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THE EFFECT OF VARYING HALOGEN SUBSTITUENT PATTERNS ON THE CYTOCHROME P450 CATALYSED DEHALOGENATION OF 4-HALOGENATED ANILINES TO 4-AMINOPHENOL METABOLITES

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Abstract—The cytochrome P450 catalysed biotransformation of 4-halogenated anilines was studied *in vitro* with special emphasis on the dehalogenation to 4-aminophenol metabolites. The results demonstrated that a fluorine substituent at the C4 position was more easily eliminated from the aromatic ring than a chloro-, bromo- or iodo-substituent. HPLC analysis of *in vitro* biotransformation patterns revealed that the dehalogenation of the C4-position was accompanied by formation of non-halogenated 4-aminophenol, without formation of NIH-shifted metabolites. Changes in the apparent V_{max} for the microsomal oxidative dehalogenation appeared to correlate with the electronegativity of the halogen substituent at C4, the fluorine substituent being the one most easily eliminated. A similar decrease in the rate of dehalogenation from a fluoro- to a chloro- to a bromo- to an iodo-substituent was observed in a system with purified reconstituted cytochrome P450 IIB1, in a tertiair butyl hydroperoxide supported microsomal cytochrome P450 system as well as in a system with microperoxidase 8. This microperoxidase 8 is a haem-based mini-enzyme without a substrate binding site, capable of catalysing cytochrome P450-like reaction chemistry. Together, these results excluded the possibility that the difference in the rate of dehalogenation with a varying C4-halogen substituent arose from a change in the contribution of cytochrome P450 enzymes involved in oxidative dehalogenation with a change in the halogen substituent. Rather, they strongly suggested that the difference was indeed due to an intrinsic electronic parameter of the various C4 halogenated anilines dependent on the type of halogen substituent. Additional *in vitro* experiments with polyfluorinated anilines demonstrated that elimination of the C4-fluorine substituent became more difficult upon the introduction of additional electron withdrawing fluorine substituents in the aniline-ring. ^{19}F-NMR analysis of the metabolite patterns showed that the observed decrease in 4-aminophenol formation was accompanied by a metabolic switch to 2-aminophenols and *N*-hydroxyanilines, while products resulting from NIH-type mechanisms were not observed. For a C4-chloro-, bromo-, or iodo-substituted 2-fluoroaniline the V_{max} for the oxidative dehalogenation was reduced by the additional electron withdrawing fluorine substituent at the C2 position in a similar way. In conclusion, the results of the present study strongly indicate that the possibilities for cytochrome P450 mediated dehalogenation of 4-halogenated anilines to 4-aminophenol metabolites are dependent on: (i) the characteristics of the halogen that has to be eliminated, the most electronegative and smallest halogen being the one most easily eliminated, and (ii) the electron-withdrawing capacities of other substituents in the aromatic ring, electron-withdrawing substituents decreasing the relative rate of the reaction. Together these data lead to the conclusion that the halogen is eliminated as a halogen anion.

Key words: cytochrome P450; oxidative dehalogenation; halogenated anilines; aromatic hydroxylation; N-hydroxylation; microperoxidase 8

The accumulation of halogen-containing aliphatic and aromatic compounds is a major factor adding to environmental pollution. The phenomenon originates in the widespread use of halogenated compounds in industry, commerce and medicine and in the relatively high persistence of these xenobiotics. Removal of halogen substituents is considered to be a crucial step in the bioconversion and biodegradation of halogenated compounds [1–5]. In mammals and microorganisms cytochrome P450 are important enzymes involved in biodehalogenation processes [6, 7].

In our previous studies on the dehalogenation of 4-fluorinated anilines and phenols a reaction mechanism was proposed for the cytochrome P450 mediated oxidative defluorination of 4-fluoroanilines to 4-aminophenol metabolites and of pentafluorophenols to tetrafluorohydroquinone metabolites [8, 9]. Figure 1 schematically presents this pathway. The reaction proceeds by formation of chemically reactive benzoquinone(imine) as the primary reaction product. Because of this possible formation of reactive primary reaction products the cytochrome P450 mediated oxidative dehalogenation

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Abbreviations: MP-8, microperoxidase 8; HXO, hypohalous acid (for example, HClO = hypochlorous acid); tBuOOH, *tert*-butyl hydroperoxide; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

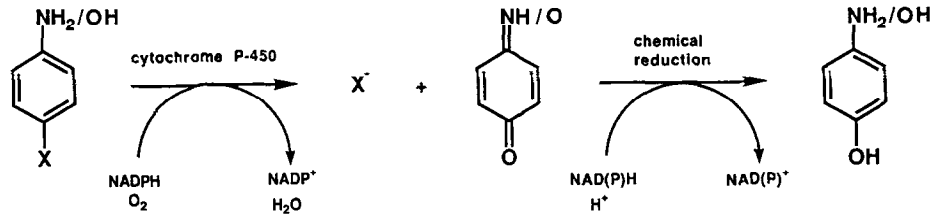

Fig. 1. Proposed reaction pathway for the cytochrome P450 catalysed dehalogenation of 4-halogenated anilines and phenols. X represents a fluoro atom [8, 9].

of aromatic compounds is of considerable interest [8–10]. Subsequent chemical reduction of benzoquinone(imine) leads to the formation of the final 4-aminophenol (or hydroquinone) metabolite (Fig. 1). Based on the electron balance of the cytochrome P450 reaction and formation of benzoquinone(imine) as the primary reaction product, the fluorine substituent is supposed to be eliminated as an anion.

Studies on the cytochrome P450-catalysed dehalogenation of aliphatic-halogenated hydrocarbons demonstrated the ease with which halogen elimination decreased in the order iodine > bromine > chlorine >fluorine [3, 5, 11–13]. The strength of the carbon–halogen bond is known to increase in the same order [1, 7]. As a result, a C–F bond is generally considered inert and difficult to break, suggesting that dehalogenation of chlorinated, brominated and iodinated compounds is easier than dehalogenation of the fluorinated analogues. Incorporation of a fluorine substituent into drugs or agrochemicals has even been suggested as a means of blocking biodegradation or bioactivation of the compounds [14–20].

Daly *et al.* [21], however, studying the conversion of various aniline derivatives, reported 4-chloroaniline to be less readily converted to 4-aminophenol than 4-fluoroaniline, although the phenomenon was not studied in more detail. In addition, in a previous study on the conversion of pentafluorochlorobenzene, the cytochrome P450 catalysed reaction appeared to preferentially eliminate the C4 fluorine substituent and not the chlorine substituent [10], again suggesting that for an aromatic compound and in contrast to the aliphatic compounds, the elimination of a fluorine might occur more readily than elimination of a chlorine substituent. Furthermore, the observation reported by Li *et al.* [22] of a much lower net rate of 2-dehalogenation of 2-bromoestradiol compared with that of 2-fluoroestradiol also suggests the relatively easier elimination of an aromatic fluorine substituent than of other aromatic halogen substituents. These authors attributed their observation to the inability of the brominated substrate to bind to the cytochrome P450 enzyme due to steric hindrance, but the phenomenon might also be due to electronic differences between the fluoro- and bromo-substituent.

The objective of the present study was to investigate the influence of the nature and number of the halogen substituents on the cytochrome P450 catalysed biodehalogenation of aromatic compounds in more detail. The results are expected to provide additional information on the importance of halogen characteristics for their possible elimination from an aromatic molecule in a cytochrome P450 catalysed reaction and, thus, on the mechanism of the reaction. Furthermore, an extension of the studies from fluorinated to other halogenated aromatics is of importance considering the more widespread use of chlorinated and brominated aromatics than of their fluorinated analogues.

4-Halogenated anilines were taken as the model compounds because defluorination of 4-fluoroaniline was already extensively demonstrated to occur in previous studies [23–25].

EXPERIMENTAL PROCEDURES

Chemicals. Aniline, 2-fluoro-, 3-fluoro-, 4-fluoro-, 4-chloro-, 4-bromo- and 4-iodoaniline were purchased from Janssen Chimica (Beerse, Belgium). All di- and polyfluoroanilines and nitrobenzenes were obtained from Fluorochem (Derbyshire, UK). 2-Aminophenol and 4-fluoronitrobenzene were from Aldrich and 4-aminophenol was from Merck (Darmstadt, Germany). The purity of all compounds was >98%.

Nitrosobenzene was purchased from Aldrich. 4-Fluoro-, 4-chloro-, 4-bromo- and 4-iodonitrosobenzene were synthesized according to Kennedy and Stock [26]. For 3 min 25 mL 15% (w/v) potassium peroxomonosulphate (minimal 4.5% active oxygen, Janssen Chimica) were added to a solution of 10 mmol C4-halogenated aniline in 20 mL glacial acetic acid, cooled in an ice bath. After 2 min mixing, the reaction mixture was immediately extracted twice with 25 mL hexane. The nitrosobenzene was further purified on a LiChroprep Si60 column using an ISCO HPLC system with hexane as the eluent. The flow-rate was 6.0 mL/min and fractions of 10 mL were collected. Detection was at 295 nm. The characteristic green-coloured nitrosobenzene containing fractions were collected and concentrated to a volume of 5 mL by evaporation of the solvent. In order to prevent the decomposition of the unstable nitrosobenzenes this fraction was not evaporated to dryness. The concentration of the C4-halogenated nitrosobenzene was determined by ^{1}H NMR on a Bruker AC 200 spectrometer using dichloromethane as the internal standard. The stock

solutions of the nitrosobenzene derivatives thus obtained were immediately used for the determination of molar extinction coefficients in the chemical assay for N-oxidation products (see below).

Synthesis of the fluorinated *N*-hydroxyanilines was performed by the chemical reduction of the nitrobenzene analogue essentially as described by Vogel [27] and Coleman *et al.* [28] or by the chemical reduction of the synthetized nitrosobenzenes in 0.1 KPi buffer (pH 7.6) containing 20 mM ascorbic acid.

3-Fluoro-4-aminophenol, 2-fluoro-4-aminophenol and 5-fluoro-2-aminophenol were synthesized as described before [24].

2-Amino-3,5-difluorophenol, 2-amino-5-chlorophenol, 2-amino-5-bromophenol and 2-amino-5-iodophenol were synthesized according to Boyland and Sims [29] using 2,4-difluoroaniline, 4-chloroaniline, 4-bromoaniline and 4-iodoaniline, respectively, as the starting material. In short, 15 g potassium persulphate (Janssen Chimica) were added to a solution of 5 g 4-halogenated aniline in 20 mL ethanol, 250 mL water and 50 mL 2N potassium hydroxide for 8 hr under continuous stirring. The mixture was kept overnight and filtered. After washing with ether, the solution was acidified with 2N sulphuric acid and the precipitate separated from the mixture by filtration. The filtrate was neutralized with 2N potassium hydroxide and the water content reduced to 25–50 mL by freeze-drying. The solution was extracted three times with 100 mL butanol, the collected butanol phases were evaporated under reduced pressure and the residue obtained was crystallized from 90% ethanol. The sulphate esters obtained were hydrolysed for 45 min at 100° in 3N HCl. After cooling, the solution was neutralized and the 2-aminophenols extracted from the mixture with diethylether. After evaporation of the solvent, the brownish precipitate was dissolved in 3 mL dichloromethane and applied to a LiChroprep Si60 column (310 mm × 25 mm, particle size 40–63 μM) (Merck) using an ISCO HPLC system with 2% (v/v) ethanol in dichloromethane as the eluen. The flow-rate was 5 mL/min and fractions of 10 mL were collected. Detection was at 295 nm using an ISCO absorbance detector. The fractions were analysed for 2-aminophenol content by monitoring their reaction in a chemical assay for 2-aminophenols as described elsewhere [30].

The 2-aminophenol-containing fractions were collected and after crystallization the purity of these aminophenols was verified using a Kratos 400 HPLC system, with a LiChrosorb C_8 column (100 mm × 3 mm) (Chrompack, Middelburg, The Netherlands). After eluting for 5 min with nanopure water, a linear gradient from 0 to 80% (v/v) methanol in 22 min followed by 80% (v/v) methanol for 6 min was applied. Detection was at 240 nm using a Waters™ 996 photodiode array detector. From the elution patterns obtained, the purity of the synthesized compounds was judged to be >95%.

The identity of the synthetized 2-aminophenols was derived from their ^{19}F-NMR and/or ^{1}H-NMR spectral characteristics. 5-Fluoro-2-aminophenol: ^{19}F-NMR (0.1 M potassium phosphate pH 7.6, relative to $CFCl_3$) (ppm): −127.5 (F5) (m, $^{3}J_{F5\text{-}H4}$ = 12.0 Hz, $^{3}J_{F5\text{-}H6}$ = 12.0 Hz, $^{4}J_{F5\text{-}H3}$ = 9.0 Hz) and ^{1}H-NMR (acetone) (ppm): 9.29 (H3) (tr, $^{3}J_{H3\text{-}H4}$ = 9.0 Hz, $^{4}J_{H3\text{-}F5}$ = 9.0 Hz), 9.22 (H4) (dd, $^{3}J_{H4\text{-}F5}$ = 12.0 Hz, $^{3}J_{H4\text{-}H3}$ = 9.0 Hz), 9.07 (H6) (d, $^{3}J_{H6\text{-}F5}$ = 12.0 Hz); 5-chloro-2-aminophenol: ^{1}H-NMR (acetone) (ppm): 9.42 (H6) (s), 9.33 (H4) (d, $^{3}J_{H4\text{-}H3}$ = 8.3 Hz), 9.29 (H3) (d, $^{3}J_{H3\text{-}H4}$ = 8.3 Hz); 5-bromo-2-aminophenol: ^{1}H-NMR (acetone) (ppm): 9.55 (H6) (s), 9.42 (H4) (d, $^{3}J_{H4\text{-}H3}$ = 8.5 Hz), 9.29 (H3) (d, $^{3}J_{H3\text{-}H4}$ = 8.5 Hz); 5-iodo-2-aminophenol: ^{1}H-NMR (acetone) (ppm): 9.71 (H6) (s), 9.60 (H4) (d, $^{3}J_{H4\text{-}H3}$ = 8.5 Hz), 9.19 (H3) (d, $^{3}J_{H3\text{-}H4}$ = 8.5 Hz); 3-fluoro-2-aminophenol: ^{19}F-NMR (0.1 M potassium phosphate pH 7.6, relative to $CFCl_3$) (ppm): −136.9 (F3) (dd, $^{3}J_{F3\text{-}H4}$ = 10.0 Hz, $^{4}J_{F3\text{-}H5}$ = 8.5 Hz) and ^{1}H-NMR (acetone) (ppm): 9.25 (H5) (m, $^{3}J_{H5\text{-}H6}$ = 8.5 Hz, $^{3}J_{H5\text{-}H4}$ = 8.5 Hz, $^{4}J_{H5\text{-}F3}$ = 8.5 Hz), 9.11 (H6) (d, $^{3}J_{H6\text{-}H5}$ = 8.5 Hz), 9.02 (H4) (dd, $^{3}J_{H4\text{-}H5}$ = 8.5 Hz, $^{3}H_{H4\text{-}F3}$ = 10.0 Hz); 3,5-difluoro-2-aminophenol: ^{19}F-NMR (0.1 M potassium phosphate pH 7.6, relative to $CFCl_3$) (ppm): −134.5 (F3) (d, $^{3}J_{F3\text{-}H4}$ = 9.0 Hz), −127.1 (F5) (tr, $^{3}J_{F5\text{-}H6}$ = 9.0 Hz, $^{3}J_{F5\text{-}H4}$ = 9.0 Hz) and ^{1}H-NMR (acetone) (ppm): 9.17 (H6) (d) ($^{3}J_{H6\text{-}F5}$ = 9.0 Hz), 9.10 (H4) (tr) ($^{3}J_{H4\text{-}F3}$ = 9.0 Hz, $^{3}J_{H4\text{-}F5}$ = 9.0 Hz).

All di- and polyfluorinated 4-aminophenols were prepared by biosynthesis, purified and their identity derived from ^{19}F-NMR and ^{1}H-NMR spectral characteristics essentially as described previously [31].

Preparation of microsomes. Microsomes were prepared from the perfused livers of male Wistar rats (±400 g) pretreated with isosafrole (Janssen Chimica) as described previously [24]. For the *in vitro* experiments liver microsomes of isosafrole pretreated rats were used, since this type of microsome was demonstrated to possess the highest capacity to convert 4-fluoroaniline to its 4-aminophenol [25]. This was also the case for other 4-halogenated anilines (unpublished results). Cytochrome P450 content of the microsomes was measured as described by Omura and Sato [32].

Purification of MP-8. MP-8 was purified by proteolytic digestion of horse heart cytochrome c (Sigma, St Louis, MO, USA) essentially as described in the literature [33]. The sample was more than 96% pure based on HPLC analysis [33] performed on a Waters™ 600 Controller HPLC with a Baker bond WPC 4 column (25 cm × 4.6 mm). A linear gradient from 0.1% trifluoroacetic acid in water to 50%, 0.1% trifluoroacetic acid in water and 50%, 0.1% trifluoroacetic acid in acetonitrile was applied in 50 min, which was continued isocratically for 5 min. Detection at 214 nm was performed on an ISCO V^4 absorbance detector and detection at 395 nm was performed on an Applied Biosystems 757 absorbance detector. The haem content was determined essentially as described previously [34].

Purification of cytochrome P450 IIB1 and NADPH-cytochrome P450 reductase. Cytochrome P450 IIB1 and NADPH-cytochrome P450 reductase were purified from liver microsomes of phenobarbital (Brocacef BV, Maarssen, The Netherlands) (0.1% in drinking water for 7 days) pretreated male and female Wistar rats essentially as previously described by Boersma *et al.* [35].

In vitro *incubations*. Cytochrome P450-dependent conversion was studied *in vitro* in microsomal incubations containing (final concentrations) 0.1 M potassium phosphate pH 7.6, between 0 and 15 mM of the halogenated aniline (as indicated) added as 1% (v/v) of a 100-fold concentrated stock solution in dimethylsulphoxide and 1 μM microsomal cytochrome P450. The reaction was started by the addition of NADPH (1 mM final concentration) or tBuOOH (10 mM final concentration) and carried out at 37° for 10 min. The incubations with tBuOOH as the artificial oxygen donor additionally contained NADH (1 mM final concentration) in order to reduce benzoquinoneimine to 4-aminophenol. The conversion of halogenated anilines to their 4-aminophenol metabolites is linear for at least 10 min. Incubations with purified, reconstituted cytochrome P450 IIB1 were conducted using similar final incubation conditions as described for the microsomal incubations. Instead of microsomes, the incubations contained 0.5 nmol cytochrome P450 IIB1, 1.24 units NADPH-cytochrome reductase and 20 μg dilauroyl phosphatidylcholine (Sigma) per mL incubation mixture and were preincubated for 6 min at 37°.

MP-8 catalysed conversion was studied in incubations containing (final concentrations) 0.1 M potassium phosphate pH 7.6, with between 0 and 12.5 mM of the halogenated aniline (as indicated) added as 1% (v/v) of a 100-fold concentrated stock solution in dimethylsulphoxide, 7.5 μM MP-8 and 1 mM NADH in order to reduce the primary metabolite 4-benzoquinoneimine to 4-aminophenol. The reaction was started by the addition of H_2O_2 (2.5 mM final concentration) and carried out at 37° for 1 min.

For chemical analysis of 4-aminophenol or *N*-hydroxyaniline the reaction was terminated by the addition of 0.8 mL of the incubation mixture to 0.24 mL of 20% (w/v) trichloroacetic acid. Upon mixing and centrifugation (5 min, 13,000 rpm) the supernatant was used for the chemical determination of 4-aminophenol or *N*-hydroxyaniline metabolites as described hereafter. For the chemical analysis of 2-aminophenol metabolites (described below) the reaction was terminated by freezing the sample into liquid nitrogen.

Incubations for HPLC and ^{19}F-NMR analysis additionally contained 1 mM ascorbic acid to prevent autoxidation of the aminophenols especially during the overnight ^{19}F-NMR measurement. These incubations were terminated by freezing the samples into liquid nitrogen. Samples were stored at −20°, thawed and centrifuged (5 min, 13,000 rpm). HPLC analysis of these incubations was performed essentially as described above for the check on purity of the synthesized aminophenols. The samples for the ^{19}F-NMR measurements were made oxygen-free by four cycles of evacuating and filling with argon.

Chemical determination of hydroxylated metabolites. 4-Aminophenols were determined essentially as described by Brodie and Axelrod [36]. In short, 100 μL phenol reagent (5% w/v phenol in 2.5 N NaOH) and 200 μL 2.5 M Na_2CO_3 were added to 1 mL trichloroacetic acid precipitated supernatant. After 60 min at room temperature absorbance at 630 nm was measured. The presence of a halogen in the indophenol formed in this assay influences its molar extinction coefficient at 630 nm [37]. For this reason the molar extinction coefficient of the halogenated aminophenol-derived indophenols was determined to allow quantification of the 4-aminophenol metabolites from the various aniline derivatives. Molar extinction coefficients were 30.5, 26.7, 26.3, 14.5, 20.0, 16.8, and 12.5 mM^{-1} cm^{-1} for the indophenols derived from 4-aminophenol, 3-fluoro-4-aminophenol, 2-fluoro-4-aminophenol, 3,5-difluoro-4-aminophenol, 2,6-difluoro-4-aminophenol, 2,3-difluoro-4-aminophenol and 2,3,5-trifluoro-4-aminophenol, respectively.

Products of C2 hydroxylation of the 4-halogenated anilines were determined by a chemical assay developed for the detection of 2-aminophenols. This newly developed assay is described in detail elsewhere [30]. In short, a 1 mL microsomal incubation, frozen into liquid nitrogen to stop the reaction, was thawed and centrifuged at 13,000 rpm for 10 min at 4°. The supernatant was extracted three times with 3 mL of diethylether. After evaporation of the diethylether, 300 μL of 20% (w/v) trichloroacetic acid, 1 mL demineralized water and 100 μL 40 mM ammonium iron(III) sulphate dodecahydrate (Janssen Chimica) were added to the residue. After 45 min at room temperature, the reaction mixture was extracted with 2 mL chloroform and the absorbance of the chloroform phase was measured at λ_{max} of the halogenated 2-aminophenol-derived yellow derivative. The values for the 2-aminophenol concentrations of the samples were determined from the calibration curves of 2-aminophenol, 2-amino-5-fluorophenol, 2-amino-5-chlorophenol, 2-amino-5-bromophenol and 2-amino-5-iodophenol.

The chemical determination of N-oxidation products (*N*-hydroxyaniline plus nitrosobenzene derivatives) was performed essentially as described by Herr and Kiese [38]. Potassium ferricyanide (0.3 mL, 10% (w/v)) in 1 N HCl and 1 mL demineralized water were added to 1 mL trichloroacetic acid precipitated microsomal supernatant in order to oxidize the *N*-hydroxyaniline to the corresponding nitrosobenzene. These samples were extracted with 2 mL CCl_4 and the CCl_4 phases washed twice with 5 N H_2SO_4. Glacial acetic acid (1.0 mL) and 50 μL 20% (w/v) sodium nitrite were added to 1.0 mL of the washed CCl_4 phases. After 15 min, 100 μL 50% (w/v) ammonium sulphamate (Janssen Chimica) were added and the samples mixed for 10 min. Finally, the colour formation was started by the addition of 250 μL 80% (v/v) acetic acid and 50 μL *N*-(1-naphthyl)ethylenediamine dihydrochloride (Aldrich, Steinheim, Germany). After 2 hr in the dark the absorbance of the water phase at 555 nm was measured. Using nitrosobenzene and synthesized 4-fluoro-, 4-chloro-, 4-bromo- and 4-iodo-nitrosobenzene, molar extinction coefficients of 40.4, 32.9, 41.3, 40.9 and 42.2 mM^{-1} cm^{-1} respectively, were determined.

Kinetic analysis. The apparent V_{max} and K_m values for C4-, C2- and *N*-hydroxylation were determined by fitting the data to the standard Michaelis–Menten equation: $v = V_{max}*[S]/(K_m + [S])$ with the program

of KaleidaGraph, version 2.0.2 (Abelbeck Software). The correlation coefficient of the fits was above 0.97 in all cases.

NMR measurements. ^{19}F-NMR measurements were performed on a Bruker AMX 300 NMR spectrometer essentially as described previously by Vervoort *et al.* [39]. Chemical shifts are reported relative to $CFCl_3$. Quantification of the fluorinated compounds in the samples was achieved by comparison of the integrals of their ^{19}F-NMR resonances to the integral of the resonance of 4-fluorobenzoic acid added as an internal standard. The splitting patterns of the ^{1}H-NMR and ^{19}F-NMR resonances of the (bio)synthesized 2- and 4-aminophenols dissolved in acetone-d_6 were measured on a Bruker AMX 500 MHz. ^{19}F-NMR measurements were performed using a ^{1}H probehead tuned to the ^{19}F frequency (470.5 MHz). ^{1}H-NMR chemical shifts are reported relative to acetone, present in acetone-d_6, used for locking the magnetic field.

RESULTS

Microsomal cytochrome P450 catalysed conversion of 4-halogenated anilines

The influence of the type of C4 halogen substituent in an aniline molecule on cytochrome P450 catalysed dehalogenation to 4-aminophenol was investigated by HPLC analysis of microsomal incubations.

Figure 2 presents HPLC chromatograms of the microsomal metabolite patterns of the four 4-halogenated anilines and—for comparison—aniline itself. The reaction is cytochrome P450 mediated as no conversion was observed without NADPH, without microsomes or in the presence of CO [25]. Peaks were identified on the basis of their retention times compared to the retention times of the synthesized reference compounds and the similarity of the absorption spectra provided by the diode array detector. From these results it follows that microsomal cytochrome P450 catalysed conversion of the anilines results in the formation of 4-aminophenol, (halogenated) 2-aminophenols and/or N-oxidation products (*N*-hydroxyaniline and nitrosobenzene derivatives). Formation of (halogenated) 3-aminophenols or NIH-shifted metabolites is not observed. In contrast, metabolites resulting from hydroxylation at C4 accompanied by dehalogenation are readily observed, especially for 4-fluoroaniline and 4-chloroaniline. These results support the hypothesis that cytochrome P450 catalysed C4-hydroxylation of 4-halogenated anilines proceeds by dehalogenation of the aromatic ring rather than by hydroxylation at C4 accompanied by an NIH shift of the halogen to the adjacent C3 or C5 position.

In addition to the HPLC experiments, metabolite formation in microsomal incubations was quantified by chemical analysis using specific methods for detection of 2-aminophenol, 4-aminophenol and N-oxidation products (*N*-hydroxyaniline- plus nitrosobenzene-derivatives). Table 1 presents the kinetic parameters obtained for the microsomal conversion of aniline and the four halogenated anilines. The apparent V_{max} for the formation of 4-aminophenol decreases to 66.8% of the value of

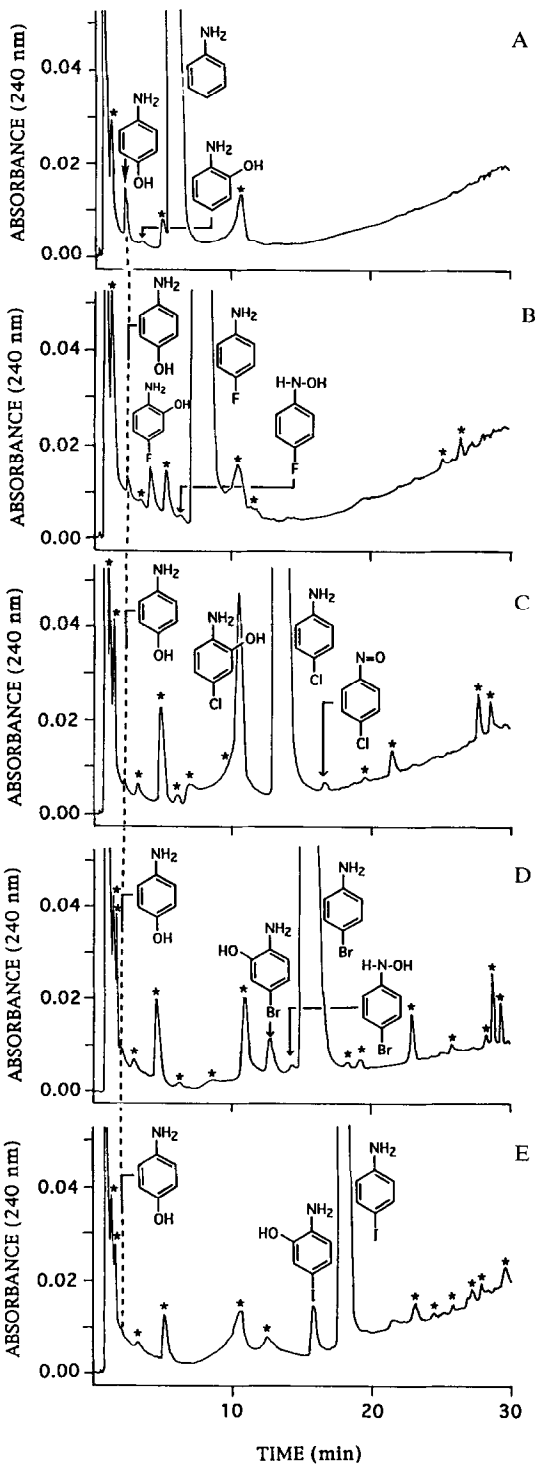

Fig. 2. Reversed-phase HPLC chromatograms of the microsomal conversion of (A) aniline, (B) 4-fluoroaniline, (C) 4-chloroaniline, (D) 4-bromoaniline, and (E) 4-iodoaniline with detection at 240 nm. The peaks marked with an asterisk were also present in the chromatograms of control incubations carried out in the absence of NADPH, in the absence of microsomes, or in the absence of the aniline derivatives.

Table 1. Kinetic characteristics of the cytochrome P450 catalysed aromatic C4-hydroxylation of aniline and its 4-halogenated derivatives determined using liver microsomes from isosafrole pretreated male Wistar rats (N = 2–4)*

Compound	Apparent V_{max} (nmol product/min/nmol cyt P450)	Apparent K_m (mM)
Aniline	5.55 ± 0.20 (100%)	17.0 ± 2.0
4-Fluoroaniline	3.71 ± 0.28 (66.8%)	9.3 ± 1.7
4-Chloroaniline	0.54 ± 0.09 (9.7%)	1.2 ± 0.2
4-Bromoaniline	0.39 ± 0.10 (7.0%)	0.7 ± 0.2
4-Iodoaniline	0.38 ± 0.16 (6.8%)	0.3 ± 0.1

* Values presented are the mean ± standard error of the mean. Values in parentheses represent the % relative to the value for C4-hydroxylation of aniline.

Table 2. Apparent V_{max} values of cytochrome P450 catalysed C2 hydroxylation and N-hydroxylation and the total conversion of aniline and its 4-halogenated derivatives determined using liver microsomes from isosafrole pretreated male Wistar rats (N = 2–4)*

Compound	Apparent V_{max} (nmol product/min/nmol cyt P450)		V_{max} for total conversion†
	2-Aminophenol	*N*-Hydroxyaniline	
Aniline	1.13 ± 0.12	1.42 ± 0.07	8.10 ± 0.39
4-Fluoroaniline	2.27 ± 0.07	3.34 ± 0.09	9.32 ± 0.44
4-Chloroaniline	2.93 ± 0.12	4.35 ± 0.09	7.82 ± 0.30
4-Bromoaniline	2.67 ± 0.04	4.39 ± 0.09	7.45 ± 0.23
4-Iodoaniline	2.93 ± 0.47	2.93 ± 0.17	6.24 ± 0.80

* Values presented are the mean ± standard error of the mean.
† Calculated as the sum of apparent V_{max} for C2-, N- and C4-hydroxylation (Table 1).

aniline when the hydrogen at C4 is replaced by a fluorine. Replacement of the hydrogen at C4 by a chlorine, bromine or iodine substituent even further decreases the apparent V_{max} of this reaction. However, the apparent V_{max} values observed for 4-chloro-, 4-bromo- and 4-iodo-aniline vary only slightly (Table 1).

To verify whether the observed change in apparent V_{max} for dehalogenation to C4 aminophenols was not the result of a general decrease in metabolism from fluorine > chlorine > bromine > iodine, the conversion rates for formation of metabolites resulting from C2 and N-hydroxylation were also determined. Table 2 presents the apparent V_{max} values for microsomal C2 and N-hydroxylation and also the total maximal conversion rate, calculated as the sum of the V_{max} values obtained for the conversion to 2-aminophenol, 4-aminophenol and *N*-hydroxyaniline metabolites. The results in Table 2 demonstrate that the total V_{max} for the C2, C4 plus N-hydroxylation is not significantly influenced by the type of C4 halogen substituent. The decrease in the apparent V_{max} for the 4-hydroxylation is accompanied by an increase in hydroxylation of the 4-halogenated anilines at their C2- and N-position. Thus, the results in Table 2 indicate that the observed change in dehalogenation is not a result of a reduced overall capacity of cytochromes P450 to convert the 4-halogenated anilines.

Dehalogenation of 4-halogenated anilines by reconstituted cytochrome P450 IIB1, by a model haem-based catalyst and by a tertiair hydroperoxide supported microsomal cytochrome P450 system

To demonstrate that the change in the extent of C4 hydroxylation with a change in the type of halogen substituent is not due to a change in cytochrome P450 enzymes actually contributing to oxidative dehalogenation, experiments with purified reconstituted cytochrome P450 IIB1 and MP-8 were performed. MP-8 is a haem-containing mini-enzyme without a substrate binding site. This mini-enzyme, consisting of a protoporphyrin IX haem covalently bound to an oligopeptide of eight amino acids, has been reported to form an $(FeO)^{3+}$ intermediate similar to the cytochrome P450 enzyme and to catalyse P450-like reactions [40, 41]. Table 3 presents the results for cytochrome P450 IIB1 and MP-8 mediated conversion of aniline, 4-fluoro-, 4-chloro-, 4-bromo- and 4-iodoaniline to 4-aminophenol. The results clearly demonstrate, for both systems, a decrease in 4-aminophenol formation from aniline > 4-fluoroaniline > 4-chloroaniline > 4-bromoaniline > 4-iodoaniline. Moreover, for cytochrome IIB1 as well as for MP-8, the same dependency in reduction of the rate of 4-aminophenol formation exists as is observed for the conversion by liver microsomes of isosafrole pretreated rats,

Table 3. Apparent maximal reaction rates for the microperoxidase 8, purified reconstituted cytochrome P-450 IIB1 and microsomal tBuOOH supported catalysed C4-hydroxylation of aniline and its 4-halogenated derivatives*

Compound	Apparent V_{max} (nmol product/min/nmol cyt P450)		
	MP-8	Cytochrome P450 IIB1	Microsomal tBuOOH supported cytochrome P450
Aniline	3.14 ± 0.06 (100%)	0.97 ± 0.07 (100%)	5.76 ± 0.42 (100%)
4-Fluoroaniline	0.42 ± 0.01 (13.4%)	0.28 ± 0.02 (28.9%)	1.94 ± 0.05 (33.7%)
4-Chloroaniline	0.18 ± 0.01 (5.7%)	0.12 ± 0.01 (12.4%)	0.35 ± 0.05 (6.1%)
4-Bromoaniline	0.15 ± 0.01 (4.8%)	0.07 ± 0.00 (7.2%)	0.21 ± 0.02 (3.6%)
4-Iodoaniline	0.14 ± 0.01 (4.5%)	0.04 ± 0.01 (4.1%)	0.34 ± 0.02 (5.9%)

* Values presented are the mean ± standard error of the mean. Values in parentheses represent the % relative to the value for C4-hydroxylation of aniline.

containing mainly cytochrome P450 IA1/IA2 [42] (Table 1).

Additional experiments were performed to investigate whether the change in the extent of C4 hydroxylation with a change in the type of halogen substituent is not due to a change in rate-limiting steps in the P450 catalysis. The use of the alternative oxygen donor tertiair butyl hydroperoxide is known to provoke a short-cut in the reaction cycle, thereby circumventing several possible rate-limiting reaction steps in a NADPH/oxygen supported reaction [43]. Table 3 presents the results for the tertiair butyl hydroperoxide supported microsomal cytochrome P450 mediated C4 hydroxylation of aniline, 4-fluoro-, 4-chloro-, 4-bromo- and 4-iodoaniline to 4-aminophenol. The results show a similar decrease in 4-aminophenol formation from aniline to 4-iodoaniline as observed for the three other systems used in this study. This observation strongly suggests that, for the oxidative dehalogenation of the 4-halogenated anilines occurring in this study, the rate-limiting step might indeed be related to the actual step in which the substrates are converted, and, thus, not, for instance, to electron-donating steps.

Effect of additional electronegative substituents on the microsomal C4 hydroxylation of 4-fluorinated aniline derivatives

To gain insight into the influence of electronic characteristics of the substrate on the dehalogenation of 4-halogenated anilines to 4-aminophenol derivatives, we investigated whether the presence of additional electron withdrawing substituents would affect the elimination of fluorine from the C4 position of the aniline substrate. When electron withdrawing possibilities of the halogen atom to be eliminated are a main factor in determining whether aromatic dehalogenation can take place, it can be expected that additional electron withdrawing fluorine substituents at C2, C3, C5, and/or C6 might hamper C4-defluorination. Furthermore, in addition to the electronegative characteristics of halogen substituents, the observed decrease in C4 hydroxylation with a change in the type of C4 halogen-substituent (Table 1) might be the result of (i) a change in the reaction mechanisms for dehalogenation with a change in the type of halogen atom, or (ii) increased steric hindrance by the halogen substituent hampering the initial attack of the cytochrome P450 $(FeO)^{3+}$ intermediate at the substituted C4 position of the aniline. However, the van der Waals radius of a fluorine atom almost equals that of a hydrogen atom, resulting in minimal steric influences when the dehalogenation of a series of polyfluorinated anilines is investigated.

Table 4 presents the results obtained. From the data it is clear that the relative ease of fluorine elimination from the C4-fluorinated anilines decreases with an increase in the number of fluorine substituents. Thus, the presence of additional fluorine substituents makes elimination of the fluorine at C4 somewhat more difficult. The data also demonstrate that an additional fluorine substituent at C3 results in stronger reduction of the relative apparent V_{max} than an additional fluorine at C2. This observation is in accordance with the more pronounced effect of the electron withdrawing fluorine when it is in an *ortho* rather than *meta* position with respect to the fluorine to be removed.

Metabolite profiles of the microsomal cytochrome P450 catalysed conversion of fluorinated aniline-derivatives

To characterize the overall conversion characteristics of the fluorinated anilines for the apparent V_{max} studies on C4 hydroxylation ^{19}F-NMR spectra of the microsomal incubations were determined.

Figure 3 presents the ^{19}F-NMR spectra of the microsomal conversion of the fluorinated anilines. The ^{19}F-NMR resonances of the metabolites were identified on the basis of (synthesized) reference compounds. For metabolites 5,6-difluoro-2-aminophenol (Fig. 3D), 4,5-difluoro-2-aminophenol (Fig. 3D), 4,6-difluoro-2-aminophenol (Fig. 3F) and 4,5,6-trifluoro-2-aminophenol (Fig. 3F) ^{19}F-NMR resonances were identified on the basis of a characteristic shift of the ^{19}F-NMR resonance known to exist upon the introduction of a hydroxyl moiety at positions *ortho*, *meta* or *para* with respect to the fluorine substituent [43, 44].

The ^{19}F-NMR spectra presented in Fig. 3 clearly demonstrated that the fluorine substituent at C4 is

Table 4. Apparent V_{max} values for the C4-hydroxylation of 4-fluorinated anilines compared to the apparent V_{max} values of their non-C4 fluorinated analogues, determined using liver microsomes from isosafrole pretreated male Wistar rats (N = 2–4)*

Substrate	Apparent V_{max} (nmol 4-aminophenol/min/nmol cyt P450)	Relative apparent V_{max} as % of the non-C4-fluorinated analogue
Aniline	5.55 ± 0.20	
4-Fluoroaniline	3.71 ± 0.28	66.8
2-Fluoroaniline	6.23 ± 0.20	
2,4-Difluoroaniline	2.99 ± 0.07	48.0
2,6-Difluoroaniline	8.89 ± 0.05	
2,4,6-Trifluoroaniline	3.21 ± 0.16	36.1
3-Fluoroaniline	4.85 ± 0.12	
3,4-Difluoroaniline	0.90 ± 0.03	18.6
2,3-Difluoroaniline	9.46 ± 0.39	
2,3,4-Trifluoroaniline	1.18 ± 0.04	12.5
3,5-Difluoroaniline	6.82 ± 0.39	
3,4,5-Trifluoroaniline	0.42 ± 0.02	6.2
2,3,6-Trifluoroaniline	3.23 ± 0.12	
2,3,4,6-Tetrafluoroaniline	0.66 ± 0.16	20.3

* Values presented are the mean ± standard error of the mean.

eliminated from the aromatic aniline-ring upon cytochrome P450 catalysed hydroxylation to fluorinated 4-aminophenols. Metabolites resulting from C4 hydroxylation accompanied by an NIH shift of the fluorine substituent at C4 are not observed. Furthermore, the metabolic patterns show that, as for 4-fluoro-, 4-chloro-, 4-bromo- and 4-iodoaniline, the incorporation of a halogen atom at the C4 position in the (poly)fluoroanilines leads to a metabolic switch from formation of 4-aminophenol to 2-aminophenol and *N*-hydroxyaniline metabolites.

Effect of an additional electron-withdrawing substituent on the oxidative dehalogenation of C4 chlorinated, brominated or iodinated aniline derivatives

Analogous to the experiments presented in Table 4, the influence of an additional fluoro-substituent on the oxidative dehalogenation of a C4-chlorinated, brominated and iodinated aniline was studied. This was done to investigate whether an electron withdrawing substituent would also affect oxidative dehalogenation in the case of a chloro-, bromo-, or an iodo-substituent. Table 5 presents microsomal cytochrome P450 catalysed conversion of 2-fluoro-4-halogenated anilines to 3-fluoro-4-aminophenol. The results are in accordance with the observations for the defluorination of the di- and poly-fluorinated anilines. Firstly, the formation of 3-fluoro-4-aminophenol decreases in the order hydrogen >> fluoro >> chloro ≈ bromo ≈ iodo. Secondly, the presence of the additional fluoro substituent hampers oxidative elimination of the halogen at C4 as compared with the non-C2-fluorinated analogues for which the V_{max} of their conversion was already presented in Table 1.

DISCUSSION

The mechanism of dehalogenation of halogenated aromatic xenobiotics is still a matter of considerable debate. Recently, we proposed a mechanism for the cytochrome P450 catalysed oxidative defluorination of 4-fluorinated anilines and phenols proceeding by the mechanism depicted in Fig. 1. This reaction scheme was based on the formation of a primary reaction product that could be reduced through a two electron step to the hydroxyaniline or phenol indicating it to be the reactive benzoquinone(imine) [8, 9]. Elimination of the halogen as a halogen anion then follows from the net two electron balance of the cytochrome P450 reaction. Results of the present study demonstrate that the cytochrome P450 catalysed 4-hydroxylation of 4-halogenated anilines proceeds by dehalogenation rather than by hydroxylation accompanied by an NIH-shift. A fluorine substituent is significantly more easily eliminated than a chlorine, bromine or iodine substituent, the dehalogenation rate of 4-chloro-, 4-bromo- and 4-iodoaniline being much more similar. These findings are in accordance with the observations of Ullrich *et al.* who studied the conversion of a comparable group of compounds, the 4-halogenated acetanilides [46].

Using purified reconstituted cytochrome P450 IIB1, tBuOOH supported microsomal cytochrome P450 catalysis and MP-8, a haem-based model system able to catalyse cytochrome P450-like chemistry [41], similar changes in the rate of oxidative dehalogenation with a change in the C4 halogen substituent were observed. Based on these observations it may be concluded that the observed decrease in dehalogenation with a change in the halogen is not dependent on (i) different P450 enzymes contributing to the reaction, nor on (ii) changes in orientation of the substrates by the protein core in the large active sites of P450. The observation of similar effects on the V_{max} of the oxidative dehalogenation in the tBuOOH driven reaction with a change in the halogen substituent

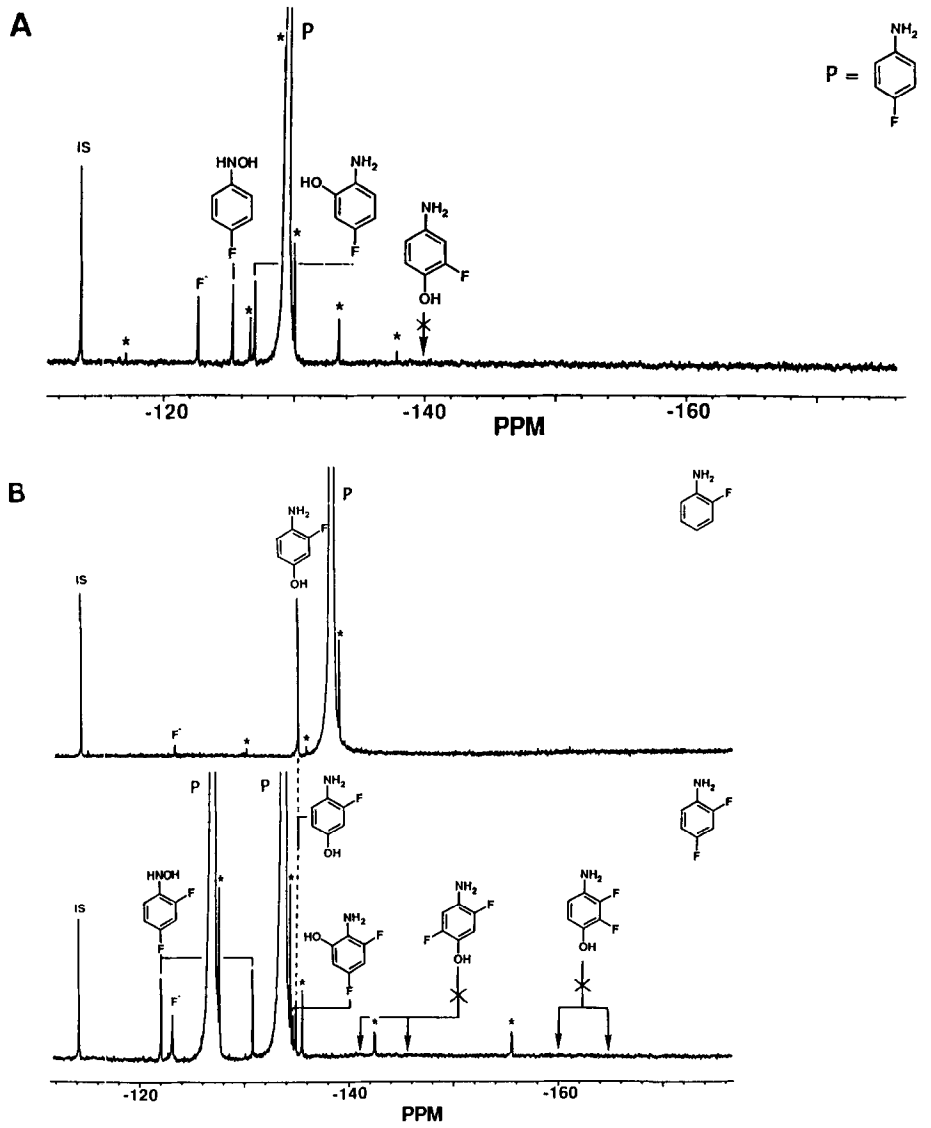

Fig. 3. ^{19}F-NMR spectra of the microsomal cytochrome P450 catalysed conversion of the non-C4 fluorinated anilines (upper part) and their 4-fluorinated analogues (lower part); (A) 4-fluoroaniline, (B) 2-fluoroaniline and 2,4-difluoroaniline, (C) 2,6-difluoroaniline and 2,4,6-trifluoroaniline, (D) 3-fluoroaniline and 3,4-difluoroaniline, (E) 2,3-difluoroaniline and 2,3,4-trifluoroaniline, (F) 3,5-difluoroaniline and 3,4,5-trifluoroaniline, (G) 2,3,6-trifluoroaniline and 2,3,4,6-tetrafluoroaniline. The resonances marked with an asterisk were also present in the ^{19}F-NMR spectra of control incubations carried out in the absence of NADPH. The resonance marked IS is from the internal standard 4-fluorobenzoic acid. The arrows in the spectrum indicate the ppm value where NIH-shifted metabolites are expected.

supports the hypothesis that the actual rate-limiting step follows formation of the activated cofactor, and might thus indeed be related to the effect of the halogen substituent on the actual dehalogenation step. Taken together, these results with the purified reconstituted cytochrome P450 IIB1 system, the tBuOOH supported microsomal cytochrome P450 and the MP-8 model compound point to the importance of the electronic characteristics of the halogen substituents for the reactivity of the C4-halogenated anilines to participate in cytochrome P450 mediated oxidative dehalogenation reactions.

For all systems tested (i.e. microsomes, purified reconstituted cytochrome P450 IIB1, tBuOOH supported microsomal cytochrome P450 and MP-8), the observed relative apparent V_{max} values for dehalogenation of the anilines correlate ($r =$ 0.95, 0.98, 0.93 and 0.97, respectively) with the electronegativity of fluorine (4.10), chlorine (2.83), bromine (2.74) and iodine (2.21). The changes in

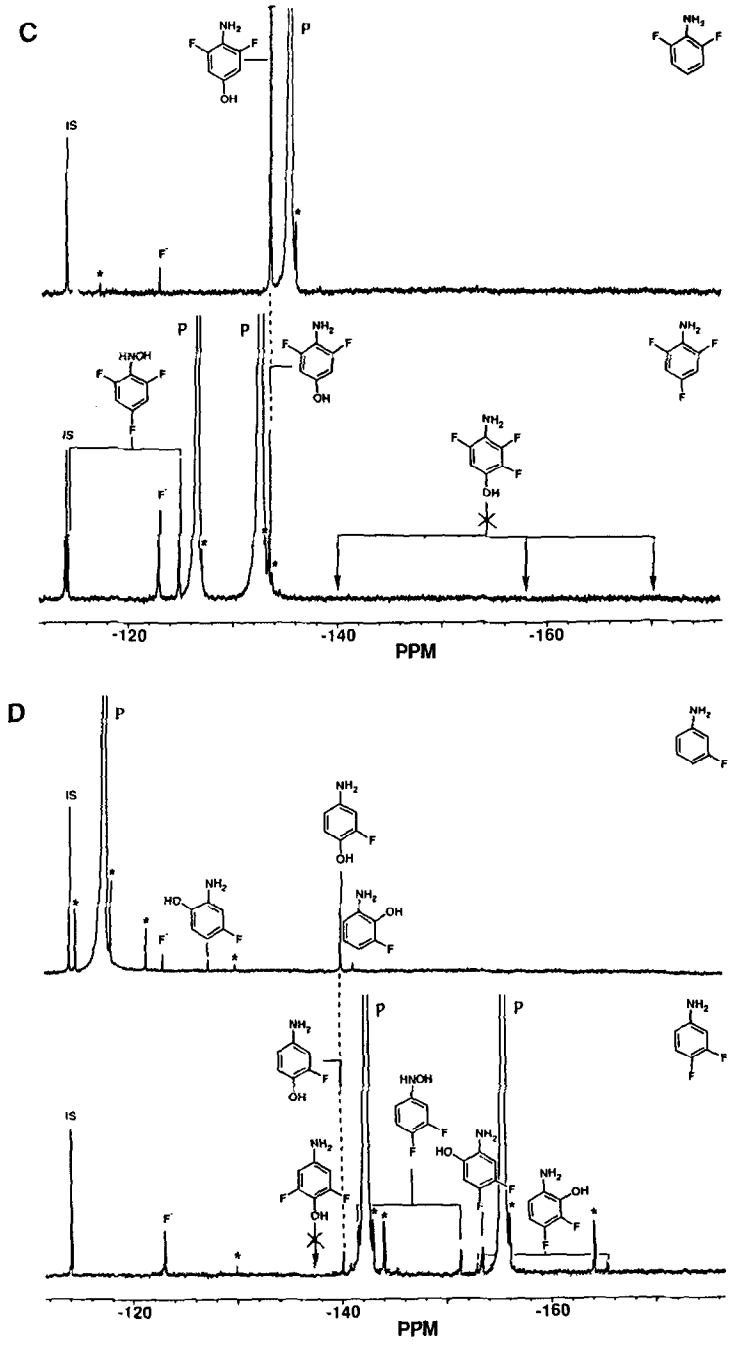

Fig. 3—*contd.*

the apparent V_{max} for C4 hydroxylation with a change in the C4 substituent do not correspond to the strength of the carbon halogen bond known to increase in the opposite order, namely, iodine < chlorine < fluorine [1, 7]. Together, these results point to the importance of electronegativity of the halogen to be eliminated in cytochrome P450 catalysed oxidative dehalogenation. This observation supports the conclusion that the halogen is eliminated as a halogen anion. However, steric hindrance of the initial cytochrome P450 $(FeO)^{3+}$ attack on C4 by the larger chloro-, but especially bromo- and iodo-substituents with Van der Waals radius of, respectively, 1.80 Å, 1.95 Å and 2.15 Å, might provide an additional explanation for the observed effects of the type of halogen on the apparent V_{max} for the dehalogenation. Such steric hindrance, in combination with the susceptibility of an iodo-

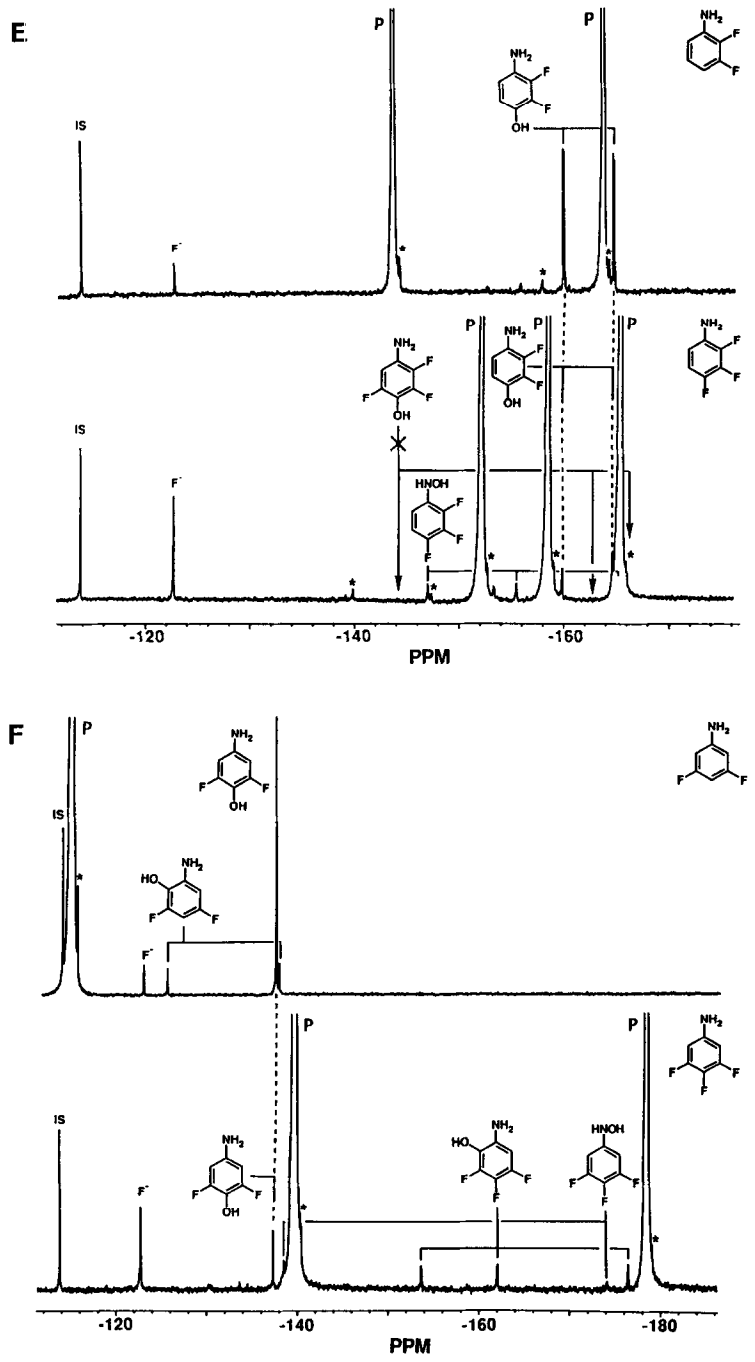

Fig. 3—*contd.*

substituent in particular to become oxidized by cytochromes P450, as demonstrated by Guengerich using 4-*tert*-butyl-2,5-bis[1-hydroxy-1-(trifluoromethyl)-2,2,2-trifluoro-ethyl]-iodobenzene [47], may even result in a different mechanism. For iodinated aromatic molecules a mechanism for cytochrome P450 catalysed deiodination has been suggested that proceeds by an interaction of the reactive cytochrome P450 $(FeO)^{3+}$ intermediate with non-bonding halogen electrons of the iodine [47, 48]. This interaction results in the formation of a haloso compound, and, finally, the formation of the hydroxylated substrate and elimination of the halogen as HXO [49]. Such a possible change in the actual mechanism of dehalogenation may add to a change in the rate of the dehalogenation reaction, especially for 4-iodoaniline. Therefore, additional dehalogenation experiments were performed to

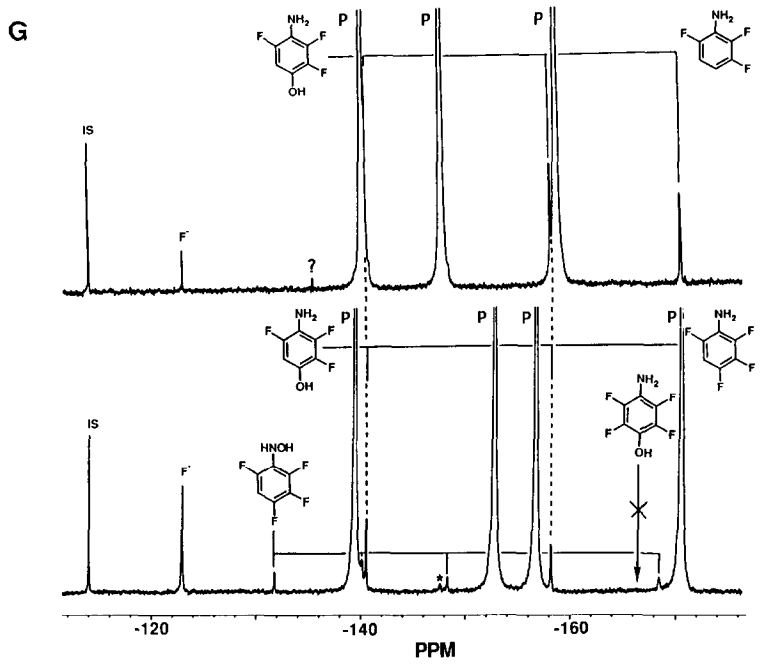

Fig. 3—*contd.*

Table 5. The cytochrome P450 catalysed biotransformation of a series of 2-fluoro-4-halogenated anilines to 3-fluoro-4-aminophenol

Compound	Apparent V_{max} (nmol 4-aminophenol/min/nmol cyt P450)	Relative apparent V_{max} as % of the non-C4 halogenated analogue
2-Fluoroaniline	6.23 ± 0.20	100.0
2,4-Difluoroaniline	2.99 ± 0.07	48.0
2-Fluoro-4-chloroaniline	0.29 ± 0.00	4.7
2-Fluoro-4-bromoaniline	0.27 ± 0.00	4.3
2-Fluoro-4-iodoaniline	0.29 ± 0.00	4.7

provide further evidence for the mechanism proceeding by elimination of the halogen as an anion. The effect of additional electron withdrawing fluorine substituents on the cytochrome P450 catalysed oxidative dehalogenation of 4-halogenated aniline-derivatives to 4-aminophenol metabolites was studied. The results obtained demonstrate that the presence of additional electron withdrawing substituents at the aromatic ring is another factor influencing dehalogenation. The introduction of additional fluorine substituents in a 4-halogenated aniline molecule lessens the possibility of its oxidative dehalogenation and, consequently, leads to a metabolic switch to fluorinated 2-aminophenol and *N*-hydroxyaniline metabolites. Formation of NIH-shifted metabolites was not observed. The decreasing effect of a fluorine substituent *ortho* with respect to the halogen to be eliminated is larger than that of an additional *meta* fluorine substituent. This observation further lends weight to a mechanism in which the fluorine elimination is dependent on (i.e. hampered by) additional electron withdrawing substituents in the aromatic ring and, thus, a mechanism proceeding by elimination of the halogen as anion.

Altogether, the results of the present paper demonstrate that the possibilities for the cytochrome P450 mediated oxidative dehalogenation of halogenated anilines do not depend exclusively on factors previously reported: i.e. the capacity of the amino moiety to lose a proton and donate electrons to create the quinoneimine primary metabolite as indicated by the mechanism depicted in Fig. 1 [9]. Additional factors influencing cytochrome P450 catalysed oxidative dehalogenation are: (i) the electronegativity of the halogen to be removed, the more electronegative fluorine being more easily eliminated than a chlorine, bromine or iodine; and (ii) the position, number and electron-withdrawing capacities of other substituents in the aromatic ring,

electron withdrawing substituents decreasing the relative rate of the reaction, with an *ortho* positioned electron withdrawing substituent being more efficient than one positioned *meta* with respect to the reaction centre.

Thus, the results of the present study demonstrate that for the dehalogenation of aromatic halogenated compounds the electronegativity of the halogen to be eliminated is more important than the bond energy of the carbon–halogen bond, known to follow the order fluoro > chloro > bromo > iodo. Surprisingly, this implies that the ease of oxidative dehalogenation of the aromatic 4-halogenated anilines follows the opposite order than that reported for dehalogenation of aliphatic halogenated hydrocarbons [3, 5, 11–13]. Previous studies on 2-fluoro- and 2-bromo-estradiol [22] and pentafluorochlorobenzene [10], suggest that for other aromatic halogenated compounds the order of elimination of the halogen substituents also decreases in the order fluorine > chlorine > bromine > iodine, and, thus, opposite the order for aliphatic halogenated hydrocarbons. This difference between aromatic and aliphatic dehalogenation might be due to a different reaction mechanism. The dehalogenation of aliphatic compounds by cytochrome P450 might proceed by one or two electron reductive pathways [3, 5, 50] or—as proposed for the conversion of dihalomethanes—by oxidative α-hydroxylation followed by the loss of a halogen as a result of nonenzymatic collapse of an unstable intermediate. In contrast, aromatic dehalogenation most likely proceeds by the mechanism for oxidative dehalogenation as presented in Fig. 1 [8, 9, 51].

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REFERENCES

1. Anders MW, Aliphatic halogenated hydrocarbons. In: *Metabolic Basis of Detoxication* (Ed. Jakoby), pp. 29–49. Academic Press, New York, U.S.A., 1982.
2. Wiersma DA, Schnellmann RG and Sipes IG, Pathways of halogenated hydrocarbon metabolism. In: *Foreign Compound Metabolism* (Eds. Caldwell J and Paulson GD), pp. 53–64. Taylor & Francis, New York, U.S.A., 1984.
3. Ahr HJ, King LJ, Nastainczyk W and Ullrich V, The mechanism of reductive dehalogenation of halothane by liver cytochrome P450. *Biochem Pharmacol* **31**: 383–390, 1982.
4. Schenk T, Müller R, Mörsberger F, Otto MK and Lingens F, Enzymatic dehalogenation of pentachlorophenol by extracts from *Arthrobacter* sp. strain ATCC 33790. *J Bacteriol* **171**: 5487–5491, 1989.
5. Castro CE, Yokoyama WH and Belser NO, Biodehalogenation; reductive reactivities of microbial and mammalian cytochrome P450 compared with heme and whole-cell models. *J Agric Food Chem* **36**: 915–919, 1988.
6. Uotila JS, Kitunen VH, Saastamoinen T, Coote T, Haggblom MM and Salkinoja-Salonen MS, Characterization of aromatic dehalogenases of mycobacterium fortuitum CG-2, *J Bacteriol* **174**: 5669–5675, 1992.
7. MacDonald TL, Chemical mechanisms of halocarbon metabolism. *CRC Crit Rev Toxicol* **11**: 85–120, 1983.
8. Rietjens IMCM and Vervoot J, Bioactivation of 4-fluorinated anilines to benzoquinoneimines as primary reaction products. *Chem–Biol Interact* **22**; 263–281, 1991.
9. Den Besten C, Van Bladeren PJ, Duizer E, Vervoort J and Rietjens IMCM, Cytochrome P450-mediated oxidation of pentafluorophenol to tetrafluorobenzoquinone as the primary reaction product. *Chem Res Toxicol* **6**: 674–680, 1993.
10. Rietjens IMCM and Vervoort J, A new hypothesis for the mechanism for cytochrome P450 dependent aerobic conversion of hexahalogenated benzenes to pentahalogenated phenols. *Chem Res Toxicol* **5**: 10–19, 1992.
11. Kubic VL and Anders MW, Metabolism of dihalomethanes to carbon monoxide. II. In vitro studies. *Drug Metab Disp* **3**: 104–112, 1975.
12. Ahmed AE and Anders MW, Metabolism of dihalomethanes to formaldehyde and inorganic halide. I. *In vitro* studies. *Drug Metab Disp* **4**: 357–361, 1976.
13. Stevens JL, Ratnayake JH and Anders MW, Metabolism of dihalomethanes to carbon monoxide. IV. Studies in isolated hepatocytes. *Toxicol Appl Pharmacol* **55**: 484–489, 1980.
14. Hecht SS, LaVoie EJ, Bedenko V, Pingaro L, Katayama S, Hoffmann D, Sardella DJ, Boger E and Lehr RE, Reduction of tumorigenicity and of dihydrodiol formation by fluorine substitution in the angular rings of dibenzo(*a,i*)pyrene. *Cancer Res* **41**: 4341–4345, 1981.
15. Hey MM, Haaf H, McLachlan JA and Metzler M, Indirect evidence for the metabolic dehalogenation of tetrafluorodiethylstilbestrol by rat and hamster liver and kidney microsomes. *Biochem Pharmacol* **35**: 2135–2139, 1986.
16. Liehr JG, Modulation of estrogen-induced carcinogenesis by chemical modifications. *Arch Toxicol* **55**: 119–122, 1984.
17. Liehr JG, 2-Fluoroestradiol: separation of estrogenicity from carcinogenicity. *Mol Pharmacol* **23**: 278–281, 1983.
18. Morgan P, Maggs JL, Page PCB and Park BK, Oxidative dehalogenation of 2-fluoro-17α-ethynyloestradiol *in vivo*. *Biochem Pharmacol* **44**: 1717–1724, 1992.
19. Oravec CT, Daniel FB and Wong LK, Comparative metabolism of 7,12-dimethylbenz[*a*]anthracene and its non-carcinogenic 2-fluoro analogue by Syrian hamster embryo cells. *Cancer Lett* **21**: 43–55, 1983.
20. Scribner JD, Scribner NK and Koponen G, Metabolism and nucleic acid binding of 7-fluoro-2-acetamidofluorene in rats: oxidative defluorination and apparent dissociation from hepatocarcinogenesis of 8-(*N*-arylamide)guanine adducts on DNA. *Chem–Biol Interact* **40**: 27–43, 1982.
21. Daly JW, Guroff G, Udenfriend S and Witkop B, Hydroxylation of alkyl and halogen substituted anilines and acetanilides by microsomal hydroxylases. *Biochem Pharmacol* **17**: 31–36, 1968.
22. Li JJ, Purdy RH, Appelman EH, Klicka JK and Li SA, Catechol formation of fluoro- and bromo-substituted estradiols by hamster liver microsomes. *Mol Pharmacol* **27**: 559–565, 1985.
23. Renson J and Bourdon V, Hydroxylation et défluorination des dérivés fluores de l'aniline. *Arch Int Pharmacodyn* **171**: 240–242, 1968.
24. Cnubben NHP, Vervoort J, Veeger C and Rietjens

IMCM, Study on the regioselectivity and mechanism of the aromatic hydroxylation of monofluoroanilines. *Chem–Biol Interact* **85**: 151–172, 1992.
25. Rietjens IMCM and Vervoort J, Microsomal metabolism of fluoroanilines. *Xenobiotica* **19**: 1297–1305, 1989.
26. Kennedy RJ and Stock AM, The oxidation of organic substances by potassium peroxymonosulfate. *J Org Chem* **25**: 1901–1906, 1960.
27. Vogel AI, In: *Vogel's Textbook of Practical Organic Chemistry* (5th edn) (Eds. Furbiss BS, Hannaford AJ, Smith PWG and Fatchell AR). John Wiley & Sons, New York, U.S.A., 1989.
28. Coleman GH, McCloskey CM and Stuart FA, Nitrosobenzene. *Organic Synthesis* **25**: 80–83, 1946.
29. Boyland E and Sims P, The oxidation of some aromatic amines with persulphate. *J Chem Soc* 980–985, 1954.
30. Cnubben NHP, Blaauboer B, Juyn S, Vervoort J and Rietjens IMCM, A spectrophotometric assay for the detection of 2-aminophenols in biological samples. *Anal Biochem* **220**: 165–171, 1994.
31. Cnubben NHP, Peelen S, Borst JW, Vervoort J, Veeger C and Rietjens IMCM, Molecular orbital based quantitative structure–activity relationship for the cytochrome P450 catalyzed 4-hydroxylation of halogenated anilines. *Chem Res Toxicol* **7**: 590–598, 1994.
32. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
33. Aron J, Baldwin DA, Marques HM, Pratt JM and Adams PA, Hemes and hemoproteins, 1: Preparation and analysis of the heme-containing octapeptide (microperoxidase 8) and identification of the monomeric form in aqueous solution, *J Inorg Biochem* **27**: 227–243, 1986.
34. Falk JE, In: *Porphyrins and Metalloporphyrins*, pp. 181, 240. Elsevier, Amsterdam, The Netherlands, 1964.
35. Boersma MG, Cnubben NHP, Van Berkel WJH, Blom M, Vervoort J and Rietjens IMCM, Role of cytochromes P-450 and flavin-containing monooxygenase in the biotransformation of 4-fluoro-*N*-methylaniline. *Drug Metab Dispos* **21**: 218–230, 1993.
36. Brodie BB and Axelrod J, The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl *p*-aminophenol and *p*-aminophenol (free and total conjugated) in biological fluids and tissues. *J Pharmacol Exp Ther* **94**: 22–28, 1948.
37. Corbett JF, Benzoquinone imines. Part VII. Mechanisms and kinetics of the reaction of *p*-benzoquinone monoamines with monohydric phenols. *J Chem Soc* 1503–1508, 1970.
38. Herr F and Kiese M, Bestimmung von nitrosobenzol im blute. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* **235**: 3510–3530, 1959.
39. Vervoort J, De Jager PA, Steenbergen J and Rietjens IMCM, Development of a ^{19}F-n.m.r. method for studies on the *in vivo* and *in vitro* metabolism of 2-fluoroaniline. *Xenobiotica* **20**: 657–670, 1990.
40. Rusvai E, Vègh M, Kramer M and Horváth I, Hydroxylation of aniline mediated by heme-bound oxy-radicals in a heme peptide model system. *Biochem Pharmacol* **37**: 4577–4581, 1988.
41. Nakamura S, Mashino T and Hirobe M, ^{18}O incorporation from $H_2{}^{18}O_2$ in the oxidation of *N*-methylcarbazole and sulphides catalyzed by microperoxidase-11. *Tetrahedron Lett* **33**: 5409–5412, 1992.
42. Ryan DE and Levin W, Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol Ther* **45**: 153–239, 1990.
43. Ortiz de Montellano PR, Oxygen activation and transfer. In: *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ed. Ortiz de Montellano PR), pp. 217–271. Plenum Press, New York, U.S.A., 1986.
44. Rietjens IMCM, Cnubben NHP, van Haandel M, Tyrakowska B, Soffers AEMF and Vervoort J, Different metabolic pathways of 2,5-difluoronitrobenzene and 2,5-difluoroaminobenzene compared to molecular orbital substrate characteristics. *Chem–Biol Interact* **94**: 49–72, 1995.
45. Wray V, Fluorine nuclei in difluorobenzene derivatives. In: *Annual Reports on NMR Spectroscopy* (Vol. 14) (Ed. Webb GA), pp. 273–281. Academic Press, 1983.
46. Ullrich V, Wolf J, Amadori E and Staudinger H, *Hoppe-Seyler's Z Physiol Chem* **349**: 85–94, 1968.
47. Guengerich FP, Oxidation of halogenated compounds by cytochrome P450 peroxidases and model metalloporphyrins. *J Biol Chem* **264**: 17198–17205, 1989.
48. Burka LT, Thorsen A and Guengerich FP, Enzymatic monooxygenation of halogen atoms: cytochrome P450 catalyzed oxidation of iodobenzene by iodosobenzene. *J Am Chem Soc* **102**: 7615–7616, 1980.
49. Van Ommen B and Van Bladeren PJ, Possible reactive intermediates in the oxidative biotransformation of hexachlorobenzene. *Drug Metab Drug Interact* **7** 214–243, 1989.
50. Nastainczyk W, Ahr HJ and Ullrich V, The reductive metabolism of halogenated alkanes by liver microsomal cytochrome P450. *Biochem Pharmacol* **31**: 391–396, 1982.
51. Rietjens IMCM, Tyrakowska B, Veeger C and Vervoort J, Reaction pathways for biodehalogenation of fluorinated anilines. *Eur J Biochem* **194**: 945–954, 1990.