate and the corresponding control (DMSO only) rate) to the amount of analogue added. The result obtained over a 30 min with 0.6 nmol perfluorododecanoic acid/mL of incubation was 6.11–0.48 = 5.67 nmol bilirubin oxidized/mL; a 9.45 excess over the halogenated compound added. This indicates that the increased oxidation of bilirubin exceeded in molar terms the amount of halogenated compound added.

### 3.2.5. *Formation of hydrogen peroxide during the reaction and effect of adding catalase or superoxide dismutase*

Previous work with model enzyme systems [17,18] or with model chemical systems [4,19,20] has indicated that, in presence of a heme compound or of non-heme iron, $H_2O_2$ is very effective at promoting bilirubin and URO'gen oxidation; 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 CYP enzymes on binding of

Table 4
Effect of dodecanoic acid or perfluorododecanoic acid added *in vitro* to a preparation of CYP102 on the rates of NADPH oxidation and oxygen uptake and on the NADPH/$O_2$ stoichiometry ratios

| Addition and final concentration | NADPH oxidation (nmol/min) | | Oxygen uptake (nmol/min) | | Apparent NADPH/$O_2$ molar stoichiometry ratio (B/D) |
|---|---|---|---|---|---|
| | Rate observed (A) | Stimulation due to substrate (B) | Rate observed (C) | Stimulation due to substrate (D) | |
| DMSO, 1.43 μL/mL | 3.5 ± 2 (3) | – | 2.9 ± 0.3 (6) | – | – |
| Dodecanoic acid, 150 μM | 102 ± 6 (3)[a] | 98 | 94 ± 0.3 (5)[b,c] | 91 | 1.08 |
| Perfluorododecanoic acid, 1.5 μM | 98 ± 7 (3)[a] | 94.5 | 44.5 ± 3 (4)[b] | 41.6 | 2.27 |

CYP102 (140 pmol/mL) were incubated with dodecanoic acid or with its perfluoro analogue at the concentrations stated, in presence of NADPH (240 μM) and the rates of NADPH oxidation and oxygen uptake were measured as indicated in subsection 2.3. Results are given as averages ± SEM of the number of observations in parentheses.
[a] $P < 0.01$, when compared to corresponding values obtained with DMSO alone.
[b] $P < 0.001$, when compared to corresponding values obtained with DMSO alone.
[c] $P < 0.001$, when compared to corresponding values obtained with perfluorododecanoic acid.

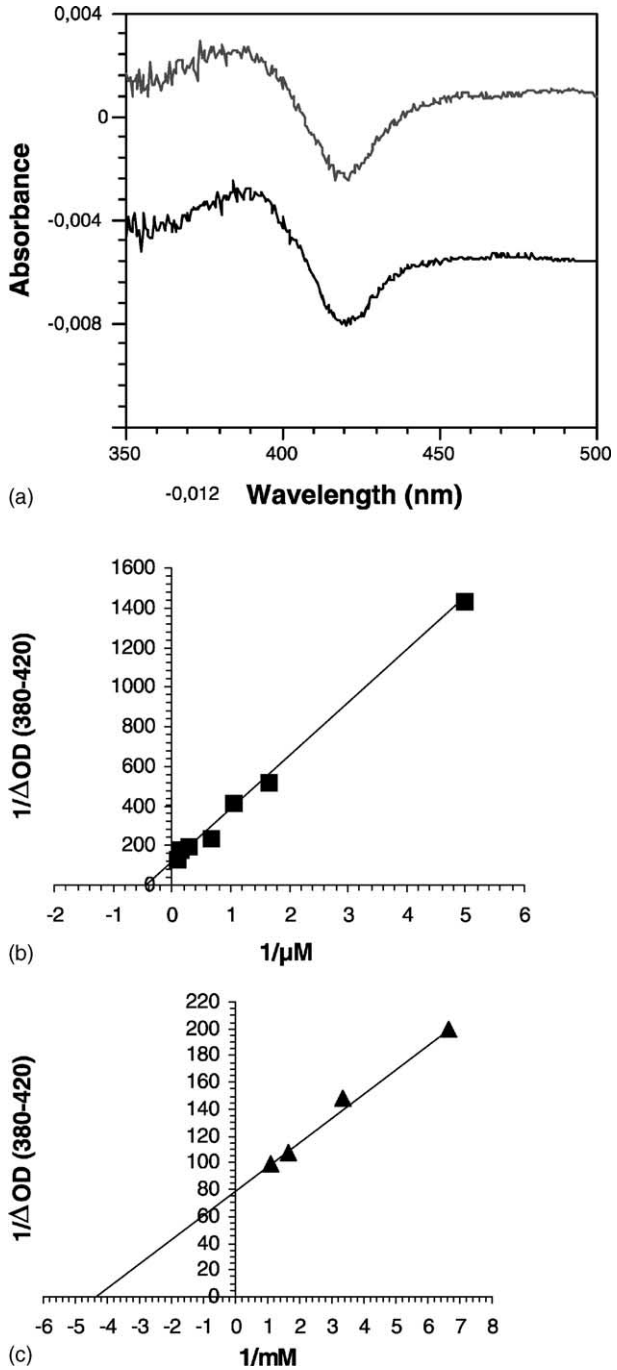


Fig. 5. (a) Type I binding spectra obtained by adding either perfluorododecanoic acid (1.5 μM, upper tracing) or dodecanoic acid (150 μM, lower tracing) to a preparation of CYP102 (180 pmol/mL). The double reciprocal plot of the spectral change observed against the concentration of ligand added is shown in (b) and (c) for perfluorododecanoic acid (■) and dodecanoic acid (▲), respectively.

substrates and are thought to arise from abortive breakdown of the oxygenated complexes of the CYP catalytic cycle [21]. Schlezinger and co-workers [22,23] have also proposed that 3,4-TCB, a potentially stable compound acting as an uncoupler, on binding CYP1A1, stimulates production of $O_2^-$ and $H_2O_2$.

We have therefore examined the formation of hydrogen peroxide during the reaction of CYP102 with either dodecanoic acid or its perfluoro analogue (under conditions, that is, where the effects of the two compounds on the molar stoichiometry ratio of NADPH/$O_2$ and on rate of bilirubin degradation were both significantly different). The amount of $H_2O_2$ recovered after incubation (Table 5) did not differ significantly between dodecanoic acid and perfluorododecanoic acid; under these conditions the recovery of added authentic $H_2O_2$ ranged from 92.6 to 101.2%. Addition of SOD to the $H_2O_2$-producing incubation may have increased the yield of the latter to a small extent in some experiments, but here again no significant difference was found between dodecanoic acid and perfluorododecanoic acid. These findings clearly contrast with the effects on bilirubin oxidation, where a very significant difference was found between substrate and analogue. This suggests that $O_2^-$ and $H_2O_2$ may have contributed to bilirubin degradation to a small extent, but the majority of the effect of the halogenated analogue in stimulating bilirubin degradation probably involved a different mechanism.

The effect of SOD and catalase on the rate of NADPH and bilirubin oxidation was also studied. The addition of SOD (1000 U/mL) did not influence significantly the rates of NADPH and bilirubin oxidation in presence of either dodecanoic acid or perfluorododecanoic acid (results not shown). Catalase (1000 U/mL) did not influence the rates of NADPH and BR oxidation in presence of dodecanoic acid (1.5 mM), but produced a significant decrease of both rates with perfluorododecanoic acid. For example, at a concentration. of perfluorododecanoic acid of 1.5 μM, rates of NADPH and bilirubin oxidation (nmol/min) were, respectively: in absence of catalase, $27.8 \pm 2$ (8); $0.9 \pm 0.03$ (6); and, in the presence of catalase, $14.5 \pm 0.8$ (4); $0.53 \pm 0.06$ (3); the values obtained in presence of catalase being statistically different ($P < 0.001$) for both oxidations. These findings may be taken as an evidence that hydrogen peroxide played a major role in the oxidation of bilirubin by the uncoupling agent. However, we tend to favour an alternative explanation for the following reasons: first, catalase did not change the rate of bilirubin degradation due to dodecanoic acid, even though this substrate stimulated hydrogen peroxide formation just as much as the perfluoro analogue. Second, the changes caused by catalase in presence of perfluorododecanoic acid affected not only bilirubin degradation, but also NADPH oxidation. If the lower NADPH consumption observed in presence of catalase was in fact due to decreased levels of hydrogen peroxide, then one might have expected NADPH consumption to be increased by the addition of $H_2O_2$ and this was not found. Rates of NADPH oxidation (averages of two observations), with concentrations of added $H_2O_2$ given in parentheses, were as follows: (no $H_2O_2$), 0.52 nmol/min; (2.6 nmol $H_2O_2$/mL), 0.4 nmol/min; (7.9 nmol $H_2O_2$/mL), 0.36 nmol/min. Perhaps catalase may adsorb some of the perfluoro analogue added, making less of it available for

Table 5
Effect of dodecanoic acid or its perfluoro analogue on the production of $H_2O_2$ by CYP102

| Addition (final concentration) | $H_2O_2$ formed | | |
|---|---|---|---|
| | nmol/mL | nmol/mL | Percent of NADPH oxidized |
| | Effect of NADPH | | |
| Dodecanoic acid (150 μM) | 2.6 ± 0.97 (3) | – | – |
| Dodecanoic acid (150 μM) + NADPH (120 μM) | 4.7 ± 1.1 (3) | 2.2 ± 0.3 (3) | 1.8 |
| Dodecanoic acid (150 μM) + NADPH (120 μM) + SOD (1000 U/mL) | 4.6 ± 1.0 (3) | 2.0 ± 0.6 (3) | 1.7 |
| Perfluorododecanoic acid (1.5 μM) | 1.4 ± 0.2 (3) | – | – |
| Perfluorododecanoic acid (1.5 μM) + NADPH (120 μM) | 3.7 ± 0.01 (3) | 2.3 ± 0.2 (3) | 1.93 |
| Perfluorododecanoic acid (1.5 μM) + NADPH (120 μM) + SOD (1000 U/mL) | 4.6 ± 2.11 (3) | 3.1 ± 2.1 (3) | 2.63 |

All samples contained CYP102 (140 pmol/mL) and additional components as indicated. Since NADPH was found to inhibit the ABTS-derived colour, the reaction was allowed to proceed until NADPH consumption was complete before adding ABTS and peroxidase and monitoring the absorbance at 405 nm (see Section 2). The percentage of NADPH utilized which was accounted for by $H_2O_2$ production was calculated relating the $H_2O_2$ formed to the total NADPH which was consumed (120 nmol). The concentrations of dodecanoic acid and perfluorododecanoic acid employed are those of the NADPH/$O_2$ utilization experiments and were selected in order to obtain a similar stimulation of the rate of NADPH oxidation. Results are given as averages ± SEM of the number of observations in parentheses.

CYP102 binding. We conclude therefore that if hydrogen peroxide has a part to play in bilirubin oxidation, its role in this effect—at least under the conditions described in this present work—is likely to be a minor one.

## 4. Discussion

This work has shown that a planar PCB, 3,4-TCB, stimulated bilirubin oxidation also with rat CYP1A1 genetically overexpressed in insect cells, whereas the non-planar di-*ortho*-substituted isomer, 2,4-TCB was almost inactive. This confirms the stereoselectivity already reported with intact microsomes and supports the hypothesis that a PCB of the appropriate configuration may first induce a CYP enzyme [24], then interact with its active site as a poor substrate, producing bilirubin-degrading (and URO'gen oxidizing) species by an uncoupling mechanism. Also in agreement with data previously obtained with induced liver microsomes [3], genetically expressed CYP1A2 was more active than CYP1A1 at oxidizing bilirubin in presence of NADPH alone and reacted to addition of 3,4-TCB with a depression, rather than a stimulation, of bilirubin oxidation. This may indicate that the CYP1A2 is already partially uncoupled and capable of producing oxidizing species in its basic state, before—that is—the addition of a halogenated substrate analogue. The stereoselectivity now confirmed, and the similarity of the *K*s values to the $EC_{50}$ values for oxidation of both NADPH and bilirubin are compatible with the concept that occupancy of the active site is involved in bilirubin oxidation by an uncoupling mechanism, a conclusion more directly supported by the findings obtained in this work with the bacterial CYP102 enzyme, in absence of lipid peroxidation and other membrane-related effects.

Evidence of perfluorododecanoic acid uncoupling CYP102 has now been provided, by studying the molar stoichiometry ratio of NADPH/$O_2$ consumption: with dodecanoic acid, this approximated, as expected, the value of 1, whereas a ratio of approximately 2 was obtained with the perfluoro analogue. This suggests full reduction of $O_2$ to $H_2O$ in this type of uncoupling, as documented for perfluorohexane [25], and other non-metabolizable halogenated chemicals [26], including 2,4,6,2′,4′,6′-hexachlorobiphenyl [27] and, in preliminary experiments reported in [7], 2,4-TCB. All of these stimulated NADPH and $O_2$ utilization by microsomes from phenobarbital-induced animals with a molar stoichiometry ratio of 2.

The relevance of these findings to the catalytic cycle of CYP enzymes is schematically illustrated in Fig. 6. Both substrates and uncouplers may be expected to stimulate the rate of formation of the intermediary ferryl-oxygen complex, but only with substrates there will also be an increased rate of its utilization (oxygen insertion), so that the intermediate will not accumulate. In contrast, with the uncoupler, the ferryl-oxygen complex will be formed at an increased rate, but broken down with difficulty, as the

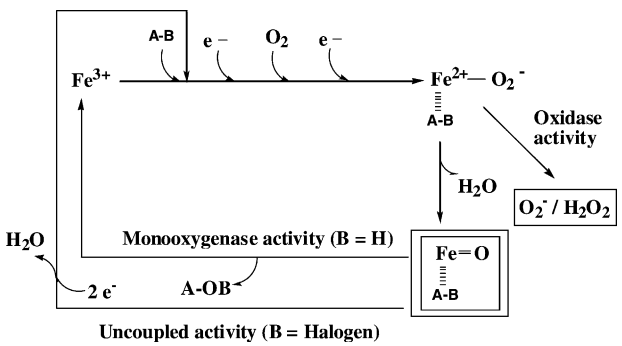


Fig. 6. Schematic representation of the catalytic cycle of cytochrome P450, showing the normal monooxygenase pathway, as well as the activity leading to increased formation of $O_2^-$ and $H_2O_2$ (labelled "oxidase activity") and the uncoupled pathway, involving full reduction of oxygen to water. The latter pathway may lead to an increased concentration of the ferryl-oxygen intermediate (see text), which can either be detoxified to $H_2O$ by reduced pyridine nucleotides or abstract electrons from biologically important molecules, among these bilirubin and URO'gen.

analogue will not be readily metabolized by oxygen insertion. There may follow an increased steady state concentration of the ferryl-oxygen intermediate, which will either (a) be detoxified to $H_2O$ by reduced pyridine nucleotides; or (b) abstract electrons from other biological molecules, among these bilirubin and URO'gen. Whether reaction (a) or (b) predominates *in vivo* will depend on the concentration of the appropriate electron-donating molecules, on how easily available they are to the oxidizing intermediate and also on their respective oxido-reduction potential. Since NADPH will be required both to produce the oxidant and to inactivate it, another important point to consider is the relative ''affinity'' for the pyridine nucleotide of each of these two steps of the uncoupling process, an aspect which will require further study. According to this concept, type (b) reactions may have important pharmacological and toxicological implications. On this basis it is not yet possible 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 of the enzyme, by the intermediary ferryl-oxygen complex [28,29] being intercepted before it has undergone full reduction to water. It remains to be determined whether this as yet hypothetical URO'gen oxidizing species can also interact with iron, and in this way explain the synergistic increases caused by iron of PCB-induced uroporphyria, genotoxicity and cancer [30,31].

Alternatively, it is still possible to ascribe to hydrogen peroxide the toxic manifestations induced by iron, when this is given in excess together with polyhalogenated chemical uncouplers. Even though the amount of hydrogen peroxide produced has now been shown to represent a small proportion of the NADPH oxidized, nevertheless a polyhalogenated uncoupler may still be expected to cause a sustained production of hydrogen peroxide *in vivo*, at levels greater than caused by the corresponding substrates, for the following reasons. Many of the halogenated uncouplers would also be expected to act as inducers of the CYP enzymes [24], thus increasing their ultimate CYP site of binding, and once bound to the active site they would probably induce a long lasting effect on account of their chemical stability. This may then predispose to the toxic effects of excess iron, provided that the individual exposed to both the halogenated uncoupler and to excess iron possesses susceptibility genes [32], which modulate not only the expression of CYP1A but also, in some way, the response to iron.

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 site of CYP enzymes may lead to increased production of oxidative species by an uncoupling mechanism. These findings may help develop a treatment for severe jaundice due to unconjugated bilirubin and may also contribute to elucidate the mechanism of induction of hepatic uroporphyria and other types of toxicity of halogenated chemicals, for example, DNA oxidative damage and liver cancer [31].

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