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Oxidative and non-oxidative metabolism of 4-iodoanisole by rat liver microsomes

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1. The oxidative metabolism of 4-iodoanisole (**1**) by liver microsomes from β-naphthoflavone-treated rats yields 4-iodophenol (**2**) 2-iodo-5-methoxyphenol (**3**), 2-methoxy-5-iodophenol (**4**), 4-methoxyphenol (**5**), and 3-methoxyphenol (**6**) in relative yields of 5:2:4:1:1 respectively.

2. [3 5-$^2H_2$]-**1** was converted to the same five metabolites in the same proportions; formation of **2**, **4** and **5** involved no loss of deuterium, but formation of **3** and **6** involved respectively 55 and 28% loss of one deuterium.

3. When metabolism of **1** was carried out in buffers containing D_2O or $H_2{}^{18}O$, no incorporation of these isotopes into **2–6** could be detected. Nor was it possible to detect formation of iodinating intermediates derived from **1** by trapping with 2,6-dimethylphenol.

4. The P450-catalysed hydroxylative de-iodination of **1–5** and **6** is suggested to involve C–O bond formation via attack of the ferryl moiety on the aromatic ring followed by reductive cleavage of the C–iodine bond, with electrons coming from P450 reductase.

Introduction

Aromatic hydroxylation is a major pathway in the biotransformation of numerous drugs and other xenobiotics, and its mechanism has been studied extensively (Guroff *et al.* 1967, Daly *et al.* 1972, Tomaszewski *et al.* 1975, Hanzlik *et al.* 1984, Korzekwa *et al.* 1985, 1989, Fitzpatrick 1994. In general, this reaction can be viewed formally as an electrophilic substitution of HO^+ for H^+, and whereas the regioselectivity of many aromatic hydroxylations is in accord with this view, many other mechanistic details are not, most notably the NIH-shift phenomenon (Guroff *et al.* 1967, Daly *et al.* 1972). In the NIH-shift process a substituent, usually deuterium or tritium but occasionally a chlorine or methyl group, migrates from the site of hydroxylation to the adjacent (*ortho*) carbon. A mechanism involving a rearranged cyclohexadienone intermediate, possibly but not necessarily formed from an arene oxide intermediate, has been proposed to account for NIH-shift phenomena. Occasionally certain aromatic substituents including ^2H-, ^3H- and halogens may be lost completely (i.e. replaced by OH), with no sign of an NIH-shift during aromatic hydroxylation; this has been defined as 'direct' aromatic hydroxylation (Hanzlik *et al.* 1984). All three of these processes are exemplified in the metabolism of 2,4,5,2′,4′,5′-hexachlorobiphenyl of dog liver microsomes (figure 1) (Ariyoshi *et al.* 1992).

In an early study of aromatic hydroxylation by liver microsomes from rats induced with 3-methylcholanthrene, Daly (1970) reported an unusual loss of an iodo substituent from a carbon adjacent to rather than at the site of hydroxylation (*viz.* conversion of **1** to **6** in figure 2). Because of the novelty of this reaction, which

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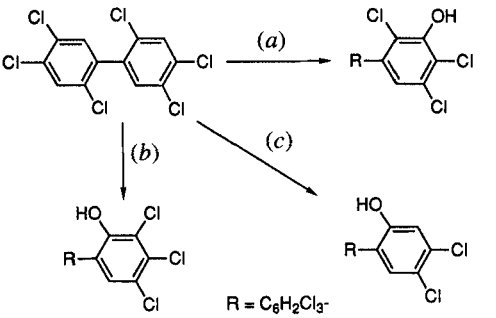

Figure 1. Direct aromatic hydroxylation (*a*), NIH-shift hydroxylation (*b*), and hydroxylative dehalogenation (*c*) in the metabolism 2,4,5,2′,4′,5′-hexachlorobiphenyl by dog liver microsomes.

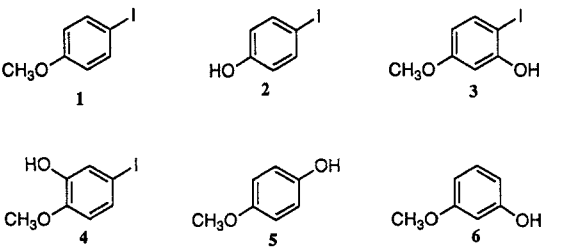

Figure 2. Structures of 4-iodoanisole (**1**) and its metabolites (**2–6**).

to our knowledge is unique, and because of the continued strong interest in mechanisms of aromatic hydroxylation, we reinvestigated the metabolism of 4-iodoanisole using a deuterium- and ^{18}O isotopic labelling approach. In this work we confirm that 3-hydroxyanisole is indeed a true metabolite of 4-iodoanisole, and propose a mechanism for its formation.

Materials and methods

Chemicals

4-Iodoanisole, 4-iodophenol, 3- and 4-methoxyphenol, 2,6-dimethylphenol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were obtained from commercial sources. 4-Chloro-2,6-dimethylphenol was synthesized as described by Mico *et al.* (1981). 4-Iodo-2,6-dimethylphenol was synthesized by treatment of 2,6-dimethylphenol in phosphate buffer (0·1 M, pH 7·4) containing 15% ethanol (v/v) with 1 equivalent of I_2 at room temperature overnight; the product was extracted with hexane (80% yield). The identity and purity ($\geq 95\%$) of these phenols was confirmed by GC-MS analysis (see below).

[3,5-$^2H_2$]-4-iodoanisole

p-Anisidine hydrochloride (1 g) was dissolved in D_2O (15 ml), heated to reflux for 48 h, cooled and lyophilized; this sequence was repeated twice to exchange the ring protons *ortho* to the amine group. Examination of the *p*-anisidine recovered by acid–base extraction by ^1H-nmr (in $CDCl_3$) showed $\delta = 3{\cdot}75$ (s, 3H), 6·74 (s, 2H) and a very small peak at 6·68 ppm representing residual unexchanged hydrogens *ortho* to the amine group. The deuterated *p*-anisidine (0·5 g, 4 mmol) was dissolved in 1·5 ml 6 N HCl, cooled to 0–3°C, and a cold solution of $NaNO_2$ (265 mg, 4·2 mmol) in 5 ml water was added dropwise. After stirring for 10 min, 60 mg urea was added, followed after 10 min by a cold solution of KI (665 mg, 0·4 mol) in 5 ml water. After stirring overnight at room temperature the reaction mixture, which was very dark and consisted of two phases, was partitioned between CH_2Cl_2 and water containing enough NaOH to make the pH alkaline and enough $NaHSO_3$ to reduce the small amount of iodine formed by air oxidation of the excess iodide present. Chromatography of the CH_2Cl_2-soluble material over silica gel yielded 274 mg (30%) of **1**-d_2 as a white crystalline solid. GC-MS analysis (see below) indicated a deuterium content of 83 mol% d_2, 14 mol% d_1 and 3 mol% d_0.

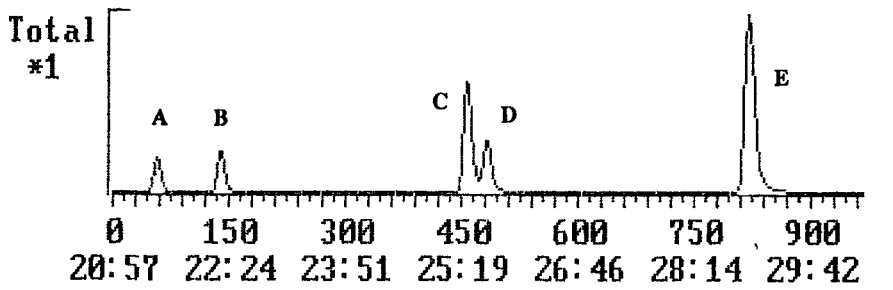

Figure 3. Total ion chromotogram of 4-iodoanisole metabolites produced by BNF-induced rat liver microsomes. The ordinate represents ion current current in arbitrary units, and the abscissa shows scan numbers and retention times in minutes and seconds. Peaks A–E are identified in the text. The chromatographic separation employed a DBwax capillary column (0·25 mm × 30 m, J.&W. Scientific, Folson, CA, USA) with oven temperature programmed from 120 to 250°C at a rate of 5°C/min. Scans numbered 0–900 correspond to the retention times shown. The substrate 4-iodoanisole had a retention time of 14·4 min in this system.

Biochemical procedures

Procedures for preparation of microsomes, conduct of incubations and metabolite isolation and analysis were as described previously (Narasimhan *et al.* 1988, Riley and Hanzlik 1994). Briefly, liver microsomes were prepared from male Sprague–Dawley rats (*c.* 200 g) pretreated with sodium phenobarbital (PB; 50 mg/kg × 3 days, i.p. in saline) or β-naphthoflavone (BNF; 50 mg/kg × 4 days; i.p. in corn oil). Typical incubations contained microsomes (10 mg protein in 5 ml buffer), NADPH-regenerating system (consisting of 4·1 mg $NADP^+$, 14·1 mg glucose 6-phosphate and 5 IU glucose 6-phosphate dehydrogenase in 0·5 ml buffer) and substrate (10 μmol in 10 μl acetonitrile solvent) giving a total volume of 5·51 ml. After incubating for 60 min at 37°C, a saturating amount of solid NaCl was added and the products were extracted using ether/pentane (1:4; 4 × 1·5 ml). The extracts were dried over anhydrous Na_2SO_4, carefully evaporated to dryness (≤ 40°C, 25–40 Torr), and the residues redissolved in CH_2Cl_2 for analysis by GC or GC-MS (Riley and Hanzlik 1994). Deuterium analyses by GC-MS were carried out as described (Hanzlik and Ling 1993). A typical GC-MS profile of the metabolites of 4-iodoanisole is shown in figure 3. For incubations in buffers containing D_2O or $H_2{}^{18}O$, an appropriate aliquot of normal buffer was lyophilized and reconstituted in water enriched in the desired isotope, allowing for modest further dilution by the ordinary water in which the concentrated microsomal suspension was prepared.

Attempts to trap iodinating intermediates arising during microsomal metabolism of 4-iodoanisole

Because one potential mechanism for formation of **5** from **1**, and conceivably **6** from **1**, involves loss of iodine as 'I^+' (or HOI), we employed 2,6-dimethylphenol (DMP) as a scavenger for trapping active halogenating intermediates. This work was patterned after the work of Mico *et al.* (1981) who used DMP to trap electrophilic-chlorinating intermediates produced during oxidative metabolism of CCl_4 by rat liver microsomes. Thus DMP (1 mM) was added to standard incubations of **1** with BNF-induced microsomes, and the incubations carried out and worked up in the usual way. In addition two important control incubations were also run. In one, Chloramine-T (0·5 μmol) was added to an incubation system containing **1** (20 μmol), DMP (10 μmol), KI (0·5 μmol), microsomal protein (20 mg) and an NADPH-generating system in an 11 ml reaction volume; the mixture was incubated and extracted as usual, and the extract analysed for 4-iodo-2,6-dimethylphenol by GC-MS. In the other control, CCl_4 (5 mM) and DMP (1 mM) were incubated with PB and BNF-induced microsomes as described for **1**, and the metabolites extracted and analysed by GC-MS.

Results

4-Iodoanisole (**1**) was observed to be a good substrate for metabolism by microsomes from rats induced with β-naphthoflavone, and five metabolites were produced (figure 3). Metabolite peaks labelled A, B and E were identified as compounds **5**, **6**, and **2** respectively, based on GC-MS comparison with authentic standards. Peaks C and D showed molecular ions at *m/z* 250, corresponding to mono-oxygenation of **1**. Specific assignments of peak C as **4** and peak D as **3** were based on differences in retention versus loss of deuterium when [3,5-$^2H_2$]-**1** was used

Table 1. Retention of deuterium during microsomal metabolism of [3,5-$^2H_2$]-4-iodoanisole.

	Deuterium content (mol%)					
	Substrate	Metabolites				
Species	1	2	3	4	5	6
d_0	3	2	3	2	2	3
d_1	14	14	60	16	14	37
d_2	83	84	37	82	84	60

(see below). However, the relative yields of these metabolites as suggested by their respective TIC peak areas (figure 3) were quite consistent, and GC analysis using a flame ionization detector instead of a mass selective detector gave a similar pattern of metabolite peak areas (data not shown). These observations confirm the metabolite identifications orginally reported by Daly, including that of the novel metabolite **6**.

To gain insight into the mechanism of formation of these metabolites, and especially that of **6**, [3,5-$^2H_2$]-**1** was incubated with BNF-induced rat liver microsomes. There was no apparent change in the relative yields of the five metabolites, but there were pronounced differences in their retention or loss of deuterium, as shown by the data in table 1. As expected, no deuterium was lost in the *O*-demethylation of **1** to **2**, in the hydroxylative de-iodination of **1** to **5**, or in the formation of the mono-oxygenation product represented in peak D. In contrast, the formation of the mono-oxyenation product represented in peak C involved 55% loss of one deuterium, implying that C is formed by hydroxylation at a deuterium-bearing site in **1**. Since C and D are isomers, they are assigned structures **3** and **4** respectively. Finally, formation of the unique product **6** also involved the partial (28%) loss of one deuterium.

When the metabolism of **1**-d_0 was carried out in buffers containing D_2O (*c.* 80 mol%), no deuterium was detected in any of the metabolites. Similarly, when the metabolism of **1** was carried out in buffers containing $H_2{}^{18}O$ (20 mol%), no excess ^{18}O was detected in any of the metabolites.

When DMP was included in incubation of **1** with BNF-induced microsomes, formation of 4-iodo-DMP could not be detected, even using selected ion monitoring to enhance sensitivity. As a positive control, when DMP-containing incubation mixtures were supplemented with Chloramine-T and KI (0·025 equivalents of each with respect to **1**) to generate a positive iodinating species *in situ*, 4-iodo-DMP was readily detected by GC-MS. Similarly, when DMP and CCl_4 were incubated with PB-induced microsomes as described by Mico and Pohl, 4-chloro-DMP ws formed; however, it was not formed if BNF-induced microsomes were used.

Discussion

The substrate 4-iodoanisole offers six potential sites for attack by P450 enzymes, i.e. the methyl group, C(1), C(2/6), C(3/5), C(4) and the iodine atom itself. *O*-dealkylation of aryl methyl ethers is a very common metabolic process, and we assume that formation of *p*-iodophenol (**2**) occurs by the typical mechanism of C-hydroxylation on the methyl group (Ohe *et al.* 1994). All other metabolites of **1** involve the introduction of an OH group on to the aromatic ring.

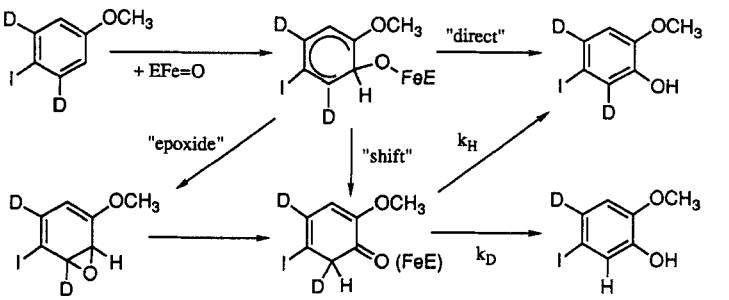

Figure 4. Proposed mechanism for 2-hydroxylation of 4-iodoanisole. EFe = O represents the ferryl form of P450.

The 2-hydroxylation of 4-iodoanisole occurs with no effect on deuterium present at the adjacent C(3) position. Aromatic hydroxylation by P450 enzymes is generally considered to involve mechanisms such as those shown in figure 4 (Tomaszewski *et al.* 1975, Hanzlik *et al.* 1984). If an epoxide or cyclohexadienone intermediate were involved in the 2-hydroxylation of 4-iodoanisole, an easily detectable loss of *c.* 20% of one deuterium atom would have been expected, based on an isotope effect (k_H/k_D) of 4 as observed for related processes (Hanzlik *et al.* 1984, Hinson *et al.* 1985). Since no loss of deuterium is observed, the formation of **4** must occur almost entirely by 'direct' aromatic hydroxylation with no arene oxide or cyclohexadienone intermediate, i.e. with no NIH-shift of H from C(2) to C(3).

It is known that P450 can oxygenate organic iodides to iodosyl compounds (R–I = O) (Guengerich 1989), but, in contrast with aliphatic iodoso compounds (Macdonald 1982), aromatic iodoso compounds do not undergo carbon-iodine bond cleavage reactions, presumably because the formation of a phenyl-type cation is extremely unfavorable energetically. This, plus the observation that no ^{18}O is incorporated into 4-methoxyphenol (**5**) from water, implies that its phenolic oxygen originated from molecular oxygen via the ferryl group of P450, which is known not to be susceptible to exchange with water (Blake and Coon 1989).

It is important to note here that formally, the conversion of **1** to **5** is not an oxidative process unless the iodine is lost as iodonium ion (I^+) or its equivalent (figure 5, path a). For the iodine to be lost as iodide ion (I^-) would require a

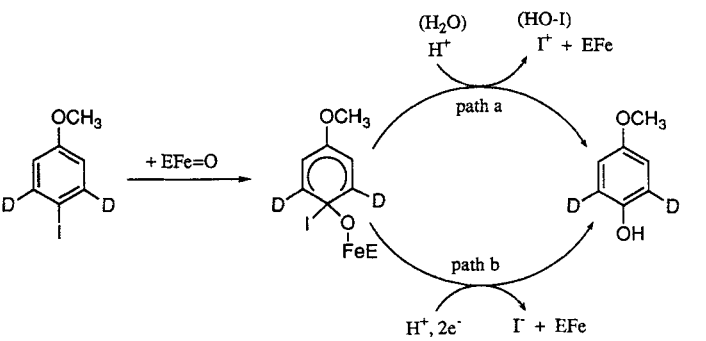

Figure 5. Proposed mechanism for 4-hydroxylation of 4-iodoanisole. EFe = O represents the ferryl of P450.

nucleophilic displacement by water or hydroxide. The metabolic formation of 4-hydroxybenzoic acid from 4-chlorobenzoic acid (via its coenzyme A ester) is an example of such a substitution process (Groenewegen *et al.* 1992). Here the driving force for substitution of chlorine by hydroxide (delivered via an enzymatic glutamate side chain) is the electron-withdrawing effect on the thioester group, which activates the ring for nucleophilic attack. The methoxy group in **1**, however, has the opposite electronic effect, which predisposes the molecule to electrophilic rather than nucleophilic attack.

We were unable to detect the formation of iodinating intermediates during the metabolism of **1**, despite success with such trapping in positive control reactions. Similarly, it was reportedly not possible to trap chlorinating intermediates during the microsomal metabolism of pentachlorophenol to tetrachlorohydroquinone (den Besten *et al.* 1991). We therefore presume that during the formation of **5** from **1**, iodine is lost as iodide and not as iodonium as suggested in figure 5, path a. This in turn requires the input of two additional electrons, relative to the normal mono-oxygenase stoichiometry, in the overall process. We propose that this could occur as shown in figure 5, path b, with the two electrons coming from NADPH via P450 reductase. Since P450 quite effectively transfers electrons from its reductase to many other substrates having high enough oxidation potentials (Hanzlik 1981, Goeptar *et al.* 1992, Zbaida *et al.* 1994), it is not unreasonable to expect that P450 reaction intermediates might also accept electrons in this fashion. This mechanism (i.e. figure 5, path b) can also explain the hydroxylative (but non-oxidative) replacement of bromine (Zheng and Hanzlik 1992), chlorine (den Besten *et al.* 1991, Ariyoshi *et al.* 1992) and even fluorine (Daly 1970, Sullivan and Franklin 1985, Rietjens and Vervoort 1989, 1992, Rietjens *et al.* 1993) from aromatic rings. These latter halogens are even less likely than iodine to be displaced as halonium ions in a direct electrophilic substitution process.

Two different 3-hydroxylation products are formed from 4-iodoanisole, one in which iodine is retained (**3**) and one in which it is lost (**6**). In the formation of **3** only about half of the deuterium present at C(3) is lost, implying a substantial degree of NIH-shift of D from C(3) to C(2), where much of it is retained due to preferential loss of hydrogen from C(2) of the cyclohexadienone intermediate (figure 6). In addition, **3**-d_1 can also arise via 'direct' aromatic hydroxylation or via the '3,4-shift' routes as shown in figure 6. Most interesting is the very high retention of deuterium from C(3) in the formation of **6**. We propose that this retention occurs as shown in figure 6, path b, via an NIH-shift of D from C(3) to C(4) followed by reductive cleavage of the carbon–iodine bond on analogy to the reduction depicted in figure 5, path b. The fact that retention of deuterium in **6** is not complete suggested that there might have been some deuterium exchange with solvent water at some point in the process. However, this seems unlikely because no deuterium was incorporated into **6** (or any other metabolite) when incubations of undeuterated substrate were run in D_2O. The apparent partial loss of deuterium in **6** is somewhat larger than can reasonably be attributed to experimental error, but the fact that **6** is a significant metabolite, together with its overall high degree of deuterium retention (72%), seem best explained by the mechanisms shown in figure 6.

In conclusion the hydroxylative dehalogenation of aryl halides requires oxidative attack by the ferryl moiety of P450 leading to C–O bond formation, but the C–halogen bond is apparently cleaved reductively; the required electrons are suggested to be provided by NADPH P450 reductase.

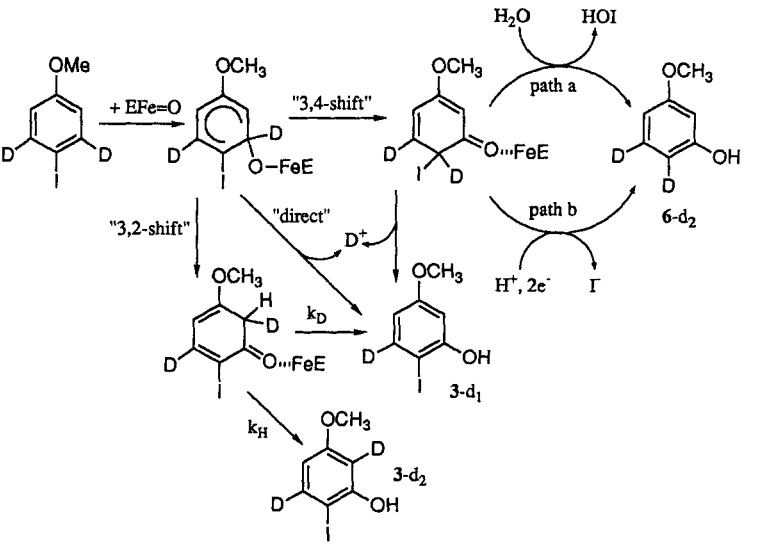

Figure 6. Proposed mechanisms for 3-hydroxylation of 4-iodoanisole, with and without retention of iodine. EFe = O represents the ferryl form of P450.

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