Regioselectivity of Cytochrome P-450 Catalyzed Hydroxylation of Fluorobenzenes Predicted by Calculated Frontier Orbital Substrate Characteristics

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ABSTRACT: In the present study, a hypothesis is presented for the prediction of the regioselectivity of cytochrome P-450 catalyzed hydroxylation of fluorobenzenes. The regioselectivity of the in vivo hydroxylation of fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene could be predicted within 6% accuracy on the basis of the substrate's frontier orbital characteristics for electrophilic attack. The in vivo regioselectivity of the hydroxylation of fluorobenzene was not significantly influenced by changes in the cytochrome P-450 enzyme pattern. This implies that the regioselectivity is not predominantly determined by the juxtaposition of the relatively small substrates in the active sites of the cytochrome P-450s catalyzing the reaction. Additional in vitro experiments using 1,2-difluorobenzene as the model substrate demonstrated that minor factors influencing the regioselectivity and possibly responsible for the 6% deviation from the calculated values in in vivo experiments might be (i) the influence of biotransformation routes occurring in vivo but not of importance in in vitro microsomal incubations and (ii) a small variation due to influences of the contribution of various cytochrome P-450 enzymes. On the basis of the results obtained, it is concluded that the aromatic hydroxylation of fluorobenzenes proceeds through an initial electrophilic attack of $(FeO)^{3+}$ on the aromatic substrate, and not through initial electron abstraction followed by attack of the $(FeO)^{2+}$ species on the substrate radical cation. The fact that the regioselectivity observed could be predicted and/or explained by the site of initial $(FeO)^{3+}$ attack also argues against epoxides as important intermediates in the formation of phenol metabolites from fluorobenzenes.

It has been proposed that the bioconversion of benzenes to toxic intermediates proceeds through formation of epoxide intermediates, ultimately giving rise to hydroxylated metabolites or mercapturic acids, and/or through formation of quinones, resulting from additional cytochrome P-450 catalyzed hydroxylation of the phenol metabolites (Brodie et al., 1971; Mitchell et al., 1971; Reid & Krishna, 1973; Hesse et al. 1980; Narashimhan et al., 1988; Buben et al., 1988; Slaughter & Hanzlik, 1991; Den Besten et al., 1991). For substituted benzenes, multiple sites for hydroxylation are present in the molecule, and hydroxylation at different sites in the molecule can result in different intermediates and metabolites. Because the various intermediates and hydroxylated metabolites formed out of a given substituted benzene may vary in their reactivities and toxic characteristics, the regioselectivity of the cytochrome P-450 dependent hydroxylation of a substituted benzene is of importance for its toxic implications. The best known examples with respect to the consequence of regioselectivity of the aromatic hydroxylation for the ultimate toxic effect of a benzene derivative are the studies on bromobenzene. For bromobenzene the route leading to 4-hydroxylation has been linked to its toxic effects, whereas it is suggested that the route leading to 2-hydroxybenzene is not linked to toxicity (Zampaglione et al., 1973; Jollow et al., 1974; Lau et al., 1980).

In the present study, an attempt was made to predict the possible regioselectivity of the aromatic hydroxylation of a series of fluorobenzenes on the basis of molecular orbital calculations in relation to the possible mechanisms suggested in the literature for the cytochrome P-450 catalyzed aromatic hydroxylation. From the literature it emerges that the mechanism for introduction of a hydroxyl group into an aromatic benzene by cytochrome P-450s is still a matter of considerable research and debate (Tomaszewski et al., 1975; Burka et al., 1983; Korzekwa et al., 1985, 1989; Ortiz de Montellano, 1986; Guengerich & MacDonald, 1990; Cnubben et al., 1992). Figure 1 presents schematically the most likely reaction pathways suggested. The first possibility is that the reaction proceeds by an initial attack of the cytochrome P-450 $(FeO)^{3+}$ species on the π-system of the aromatic ring (pathway I, Figure 1). This attack results in the formation of a so-called radical σ-complex (Burka et al., 1983; Korzekwa et al., 1989; Guengerich & MacDonald, 1990) (Figure 1). Electron abstraction by the iron atom from the substrate molecule in this radical σ-complex results in the formation of a cationic σ-complex (Korzekwa et al., 1989; Guengerich & MacDonald, 1990) (Figure 1). Alternatively, it has been proposed that the hydroxylation pathway proceeds via the initial abstraction by $(FeO)^{3+}$ of an electron from the substrate molecule (pathway II, Figure 1), followed by addition of the $(FeO)^{2+}$ to the substrate cation radical (Burka et al., 1983; Guengerich & MacDonald, 1990). This reaction sequence also results in the formation of the cationic σ-complex.

For further reaction from either the radical σ-complex or the cationic σ-complex, a number of reaction pathways have been proposed (Tomaszewski et al., 1975; Burka et al., 1983; Korzekwa et al., 1985, 1989; Ortiz de Montellano, 1986; Guengerich & MacDonald 1990) (Figure 1): (i) direct formation of the hydroxylated species; (ii) formation of a keto product that rearranges to give the hydroxylated benzene; or (iii) formation of an epoxide followed by rearrangement of the epoxide to give either the hydroxylated benzene or the keto product that rearranges to the hydroxylated benzene. Following the first two pathways, an epoxide intermediate would not be involved in the formation of the phenol metabolite, and the initial site of $(FeO)^{3+}$ or $(FeO)^{2+}$ attack would be the site of hydroxylation. However, when an epoxide intermediate

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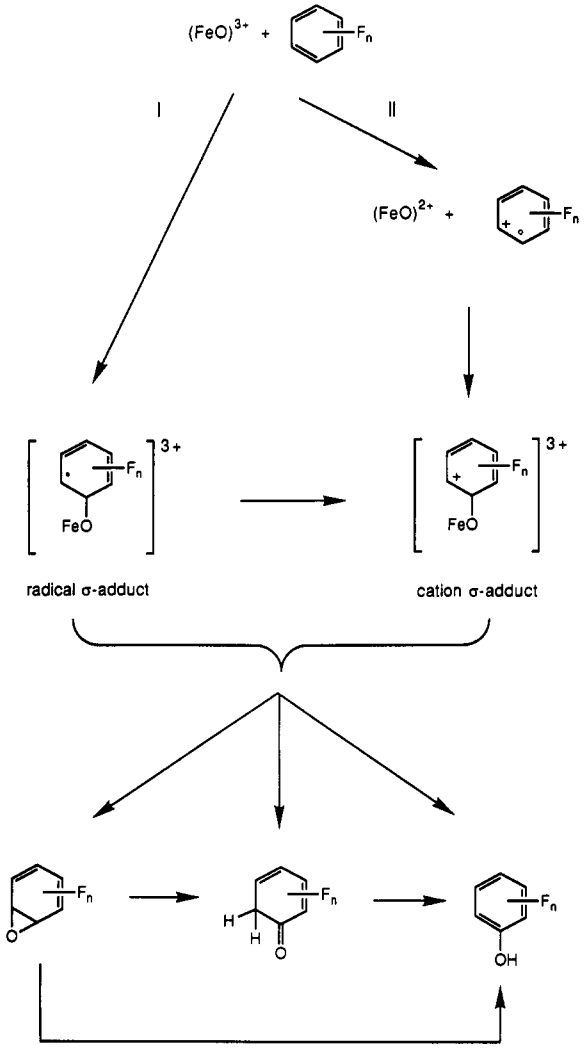

FIGURE 1: Hypotheses for the reaction pathway of the cytochrome P-450 catalyzed aromatic hydroxylation of fluorobenzenes. The haem cofactor in the active site of the cytochrome P-450 is presented as $(FeO)^{3+}$ (Ortiz de Montellano, 1986; Guengerich & MacDonald, 1990; Tomaszewski et al., 1975; Burka et al., 1983; Korzekwa et al., 1985, 1989).

is involved as a prerequisite for formation of the hydroxylated species, regioselectivities will be influenced not only by the initial site of attack but also by the extent to which the FeO–benzene adduct rearranges to either one of the two possible epoxides and the extent to which each epoxide rearranges to either one of the two possible phenols. The 2,3- and 3,4-oxides from chlorobenzene, for example, have been demonstrated to give rise to preferential formation of 2-chlorophenol and 4-chlorophenol, respectively (Selander et al., 1975a). As a consequence, the formation of 3-chlorophenol is supposed to originate from a reaction pathway without an epoxide intermediate, but with formation of the keto intermediate or direct formation of the phenol (Korzekwa et al., 1989).

In a recent study on the regioselectivity of cytochrome P-450 dependent monofluoroaniline hydroxylation (Cnubben et al., 1992), we put forward the hypothesis that the regioselectivity of the cytochrome P-450 dependent hydroxylation might be directed by the position of the frontier substrate electrons which will direct the site of initial electrophilic attack of the $(FeO)^{3+}$ on the substrate molecule. This implies that the aromatic hydroxylation is postulated to proceed by an initial interaction of the electron in the cytochrome P-450 $(FeO)^{3+}$ singly occupied molecular orbital (SOMO) [i.e., its d_{xz}–p_x or d_{yx}–p_y molecular orbital (Ortiz de Montellano, 1986)] with the frontier electrons of the benzene substrate (pathway I, Figure 1). Consequently, the frontier orbital electron distribution of the fluorobenzenes may direct and predict the regioselectivity of their aromatic hydroxylation by the cytochrome P-450s. However, when the reaction proceeds by initial electron abstraction (pathway II, Figure 1), the interaction between the $(FeO)^{2+}$ species and the singly occupied molecular orbital (SOMO) of the substrate cation radical may direct the site for attack and thus the site of hydroxylation. Finally, when epoxide intermediates determine the reaction pathway, the initial site of attack is unlikely to correlate with observed regioselectivities. In the present study, these hypotheses were tested in further detail using fluorinated benzenes as the model substrates.

It should be stressed that these working hypotheses imply that the hydrophobic active site pocket of the various cytochrome P-450s is not a major factor influencing the regioselectivity of the aromatic hydroxylation. For the monofluoroaniline molecules this proved to be the case (Cnubben et al., 1992). In the present study, fluorinated benzenes were chosen as the model substrates. This was done for several reasons. First, fluorinated benzenes are relatively small molecules, which may prevent them from regioselective binding in the hydrophobic active sites of the cytochrome P-450s. For bromo- and chlorobenzene(s), however, with relatively larger halo substituents, regioselectivity previously has been demonstrated to be dependent on the cytochrome P-450 enzyme pattern (Zampaglione et al., 1973; Selander et al., 1975b; Lau et al., 1980, Den Besten et al., 1991). Therefore, the influence of changes in the cytochrome P-450 enzyme pattern on the regioselectivity of the aromatic fluorobenzene hydroxylation was also investigated in the present study.

Furthermore, the van der Waals radius of a fluorine substituent is known to be almost similar to that of a hydrogen substituent. This eliminates the significant influence of sterical hindrance by the fluorine substituents on the regioselectivity of the aromatic hydroxylation. In addition, the fluorine substituent(s) will significantly influence the frontier orbital distributions of the benzene molecules, resulting in asymmetric frontier density distributions. Finally, the fluorine substituents make it possible to study the regioselectivity of aromatic hydroxylation using ^{19}F NMR spectroscopy. This implies that all isomeric hydroxylated products can be not only identified but also quantified by the same technique and in a single run (Vervoort et al., 1990; Rietjens & Vervoort, 1989, 1992).

MATERIALS AND METHODS

Chemicals. Fluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene were purchased from Aldrich Chemie (Steinheim, FRG). 1,2-Difluorobenzene and 1,3-difluorobenzene were obtained from Fluorochem (Derbyshire, UK). 2-Fluorophenol, 3-fluorophenol, and 4-fluorophenol were purchased from Janssen Chimica (Beerse, Belgium). 3,4-Difluorophenol, 2,4-difluorophenol, and 2,5-difluorophenol were obtained from Aldrich (Steinheim, FRG). 2,3-Difluorophenol, 2,6-difluorophenol, 3,5-difluorophenol, 2,3,4-trifluorophenol, 2,3,6-trifluorophenol, and 2,3,5-trifluorophenol were all purchased from Fluorochem (Derbyshire, UK).

In Vivo Exposure of Rats. Male Wistar rats (400 g) were exposed to 200 μmol of the desired fluorinated benzene or phenol, administered in olive oil by oral injection. After dosing, 24-h urine samples were collected. The 24-h urinary recovery was over 50% of the dose administered for all fluorobenzenes.

Analysis of Urine Samples. Urine samples were analyzed by ^{19}F NMR after 1:1 dilution in 0.2 M potassium phosphate (pH 7.6). Enzyme hydrolysis of urine samples was carried out as described previously (Rietjens & Vervoort, 1992) using either β-glucuronidase from *Escherichia coli* K12 (Boehringer, Mannheim, Germany) or arylsulfatase/β-glucuronidase from *Helix pomatia* (Boehringer, Mannheim, Germany). Samples were routinely made oxygen free by four cycles of evacuation and filling with argon.

^{19}F NMR Measurements. ^{19}F NMR measurements were performed on a Bruker AMX 300 spectrometer as described previously (Vervoort et al., 1990; Rietjens & Vervoort, 1989, 1992). Between 1500 and 50 000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal to noise ratio required. The sample volume was 1.7 mL, containing 100 μL of ^2H_2O for locking the magnetic field and 10 μL of a 8.4 mM 4-fluorobenzoic acid solution added as internal standard. For determination of the 24-h urinary recovery of 1,3-trifluorobenzene and 1,2,4-trifluorobenzene, 2,3,5,6-tetrafluorophenol (Fluorochem) was used as the internal standard because the ^{19}F NMR resonance of 4-fluorobenzoic acid overlapped with the ^{19}F NMR signal of one of the urinary metabolites.

The ethyl acetate extracts from the microsomal incubations were analyzed using an insert containing a known amount of the internal standard dissolved in ^2H_2O, instead of adding ^2H_2O and the internal standard to the 1.6-mL ethyl acetate extract in the NMR tube.

Chemical shifts are reported relative to $CFCl_3$. Concentrations of the various metabolites could be calculated by comparison of the integrals of the ^{19}F NMR resonances of the metabolites to the integral of the ^{19}F NMR resonance of 4-fluorobenzoic acid, added as an internal standard.

Preparation of Microsomes. Microsomes were prepared from the perfused livers of male Wistar rats (400 g) either untreated or treated with various cytochrome P-450 inducers as described previously (Rietjens & Vervoort, 1990). The cytochrome P-450 content of the microsomes was measured as described by Omura and Sato (1964). Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Ethoxyresorufin O-dealkylating activity and pentoxyresorufin O-dealkylating activity were measured as previously described (Balvers et al., 1992).

In Vitro Incubations. Cytochrome P-450 dependent conversion was studied in vitro in microsomal incubations containing (final concentrations) 0.1 M potassium phosphate (pH 7.6), 2 μM microsomal cytochrome P-450, 1 mM of the benzene added as 1% (v/v) of a 0.1 M cold stock solution in acetone, 1 mM ascorbic acid to prevent autoxidation of the hydroxylated reaction products, and 1.0 mM NADPH or 2.5 mM *tert*-butyl hydroperoxide as the electron and/or oxygen source. The reaction was started by the addition of the benzene and the NADPH or the *tert*-butyl hydroperoxide and was carried out at 37 °C for 10 min. The total reaction volume was 20 mL, and the reaction was carried out in a closed reaction vessel to prevent evaporation of the substrate. The reaction was terminated by the addition of 1 mL of 12 N HCl and 5 mL of ethyl acetate. Upon mixing and centrifugation (10 min, 3000 rpm), the ethyl acetate layer was collected, and 1.6 mL of the ethyl acetate extract was analyzed by ^{19}F NMR as described above.

Purification of Phenol Hydroxylase from Trichosporon cutaneum. Phenol hydroxylase was isolated from the yeast *Trichosporon cutaneum* essentially as described by Sejlitz and Neujahr (1987). The final preparation had a specific activity of 5 units/mg of protein.

Incubations with Isolated Phenol Hydroxylase. Phenol hydroxylase incubations were carried out in closed reaction vessels to prevent evaporation of the phenolic substrate and contained (final concentrations) 50 mM potassium phosphate (pH 7.6), 1 mM fluorophenol (added from a 200 mM stock solution in water), 0.1 μM phenol hydroxylase, 0.1 mM EDTA, 10 μM flavin adenine dinucleotide (FAD), and 1 mM ascorbic acid to prevent autoxidation of the fluorodihydroxybenzenes formed. The incubations were started by the addition of 0.17 mM (final concentration) NADPH. Reactions were carried out at 25 °C for 15 min and terminated by freezing the samples in liquid nitrogen.

Molecular Orbital Calculations. Molecular orbital calculations were carried out on a Silicon Graphics Iris 4D/85 workstation using Quanta/Charmm (Molecular Simulations, Cambridge, UK). The semi-empirical molecular orbital method was used, applying the AM1, MNDO, or MINDO/3 Hamiltonian from the AMPAC program. All calculations were carried out with PRECISE criteria. For all calculations the self-consistent field was achieved. Geometries were optimized for all bond lengths, bond angles, and torsion angles using the Fletcher–Powell criteria. Frontier electron densities were calculated from HOMO (highest occupied molecular orbital) and HOMO–1 characteristics and from LUMO (lowest unoccupied molecular orbital) and LUMO+1 characteristics using the equation given by Fukui et al. (1954). In this study, the outcomes of the semi-empirical calculations on molecules in vacuo are related to the electronic characteristics of the substrates in the active sites of the cytochrome P-450s. Due to solvation effects and a different dielectric constant, the intrinsic properties of the compounds may be influenced upon binding to this active site. However, it is assumed that this phenomenon will not influence the relative differences in parameters between a series of closely related compounds or between centers in a given molecule to a significant extent. The outcomes of the in vacuo computer calculations can thus be used as an approach to study relative differences within a series of related compounds or relative differences between sites in one molecule.

From the HOMO/HOMO–1, LUMO/LUMO+1, or SOMO density distributions, regioselectivities for the fluorobenzene hydroxylation were calculated by summation of all orbital densities of C-atoms that, upon hydroxylation, give rise to a specific phenolic metabolite and normalization of the sum of the values obtained for the different nonfluorinated carbon sites in a fluorobenzene to 1.0.

RESULTS

Identification of Hydroxylated Metabolites. To identify the ^{19}F NMR resonances of the fluorophenols as well as the resonances of their glucuronidated and sulfated forms, rats were exposed to these fluorophenols and their urine samples were analyzed using ^{19}F NMR both without enzyme pretreatment and with treatment by β-glucuronidase or arylsulfatase/β-glucuronidase. From the disappearance and appearance of ^{19}F NMR resonances in the urine spectra upon enzyme hydrolysis and the identification of the ^{19}F NMR resonances of the fluorophenol metabolites using added reference compounds, ^{19}F NMR resonances of the various metabolites could be identified. Table I gives an example using the urine from a rat exposed to 4-fluorophenol. Upon treatment of the urine with β-glucuronidase, the resonance at

Table I: ^{19}F NMR Characteristics of Urine Samples of a Rat Exposed to 4-Fluorophenol[a]

chemical shift	% of total fluorine intensity I[b]	II[b]	III[b]	identification
−117.3	0.6	0	0	4-fluoro-2-hydroxyphenyl glucuronide
−118.6	1.5	1.5	0	4-fluoro-2-hydroxyphenyl sulfate
−120.9	45.4	47.5	0	4-fluorophenyl sulfate
−123.0	3.6	3.5	3.8	fluoride anion
−125.2	37.3	0	0	4-fluorophenyl glucuronide
−125.9	2.0	1.7	2.0	unidentified
−126.6	3.5	3.2	0	3-fluoro-6-hydroxyphenyl sulfate
−126.7	0	2.1	7.2	4-fluoro-2-hydroxyphenol
−129.1	5.7	40.6	87.0	4-fluorophenol

[a] Resonances of the fluorophenols and the fluoride anion were identified on the basis of added reference compounds. Peaks of intensity below 0.5% of the total fluorine intensity were not included. [b] I = untreated urine, II = β-glucuronidase-treated urine, and III = arylsulfatase/β-glucuronidase-treated urine.

Table II: Chemical Shifts of ^{19}F NMR Resonances of Identified Metabolites from Various Fluorinated Benzene Metabolites in Urine 1:1 Diluted with 0.2 M Potassium Phosphate (pH 7.6)[a]

compound	chemical shift (ppm) -phenol	-phenyl glucuronide	-phenyl sulfate
2-fluoro-	−141.9	−138.3	−134.9
3-fluoro-	−116.5	−115.8	−115.5
4-fluoro-	−129.1	−125.2	−121.0
2,3-difluoro-	−142.8 (F3)	−141.6 (F3)	−140.6 (F3)
	−166.9 (F2)	−162.7 (F2)	−158.3 (F2)
3,4-difluoro-	−140.9 (F3)	−140.0 (F3)	−139.5 (F3)
	−154.2 (F4)	−149.9 (F4)	−145.3 (F4)
2,4-difluoro-	−126.1 (F4)	−121.1 (F4)	−116.5 (F4)
	−137.3 (F2)	−133.2 (F2)	−129.8 (F2)
2,5-difluoro-	−122.3 (F5)	−120.9 (F5)	−121.0 (F5)
	−147.2 (F2)	−143.7 (F2)	−140.1 (F2)
2,6-difluoro-	−138.9 (F2+F6)	−132.1 (F2+F6)	−131.0 (F2+F6)
3,5-difluoro-	−114.6 (F3+F5)	−113.4 (F3+F5)	−113.1 (F3+F5)
2,3,4-trifluoro-	−152.5 (F4)	−145.8 (F4)	−141.0 (F4)
	−161.9 (F2)	−157.1 (F2)	−152.9 (F2)
	−164.5 (F3)	−162.8 (F3)	−161.9 (F3)
3,4,5-trifluoro-	−139.6 (F3+F5)		−137.9 (F3+F5)
	−177.6 (F4)		−168.2 (F4)
2,3,6-trifluoro-	−144.3 (F3)	−136.7 (F3)	−135.5 (F3)
	−148.4 (F6)	−145.2 (F6)	−144.9 (F6)
	−164.0 (F2)	−153.9 (F2)	−152.5 (F2)
2,3,5-trifluoro-	−121.9 (F5)	−119.0 (F5)	−119.0 (F5)
	−141.6 (F3)	−138.5 (F3)	−137.4 (F3)
	−172.7 (F2)	−167.5 (F2)	−162.9 (F2)
2,4,5-trifluoro-	−139.4 (F5)		−135.5 (F5)
	−143.6 (F4)		−140.6 (F4)
	−146.9 (F2)		−144.9 (F2)

[a] The chemical shifts are relative to $CFCl_3$. Chemical shifts were ascribed to specific fluorine substituents on the basis of relative peak intensities, proton-decoupled splitting patterns, and the knowledge that meta substituents influence chemical shifts far less than para or ortho substituents.

−125.2 ppm disappears giving rise to a proportional increase of the resonance at −129.1 ppm, identified as 4-fluorophenol upon addition of this compound to the urine sample. This identifies the resonance at −125.2 ppm as 4-fluorophenyl glucuronide. Treatment of the urine with arylsulfatase/β-glucuronidase resulted in the disappearance of not only the resonance of the 4-fluorophenyl glucuronide but also the resonance at −120.9 ppm, accompanied by a proportional additional increase in the resonance of 4-fluorophenol. This identifies the resonance of 4-fluorophenyl sulfate at −120.9 ppm.

Table III: Chemical Shifts of ^{19}F NMR Resonances of 2-Hydroxyphenol (Catechol) and 4-Hydroxyphenol (Hydroquinone) Metabolites from Fluorobenzenes in 50 mM Potassium Phosphate (pH 7.6) (Catechols) or in Urine Diluted 1:1 in 0.2 M Potassium Phosphate (pH 7.6) (Hydroquinones)[a]

compound	chemical shift(s) (ppm)		
3-fluoro-2-hydroxyphenol	−140.4		
4-fluoro-2-hydroxyphenol	−126.7		
3,4-difluoro-2-hydroxyphenol	−152.1	−164.0	
4,5-difluoro-2-hydroxyphenol	−152.3		
3,5-difluoro-2-hydroxyphenol	−125.8	−137.9	
3,4,5-trifluoro-2-hydroxyphenol	−153.6	−162.0	−176.0
3,4,6-trifluoro-2-hydroxyphenol	−146.0	−153.1	−171.5
2-fluoro-4-hydroxyphenol	−138.7		
2,3-difluoro-4-hydroxyphenol	−163.4		
2,6-difluoro-4-hydroxyphenol	−137.1		
2,3,5-trifluoro-4-hydroxyphenol	−144.4	−162.2	−169.7

[a] The chemical shifts are relative to $CFCl_3$. Chemical shifts of catechols were identified on the basis of results from the conversion of fluorophenols by phenol hydroxylase from *Trichosporon cutaneum*, and the chemical shifts of hydroquinones were identified on the basis of secondary metabolites formed from the fluorophenols in vivo. For further details, see text.

In addition, β-glucuronidase treatment and arylsulfatase/β-glucuronidase treatment resulted in the disappearance of the resonances at −117.3, −118.6, and −126.6 ppm, respectively, accompanied by an increase in the resonance identified as 4-fluoro-2-hydroxyphenol (4-fluorocatechol) to an extent that—within the sensitivity of the measurement—equalled the sum of the original resonances. The resonance of 4-fluoro-2-hydroxyphenol was identified on the basis of experiments with phenol hydroxylase isolated from the yeast *Trichosporon cutaneum*. This flavin-dependent enzyme, known to catalyze the hydroxylation of various phenols at the position ortho to the hydroxyl moiety (Sejlitz & Neujahr, 1987; Neujahr, 1992), converted both 3-fluorophenol and 4-fluorophenol into a metabolite with its ^{19}F NMR resonance at −126.7 ppm. Therefore, the resonances at −117.3, −118.6, and −126.6 ppm, converted to a compound with its resonance at −126.7 ppm upon respective β-glucuronidase or arylsulfatase/β-glucuronidase treatment, could be identified as belonging to a glucuronide and two sulfate conjugates of 4-fluoro-2-hydroxyphenol. On the basis of the knowledge that changes in meta substituents influence the chemical shift of a fluorine substituent much less than a similar change in an ortho or para substituent with respect to the fluorine nucleus, the resonance at −117.3 is assigned to 4-fluoro-2-hydroxyphenol glucuronide, the resonance at −118.6 is assigned to 4-fluoro-2-hydroxyphenyl sulfate, and the resonance at −126.6 is assigned to 3-fluoro-6-hydroxyphenyl sulfate.

In a similar way, the ^{19}F NMR resonances of the various other phenols and catechols and their glucuronide and sulfate derivatives were identified. Tables II and III summarize the various ^{19}F NMR resonances thus identified. Only the resonances of 3,4,5-trifluorophenol and 2,4,5-trifluorophenol and their sulfate and glucuronide derivatives could not be identified in this way because 3,4,5-trifluorophenol and 2,4,5-trifluorophenol were not commercially available. However, the resonances of 3,4,5-trifluorophenol and its sulfated derivative were assigned on the basis of the following considerations: arylsulfatase treatment of the urine of a rat exposed to 1,2,3-trifluorobenzene resulted not only in disappearance of the signals ascribed to 2,3,4-trifluorophenyl sulfate but also in a decrease in the intensity of two unidentified peaks at −137.9 and −168.2 ppm with a ratio of the decrease in intensity of 2:1, accompanied by the appearance of two new

Table IV: Regioselectivities for the in Vivo Hydroxylations of Fluorobenzenes in Male Wistar Rats[a]

benzene	carbon centers	hydroxylation ratios at these carbon centers (2 rats)
1-fluorobenzene	C2/6:C3/5:C4	0.35:0.19:0.46
		0.32:0.20:0.48
1,2-difluorobenzene	C3/6:C4/5	0.33:0.67
		0.31:0.69
1,3-difluorobenzene	C2:C4/6:C5	0.12:0.82:0.06
		0.11:0.84:0.05
1,2,3-trifluorobenzene	C4/6:C5	0.73:0.27
		0.74:0.26
1,2,4-trifluorobenzene	C3:C5:C6	0.24:0.53:0.23
		0.27:0.49:0.24

[a] The regioselectivities observed upon in vivo biotransformation were determined from the arylsulfatase/β-glucuronidase-treated 24-h urine samples from fluorobenzene-exposed rats. Values from two independent rats are presented, demonstrating that the variation in regioselectivity is minimal. Cx/y indicates the sum of hydroxylation at identical carbon centers x and y.

peaks present in a 2:1 ratio at −139.6 and −177.6 ppm. These new peaks must belong to a phenol metabolite from 1,2,3-trifluorobenzene containing two of the three fluorine atoms in identical positions and one in a different molecular environment. This phenol metabolite can only be 3,4,5-trifluorophenol. On the basis of these considerations, the resonances at −137.9 and −168.2 ppm were ascribed to 3,4,5-trifluorophenyl sulfate and the ones at −139.6 and −177.6 ppm to 3,4,5-trifluorophenol.

In a similar way, on the basis of a comparison of the ^{19}F NMR spectra of untreated and arylsulfatase/β-glucuronidase-treated urine from a rat exposed to 1,2,4-trifluorobenzene, the resonances of 2,4,5-trifluorophenol and 2,4,5-trifluorophenyl sulfate were identified. The resonances of the corresponding phenyl glucuronides could not be identified from comparison of the ^{19}F NMR spectrum of untreated urine to that of urine treated with β-glucuronidase, because—as for the other hydroxylated fluorobenzene metabolites (see below)—the phenyl glucuronides were only minor metabolites whose resonances could not be distinguished from the many other small signals in the spectrum.

Figure 2 presents the ^{19}F NMR spectra of the arylsulfatase/β-glucuronidase-treated urine samples from rats treated with the various fluorophenols. In addition to the main phenol resonances, some minor metabolites can be observed. Due to the fact that all of these minor resonances were observed in the ^{19}F NMR spectra of the urine only after arylsulfatase treatment, it can be concluded that they represent phenolic metabolites, excreted as the sulfated species. On the basis of comparison of the minor phenolic metabolites in the various spectra, some of the ^{19}F NMR resonances observed can be ascribed to hydroquinone metabolites.

The metabolite at −138.7 ppm, for example, was observed in the ^{19}F NMR spectra of the arylsulfatase-treated urine samples from rats exposed to 2-fluorophenol and 3-fluorophenol (Figure 2a,b). This identical hydroxylated metabolite could be either 3-fluoro-2-hydroxyphenol (3-fluorocatechol) or 2-fluoro-4-hydroxyphenol (2-fluorohydroquinone). Because the ^{19}F NMR resonance of 3-fluoro-2-hydroxyphenol had already been identified at −140.4 ppm on the basis of the incubations with phenol hydroxylase (Table III), the resonance at −138.7 ppm could be assigned to 2-fluoro-4-hydroxyphenol. The fact that this hydroquinone metabolite gave rise to two different sulfated metabolites in the original urine spectrum (data not shown) further supports its identification as a diol metabolite. In a similar way the resonances of other diol metabolites could be identified, and their resonances are also summarized in Table III.

In addition, the ^{19}F NMR spectra presented in Figure 2 demonstrate that metabolites resulting from in vivo hydroxylation of the various fluorophenols were only observed to a limited extent, generally between 0 (2,3,4-trifluorophenol) and 10% (4-fluorophenol). Only from fluorophenols with a fluorinated position opposite plus a nonfluorinated position ortho to the hydroxyl moiety is the formation of catechol metabolites observed. All other fluorophenols become preferentially hydroxylated at the position opposite their hydroxyl moiety, resulting in hydroquinone metabolites (Figure 2).

In Vivo Regioselectivity for the Aromatic Hydroxylation of Fluorobenzenes. Figure 3 shows the urine spectra obtained from rats exposed to fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene. From these results it can be derived that all phenol metabolites are excreted predominantly as their sulfate derivatives. Figure 4 shows the ^{19}F NMR spectra from the same urine samples, this time treated with arylsulfatase/β-glucuronidase. From the ^{19}F NMR spectra of the arylsulfatase/β-glucuronidase-treated urine samples the regioselectivity of the aromatic hydroxylation was calculated. The results are presented in Table IV. From the data presented it follows that, upon in vivo biotransformation, the five fluorobenzenes become hydroxylated at all nonhalogenated positions, leading to different fluorophenol metabolites. This aromatic hydroxylation appears to be regioselective, since not all sites are hydroxylated to the same extent.

Phenolic metabolites resulting from hydroxylation accompanied by either dehalogenation or an NIH shift were not observed at the detection limit of the measurements, which was 1–2% of the total amount of fluorinated metabolites. The phenolic metabolites resulting from hydroxylation at a nonhalogenated position make up about 50, 45, 45, 35, and 35% of the total amount of fluorinated metabolites for fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene, respectively (Figures 3 and 4). From these results it can be calculated that the phenolic metabolites resulting from hydroxylation at a nonhalogenated position make up over 95% of all phenolic metabolites formed. This observation excludes the possibility that products resulting from hydroxylation accompanied by dehalogenation or an NIH shift influence the regioselectivity of the aromatic hydroxylation of fluorobenzenes.

Influence of the Cytochrome P-450 Enzyme Pattern on the Regioselectivity of the Aromatic Hydroxylation of Fluorobenzene. The regioselectivity of bromobenzene or chlorobenzene hydroxylation is known to vary with the cytochrome P-450 enzyme pattern (Zampaglione et al., 1973; Selander et al., 1975b; Lau et al., 1980; Den Besten et al., 1991), with 3-methylcholanthrene induction favoring, for example, C2-hydroxylation of bromobenzene. Therefore, the regioselectivity of the aromatic hydroxylation was studied using fluorobenzene as a model compound with rats pretreated differently. The results of these experiments are presented in Table V, and they demonstrate that for fluorobenzene no significant effect of changes in the cytochrome P-450 enzyme pattern on the regioselectivity of its in vivo hydroxylation is observed. The observation that ethoxyresorufin O-dealkylating activity and pentoxyresorufin O-dealkylating activity were increased 30- and 60-fold in the liver microsomes from rats receiving the pretreatment with 3-methylcholanthrene or phenobarbital, respectively (data not shown), confirmed a

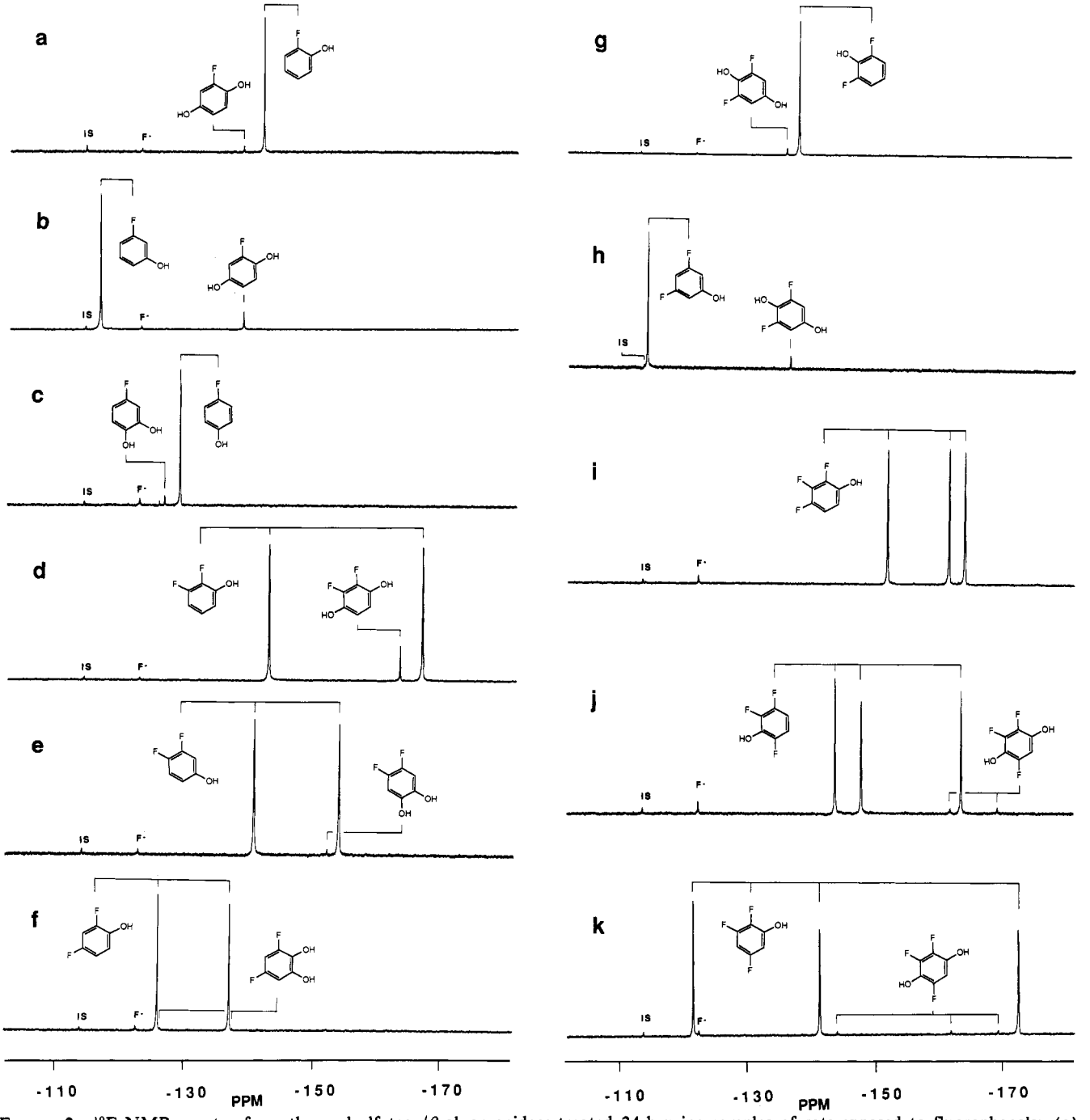

FIGURE 2: ^{19}F NMR spectra from the arylsulfatase/β-glucuronidase-treated 24-h urine samples of rats exposed to fluorophenols: (a) 2-fluorophenol; (b) 3-fluorophenol; (c) 4-fluorophenol; (d) 2,3-difluorophenol; (e) 3,4-difluorophenol; (f) 2,4-difluorophenol; (g) 2,6-difluorophenol; (h) 3,5-difluorophenol; (i) 2,3,4-trifluorophenol; (j) 2,3,6-trifluorophenol; (k) 2,3,5-trifluorophenol. Resonances were identified on the basis of ^{19}F NMR spectra of untreated urine and urine treated with either β-glucuronidase or arylsulfatase/β-glucuronidase, ^{19}F NMR spectra from the incubation of fluorophenols with phenol hydroxlase from *Trichosporon cutaneum*, formation of identical metabolites from different fluorophenols, and/or the ^{19}F NMR resonance positions of reference compounds. The resonance marked IS is from the internal standard, 4-fluorobenzoic acid. The third resonance of 2,3,5-trifluorophenol (j) is masked by the F3 signal of the parent 2,3,6-trifluorophenol.

proper cytochrome P-450 induction in the animals used.

In Vitro Regioselectivity for the Aromatic Hydroxylation of 1,2-Difluorobenzene. Because literature data on the metabolism of chloro- and bromobenzene suggested that the in vivo regioselectivity of aromatic hydroxylation could differ from the results obtained by microsomal incubations (Selander et al., 1975b; Lertratanangkoon et al., 1987), the regioselectivity of the fluorobenzene hydroxylation was also studied in microsomal incubations. For these studies, 1,2-difluorobenzene was used as the model compound because at least one of the ^{19}F NMR resonances of each of its hydroxylated metabolites was at a position significantly different from that of the parent compound, which is present in high concentrations in vitro incubations, allowing reliable quantification. Generally, the chemical shift of fluoro substituents at a position meta with respect to the chemical modification only changes to a very limited extent following the change in the molecule. Consequently, the ^{19}F NMR resonances of metabolites resulting from meta hydroxylation with respect to the fluoro substituent are not well separated from those of the parent benzene, hampering reliable quantification.

An additional problem connected to the experiments on in vitro microsomal conversion of the polyfluorobenzenes was that their conversion rates were generally below 0.5 nmol/10

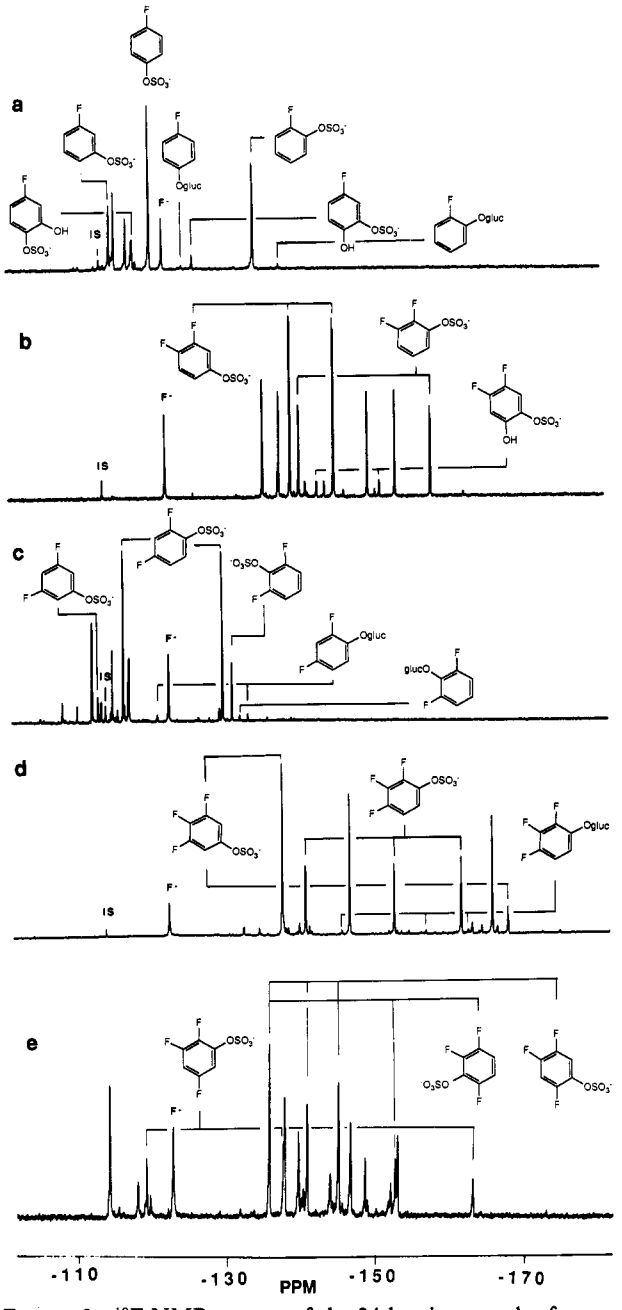

FIGURE 3: ^{19}F NMR spectra of the 24-h urine samples from rats exposed to fluorobenzenes: (a) fluorobenzene; (b) 1,2-difluorobenzene; (c) 1,3-difluorobenzene; (d) 1,2,3-trifluorobenzene; (e) 1,2,4-trifluorobenzene. Resonances were identified on the basis of the results summarized in Table II. The resonance marked IS is from the internal standard, 4-fluorobenzoic acid. The F3 + F5 resonance of 3,4,5-trifluorophenyl sulfate at −137.9 ppm overlaps with the resonance of an unknown 1,2,3-trifluorobenzene metabolite (d).

min/nmol of cytochrome P-450, resulting in metabolite concentrations below the detection limit of the ^{19}F NMR measurements. However, use of *tert*-butyl hydroperoxide as an artificial oxygen donor resulted in much higher turnover rates. Because organic peroxide stimulated cytochrome P-450 reactions have been reported to result in metabolite patterns that differ from those observed with NADPH as the electron donor and molecular oxygen (Capdevila et al., 1980), the possible (dis)similarity between a reaction supported by NADPH and one supported by *tert*-butyl hydroperoxide was checked using isosafrol microsomes, which appeared to show the highest turnover rate (Table VI). The results of this experiment are presented in Figure 5. Using isosafrol microsomes, conversion of 1,2-difluorobenzene by the NADPH-driven reaction could be detected, and the regioselectivity (C3/6:C4/5 hydroxylation = 0.21:0.79) (Figure 5a) did not

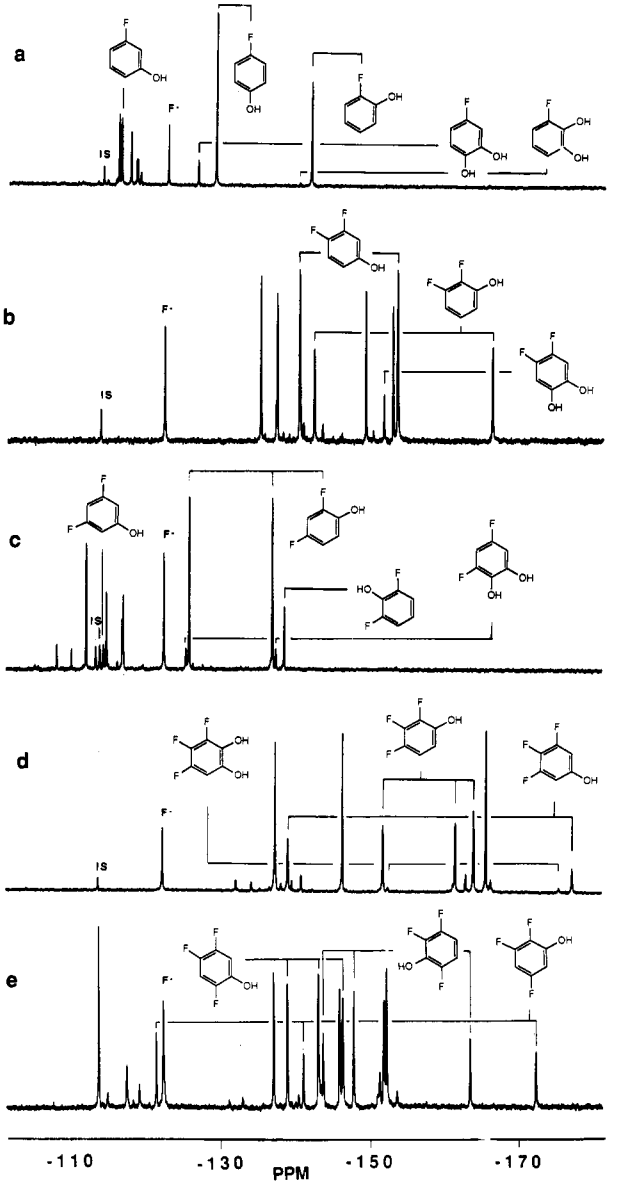

FIGURE 4: ^{19}F NMR spectra from the arylsulfatase/β-glucuronidase-treated 24-h urine samples of rats exposed to fluorobenzenes: (a) fluorobenzene; (b) 1,2-difluorobenzene; (c) 1,3-difluorobenzene; (d) 1,2,3-trifluorobenzene; (e) 1,2,4-trifluorobenzene. Resonances were identified on the basis of added reference compounds and the results summarized in Tables II and III. The resonance marked IS is from the internal standard, 4-fluorobenzoic acid.

Table V: Regioselectivities of the in Vivo Hydroxylations of Fluorobenzene Determined Using Untreated Rats or Rats Pretreated with Cytochrome P-450 Inducers[a]

inducer	hydroxylation ratios at carbon centers C2/6:C3/5:C4 (2 rats)
none	0.35:0.19:0.46
	0.32:0.20:0.48
phenobarbital	0.34:0.19:0.47
	0.34:0.18:0.48
3-methylcholanthrene	0.34:0.14:0.52
	0.34:0.16:0.50

[a] The regioselectivities observed upon in vivo biotransformation were determined from the arylsulfatase/β-glucuronidase-treated 24-h urine samples from fluorobenzene-exposed rats. Values from two independent rats are presented, demonstrating that the variation in regioselectivity is minimal.

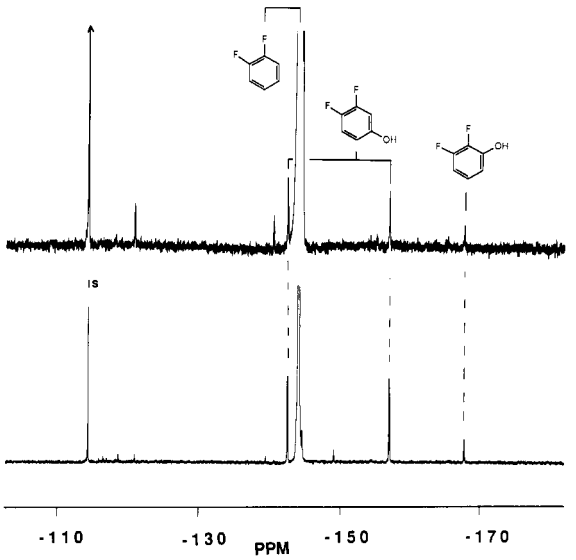

FIGURE 5: ^{19}F NMR spectra from the ethyl acetate extract from microsomal incubations with 1,2-difluorobenzene carried out using (a) NADPH and molecular oxygen and (b) *tert*-butyl hydroperoxide as the alternative oxygen donor. Resonances were identified on the basis of added reference compounds. The resonance marked IS is from the internal standard, 4-fluorobenzoic acid.

Table VI: In Vitro Regioselectivities of 1,2-Difluorobenzene Hydroxylation by Liver Microsomes from Rats Pretreated with Various Cytochrome P-450 Inducers in a *tert*-Butyl Hydroperoxide Supported Reaction[a]

inducer	hydroxylation ratio at carbon centers C3/6:C4/5 (two microsomal incubations)	total rate of conversion (nmol of C3/6+C4/5 phenol/10min/ nmol of P-450)
none	0.29:0.71 0.30:0.70	3.5 ± 0.3
phenobarbital	0.26:0.74 0.29:0.71	2.1 ± 0.3
3-methylcholanthrene	0.25:0.75 0.26:0.74	3.1 ± 0.8
isosafrole	0.21:0.79 0.22:0.78	17.2 ± 0.1
dexamethasone	0.30:0.70 0.24:0.76	1.5 ± 0.1

[a] For the regioselectivities, values from two independent incubations are presented, demonstrating that the variation in regioselectivity is minimal. For the conversion rates, the values present the mean ± SEM of these two independent incubations.

differ significantly from the regioselectivity observed with *tert*-butyl hydroperoxide (0.24:0.76) (Figure 5b).

Table VI also summarizes the results from the regioselectivity of the 1,2-difluorobenzene hydroxylation observed using microsomes from differently pretreated rats known to differ in their cytochrome P-450 enzyme pattern. The data obtained demonstrate that the absolute value of the regioselectivity observed with the different microsomal preparations varies only within ±5%. For all microsomes, 1,2-difluorobenzene became preferentially hydroxylated at its C4/5 position, the C3/6 position being hydroxylated to a lesser extent. From these results, we conclude that the active site of the cytochrome P-450 is not the major factor determining the regioselectivity of the aromatic hydroxylation of the fluorobenzenes.

Frontier Orbital and Coulomb Characteristics of the Fluorobenzenes. The in vivo and in vitro experiments described above demonstrate the effects of the fluorine substituent pattern in a benzene molecule on the regioselectivity of its aromatic hydroxylation. Molecular orbital calculations were performed to investigate whether the changes observed could be ascribed to the intrinsic electronic characteristics of the fluorobenzenes. On the basis of the frontier orbital theory (Fukui et al., 1954; Fleming, 1976), relative differences in reactivity between various sites in one molecule may be dependent on the Coulomb and/or frontier orbital characteristics of these sites in the reacting species. Because the cytochrome P-450 $(FeO)^{3+}$ species, initiating the reaction, is similar for all reaction centers, the regioselectivity of the cytochrome P-450 dependent aromatic hydroxylation of the fluorobenzenes could be dependent on the characteristics of the various sites in the fluorinated benzene molecules. The frontier orbital theory states that the relative reactivities of sites in a molecule may vary due to differences in atomic charge, resulting in varying Coulombic attractions between the reacting centers. However, from the data presented in Table VII it can be concluded that differences in net atomic charge on the various carbon sites in the polyfluorobenzenes cannot explain the regioselectivities observed.

In addition, the relative reactivities may be dependent on frontier orbital characteristics. For an electrophilic attack of the $(FeO)^{3+}$ radical species on an aromatic ring, the substrate's frontier orbital parameters of interest are the energy of the HOMO (highest occupied molecular orbital) and the relative density of this orbital on the respective aromatic reaction sites. Higher energy and higher density of the HOMO on a reaction site will both result in relatively higher reactivity. In a similar way, LUMO (lowest unoccupied molecular orbital) characteristics will influence the nucleophilic attack of a radical species on a benzene molecule, whereas an attack on a benzene radical cation will be determined by its SOMO (singly occupied molecular orbital) characteristics. Thus, for a given fluorobenzene substrate or its radical species, the HOMO, LUMO, or SOMO density distribution over its various aromatic carbon atoms will be a factor determining the relative reactivities of these sites for aromatic hydroxylation. In addition, when the HOMO–1 and LUMO+1 are very close in energy to the HOMO and LUMO, respectively, they should be taken into account using the formula for calculation of the so-called frontier orbital density presented by Fukui et al. (1954). The regioselectivity for both electrophilic and nucleophilic aromatic substitution reactions in organic chemistry have been explained in this way in the past (Fukui et al., 1954; Fleming, 1976). Table VII gives the HOMO/HOMO–1 and LUMO/LUMO+1 frontier orbital densities of the five fluorobenzenes.

The data presented in Table VII were used to predict regioselectivities for the aromatic hydroxylation of the various fluorobenzenes on the basis of the possible reaction mechanisms proposed for this cytochrome P-450 catalyzed reaction (Figure 1). For an initial electrophilic attack of the $(FeO)^{3+}$ species on the fluorobenzenes (pathway I, Figure 1), their HOMO/HOMO–1 frontier orbital densities will determine the site of attack. For a nucleophilic attack of the $(FeO)^{3+}$ on the fluorobenzenes their LUMO/LUMO+1 frontier orbital densities will be of importance, whereas for a mechanism proceeding by initial electron abstraction followed by addition of the $(FeO)^{2+}$ species to the fluorobenzene radical cation (pathway II, Figure 1), the SOMO density of the benzene radical cation determines the regioselectivity of the attack.

On the basis of the various frontier parameters and the assumption that the site of attack will be the site of hydroxylation, the regioselectivities of the fluorobenzene hydroxylations were calculated. The results are presented in Table VIII. Comparison of these data to the actual regioselectivities observed (Tables IV–VI), shows that the regi-

Table VII: Frontier Orbital and Coulomb Characteristics of Fluorobenzenes and of Their Radical Cation Forms Resulting from One-Electron Abstraction, Calculated Using the AM1 Hamiltonian

benzene	frontier orbitals	form	frontier orbital density on carbon center C1	C2	C3	C4	C5	C6
1-fluoro	HOMO/HOMO-1	parent	0.45	0.27	0.19	0.48	0.19	0.27
	LUMO/LUMO+1	parent	0.13	0.43	0.45	0.11	0.45	0.43
	SOMO	radical cation	0.29	0.10	0.05	0.31	0.05	0.10
1,2-difluoro	HOMO/HOMO-1	parent	0.41	0.41	0.12	0.32	0.32	0.12
	LUMO/LUMO+1	parent	0.23	0.23	0.53	0.22	0.22	0.53
	SOMO	radical cation	0.25	0.25	0.00	0.17	0.17	0.00
1,3-difluoro	HOMO/HOMO-1	parent	0.31	0.18	0.31	0.43	0.11	0.43
	LUMO/LUMO+1	parent	0.27	0.50	0.27	0.21	0.50	0.21
	SOMO	radical cation	0.17	0.00	0.17	0.27	0.00	0.27
1,2,3-trifluoro	HOMO/HOMO-1	parent	0.26	0.37	0.26	0.26	0.25	0.26
	LUMO/LUMO+1	parent	0.34	0.35	0.34	0.29	0.30	0.29
	SOMO	radical cation	0.09	0.35	0.09	0.04	0.22	0.04
1,2,4-trifluoro	HOMO/HOMO-1	parent	0.49	0.28	0.09	0.38	0.29	0.10
	LUMO/LUMO+1	parent	0.35	0.35	0.28	0.36	0.30	0.28
	SOMO	radical cation	0.29	0.12	0.02	0.21	0.13	0.03

form	net atomic charge on carbon center C1	C2	C3	C4	C5	C6
parent	+0.09	−0.17	−0.11	−0.15	−0.11	−0.17
radical cation	+0.28	−0.12	−0.10	+0.08	−0.10	−0.12
parent	+0.06	+0.06	−0.14	−0.12	−0.12	−0.14
radical cation	+0.21	+0.21	−0.18	+0.01	+0.01	−0.18
parent	+0.12	−0.21	+0.12	−0.18	−0.08	−0.18
radical cation	+0.24	−0.25	+0.24	0.00	−0.14	0.00
parent	+0.08	+0.02	+0.08	−0.16	−0.09	−0.16
radical cation	+0.14	+0.22	+0.14	−0.16	+0.09	−0.16
parent	+0.04	+0.09	−0.19	+0.11	−0.16	−0.12
radical cation	+0.23	+0.17	−0.22	+0.27	−0.08	−0.14

Table VIII: Possible Regioselectivities for the Aromatic Hydroxylation of Fluorobenzenes Calculated on the Basis of HOMO/HOMO-1 Frontier Orbital Characteristics or LUMO/LUMO+1 Characteristics of the Parent Substrate Molecule or on the Basis of the SOMO Characteristics of the Substrate Cation Radical Resulting from One-Electron Abstraction, All Calculated Using the Data Presented in Table VII[a]

benzene	carbon centers	calculated hydroxylation ratios at these carbon centers HOMO/HOMO-1	LUMO/LUMO+1	SOMO
1-fluoro	C2/6:C3/5:C4	0.39:0.27:0.34	0.46:0.48:0.06	0.33:0.16:0.51
1,2-difluoro	C3/6:C4/5	0.27:0.73	0.71:0.29	0.00:1.00
1,3-difluoro	C2:C4/6:C5	0.16:0.74:0.10	0.35:0.30:0.35	0.00:0.00:1.00
1,2,3-trifluoro	C4/6:C5	0.68:0.32	0.66:0.34	0.27:0.73
1,2,4-trifluoro	C3:C5:C6	0.19:0.60:0.21	0.33:0.34:0.33	0.11:0.72:0.17

[a] The regioselectivity calculated on the basis of the HOMO/HOMO-1 is representative of an electrophilic attack of the $(FeO)^{3+}$ on the fluorobenzene and that calculated on the basis of the LUMO/LUMO+1 is representative of a nucleophilic attack of the $(FeO)^{3+}$ on the fluorobenzene, whereas the regioselectivity calculated on the basis of the fluorobenzene radical cation's SOMO represents a pathway proceeding by initial electron abstraction followed by attack of the $(FeO)^{2+}$ species on the fluorobenzene cation radical.

oselectivity calculated on the basis of HOMO/HOMO-1 frontier orbital densities of the fluorobenzene substrates correlates with the observed regioselectivity for the aromatic hydroxylation for all five fluorobenzenes studied. Values predicted for the in vivo regioselectivity of the fluorobenzene hydroxylations are in accordance with the values predicted by the HOMO/HOMO-1 frontier orbital density distribution, with a mean deviation in the absolute value of 6.4 ± 0.5%. Regioselectivities predicted on the basis of LUMO/LUMO+1 or SOMO densities did not correlate with the actual regioselectivities observed for all five fluorobenzenes.

For calculation of the data presented in Table VII and VIII the AM1 Hamiltonian was used. To investigate to what extent the type of semi-empirical method used for the calculation influences the predicted regioselectivity, regioselectivities were also calculated on the basis of HOMO/HOMO-1 frontier distributions that were calculated using the MINDO/3 and MNDO Hamiltonians. Table IX summarizes the results obtained. From the data presented it follows that the calculated values vary to a small extent with the Hamiltonian used. The mean absolute deviations from the regioselectivities observed in vivo were 5.8 ± 0.9 and 5.6 ± 1.0% for the MNDO and the MINDO/3 methods, respectively.

Altogether, we conclude from the data presented above that the frontier orbital characteristics for electrophilic attack in the fluorobenzene substrate molecules predict the regioselectivities for their in vivo hydroxylation. For 1,2,4-trifluorobenzene, the HOMO/HOMO-1 frontier orbital density of the parent molecule, for example, correctly explains the preferential hydroxylation at C5 and the almost equal reactivity of C3 and C6. For 1,2,3-trifluorobenzene, formation of 2,3,4-trifluorophenol as the main metabolite can also be explained. The model also correctly predicts and explains the preferential hydroxylation of 1,3-difluorobenzene at C4/6 and even the slightly higher hydroxylation at C2 over C5 for this molecule. For 1,2-difluorobenzene, the regioselectivity observed in not only in vivo but also in in vitro experiments correlates with the regioselectivity calculated on the basis of the HOMO/HOMO-1 density of the substrate molecule. For fluorobenzene, the SOMO density distribution might explain the observed regioselectivity to an even better extent than the HOMO/HOMO-1 frontier orbital density for electrophilic

Table IX: Regioselectivities for the Aromatic Hydroxylations of Fluorobenzenes Calculated on the Basis of HOMO/HOMO–1 Frontier Orbital Characteristics of the Parent Fluorobenzene Molecules Using the AM1, MNDO, or MINDO/3 Hamiltonians

		calculated hydroxylation ratios at these carbon centers on the basis of HOMO/HOMO–1		
benzene	carbon centers	AM1	MNDO	MINDO/3
1-fluoro	C2/6:C3/5:C4	0.39:0.27:0.34	0.39:0.30:0.31	0.40:0.21:0.39
1,2-difluoro	C3/6:C4/5	0.27:0.73	0.33:0.67	0.24:0.76
1,3-difluoro	C2:C4/6:C5	0.16:0.74:0.10	0.18:0.70:0.12	0.12:0.82:0.06
1,2,3-trifluoro	C4/6:C5	0.68:0.32	0.69:0.31	0.71:0.29
1,2,4-trifluoro	C3:C5:C6	0.19:0.60:0.21	0.24:0.54:0.22	0.14:0.68:0.18

attack. However, it seems unlikely that the five closely related substrates will be converted by the cytochrome P-450s by a different mechanism. The HOMO/HOMO–1 frontier density especially overestimates the in vivo C3 hydroxylation and underestimates the C4 hydroxylation of fluorobenzene. This phenomenon might as well be explained by a small contribution of the fluorobenzene 3,4-epoxide, formed from σ-addition at C3 and eventually giving rise to hydroxylation at C4. This hypothesis provides an interesting topic for future research.

Furthermore, the results presented in Table VII demonstrate that all five fluorobenzenes contain significant frontier orbital density on their fluorinated carbon centers as well. Because the van der Waals radius of a fluorine substituent is very similar to that of a hydrogen substituent, σ-addition of the P-450 intermediate to these fluorinated centers is not prevented by sterical hindrance and has to be taken into consideration. Possible reactions following such a σ-addition are the following. First, hydroxylation accompanied by defluorination might occur, but as already indicated above, this is not observed. A second possibility would be the formation of a hydroxylated metabolite accompanied by an NIH shift of the fluorine substituent, but this also is not observed. Formation of an epoxide followed by opening of the epoxide moiety to give a product which is hydroxylated at the position ortho to the fluorinated carbon center would be a third possibility. This implies that the frontier orbital density of the fluorinated center should be included in the calculation for the amount of hydroxlation at the position ortho to this center. If this is done, for instance for 1,2-difluorobenzene, the calculated C3/6:C4/5 hydroxylation ratio would predict preferential C3/6 over C4/5 hydroxylation which is opposite to what is observed. Thus, for the fluorobenzenes, the ratios calculated on the basis of the data presented in Table VII, including the orbital densities on fluorinated centers, do not correlate with the actual regioselectivities observed. Altogether, it seems more likely that σ-addition at a fluorinated carbon center does not result in significant formation of phenolic products.

DISCUSSION

In the present study, the regioselectivity of the hydroxylation of five fluorobenzenes was studied using ^{19}F NMR for identification of the various phenols in the urine of rats exposed to these benzenes. The regioselectivities observed for the aromatic hydroxylation of the fluorobenzenes in in vivo experiments were shown to correlate with the values predicted for regioselectivity on the basis of HOMO/HOMO–1 frontier orbital density with 6% accuracy. Regioselectivities predicted on the basis of a cytochrome P-450 hydroxylation proceeding by (i) an initial nucleophilic attack on the benzene's LUMO/LUMO+1 or (ii) an initial electron abstraction followed by an attack of the $(FeO)^{2+}$ on the benzene cation radical SOMO cannot correctly predict the regioselectivities of all five fluorobenzenes tested. Because it is unlikely that these five closely related substrates will be hydroxylated by the cytochrome P-450s by different mechanisms, it is concluded from these results that the cytochrome P-450 catalyzed conversion of the fluorobenzenes proceeds by an initial electrophilic attack of the $(FeO)^{3+}$ species on the HOMO/HOMO–1 frontier orbital electrons of the fluorobenzene substrate.

This conclusion would be in accordance with that of Korzekwa et al. (1989) who, on the basis of experiments with deuterated chlorobenzenes, also concluded that the most likely mechanism for the hydroxylation of the benzene ring was through an addition–rearrangement mechanism initiated by an attack of the activated oxygen atom on the π-system of the aromatic ring (pathway I, Figure 1). The HOMO/HOMO–1 frontier orbitals of the fluorobenzenes are indeed π-type orbitals. If the benzene were to be positioned in the active site with the $(FeO)^{3+}$ axis perpendicular to its aromatic ring, the interaction of the fluorobenzene HOMO/HOMO–1 with the $(FeO)^{3+}$ d_{xz}–p_x as the SOMO orbital (Ortiz de Montellano, 1986) might occur at all carbon centers of the fluorobenzene without the requirement for fast rotation of the substrate in the active site. Furthermore, our results exclude the mechanism proceeding by initial electron abstraction (pathway II, Figure 1) suggested by Burka et al. (1983), a conclusion also in accordance with that of Korzekwa et al. (1989), stating that a mechanism proceeding by initial electron abstraction is highly unlikely for the cytochrome P-450 catalyzed hydroxylation of chlorobenzene.

In addition, the results of the present study argue against the formation of the hydroxylated fluorobenzene metabolites through intermediate epoxide formation. Although many authors generally assume the involvement of epoxides as precursors of the phenol metabolites (Lindsay Smith et al., 1972; Jerina et al., 1974; Griffeth et al., 1987; Den Besten et al., 1989, 1991), Korzekwa et al. (1989), on the basis of results from studies using deuterated chlorobenzenes, have already indicated that the epoxide mechanism for hydroxylation of the chlorobenzenes to phenol metabolites might be untenable. This conclusion is supported further by the results of the present study, because epoxide intermediates would imply that the site of attack cannot be used to predict the site of hydroxylation.

However, although the data from the present study seem to rule out the involvement of epoxides as major intermediates in the formation of the phenol metabolites, epoxide metabolites might still be formed in addition to keto or phenol metabolites. The data of the present study point in this direction as a minor amount of the in vivo metabolites observed in the urine from fluorobenzene-exposed rats could be ascribed to catechol derivatives. Because additional results from ^{19}F NMR analysis of the urine from rats exposed to fluorophenols indicated that secondary metabolism of the phenols is only a minor route and leads to hydroquinones rather than to catechols as secondary metabolites, it must be concluded that the formation of the catechol metabolites from the fluorobenzenes most likely proceeds through epoxide hydrolase catalyzed conjugation of epoxide intermediates with water, followed by aromatization

to give the catechol metabolites. This route is dominant over a second cytochrome P-450 catalyzed hydroxylation step of the phenol metabolite for in vivo formation of diol metabolites. Preferential formation of catechols in vivo through aromatization of dihydro diols instead of through secondary cytochrome P-450 metabolism has been reported previously by Miller et al. (1990) in a study on catechol metabolite formation from bromobenzene. In addition, identification of the unknown non-phenol-like metabolites observed in the ^{19}F NMR spectra of the urine of the fluorobenzene-exposed rats (Figure 3) might provide further support for the formation of epoxide intermediates in addition to keto and phenol metabolites, if these metabolites prove to be mercapturic acids, which are known to be important in vivo metabolites of halogenated benzenes and derive from glutathione conjugation of epoxide intermediates (Zampaglione et al., 1973; Jollow et al., 1974; Jerina & Daly, 1974; Monks et al., 1982; Zheng & Hanzlik, 1991).

Altogether, we conclude that the reaction pathway leading to catechol and mercapturic acid metabolites might still proceed through epoxide intermediates, whereas formation of the phenol metabolites from fluorobenzenes is unlikely to proceed through epoxide intermediates. With respect to this conclusion, the results from the theoretical calculations of Korzekwa et al. (1985) are of importance. On the basis of MNDO computer calculations, they concluded that formation of the phenol is favored over formation of the epoxide in the model cationic pathway, representative of the (FeO) cationic σ-adduct. On the other hand, formation of the epoxide was calculated to be favored over formation of the phenol metabolite for the so-called singlet biradical pathway, representative of the (FeO) radical σ-adduct. This suggests the formation of the epoxide and the phenol as independent preferential products, each from one of the two possible intermediate (FeO) σ-adducts, i.e., the radical σ-adduct or the cationic σ-adduct, respectively.

On the basis of these considerations and the results of the present study, it is postulated that these two intermediate σ-adducts are in dynamic equilibrium with each other, one giving rise to epoxide-derived metabolites and the other to phenol-derived metabolites. Finally, Figure 6 presents this working hypothesis for the reaction pathways for the cytochrome P-450 dependent conversion of the fluorobenzenes.

Additional experiments in the present study were performed to provide some insight into possible factors influencing the (small) deviation between regioselectivities for the aromatic polyfluorobenzene hydroxylation predicted on the basis of HOMO/HOMO–1 characteristics and the values actually observed in vivo. One of the factors of influence might be experimental variation in both the in vivo experiments and the semi-empirical calculations. Use of different Hamiltonians for the calculations demonstrated that data obtained by either the AM1, MNDO, or MINDO/3 Hamiltonian all varied from the regioselectivities actually observed by a mean value of about 6%. Another factor influencing differences between calculated ratios and regioselectivities observed in vivo might be the influence of additional metabolic pathways other than the cytochrome P-450 dependent hydroxylation. The 3-hydroxylation of bromobenzene, for example, has been ascribed in part to a pathway involving glutathione conjugation of the 3,4-epoxide (Lertratanangkoon et al., 1987). However, additional results of the present study demonstrated that, in in vitro incubations in which 1,2-difluorobenzene was used as the model compound, the regioselectivity of the reaction was not changed significantly compared to the regioselectivity

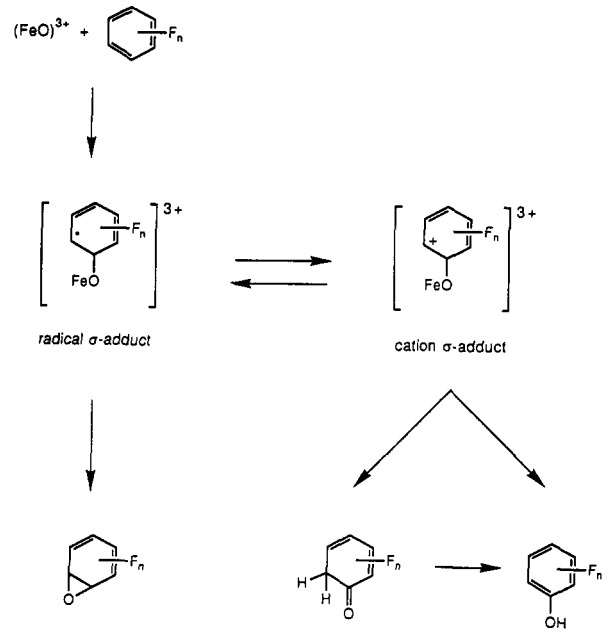

FIGURE 6: Schematic presentation of the working hypothesis for the reaction pathway for the formation of phenol metabolites from fluorobenzenes, put forward on the basis of the results from the present study and the theoretical calculations presented by Korzekwa et al. (1985). For further explanation, see the Discussion section of the text.

observed in vivo. These results of the present study indicate that the contribution of the glutathione conjugation pathway to in vivo hydroxylation of the fluorobenzenes might at most be a minor factor of influence.

Finally, a factor influencing the regioselectivity of the fluorobenzene hydroxylation in addition to the frontier electron characteristics of the substrate might be the orientation of the substrate in the active site of the cytochrome P-450 and/or the type of cytochrome P-450s actually catalyzing the reaction (Zampaglione et al., 1973; Selander et al., 1975b; Lau et al., 1980; Den Besten et al., 1991). In the present study, however, phenobarbital or 3-methylcholanthrene induction appeared to have no significant effect on the actual regioselectivity of the in vivo metabolism of fluorobenzene. Additional in vitro studies using 1,2-difluorobenzene and liver microsomes from rats pretreated with various cytochrome P-450 inducers demonstrated only a minor influence of the cytochrome P-450 enzyme pattern on the regioselectivity of the 1,2-difluorobenzene hydroxylation. Therefore, it can be concluded that the juxtaposition of the fluorobenzene molecules in the relatively large hydrophobic pocket of the cytochrome P-450(s) is not a major factor influencing the regioselectivity of their aromatic hydroxylation. This is in accordance with the results reported previously for monofluoroanilines (Cnubben et al., 1992).

In conclusion, the results of the present article demonstrate that the HOMO/HOMO–1 frontier orbital distribution of fluorobenzenes provides a basis for the prediction of the regioselectivities of their in vitro and in vivo hydroxylations. This observation points to a reaction pathway of the cytochrome P-450 catalyzed hydroxylation initiated by initial electrophilic attack of the $(FeO)^{3+}$ on the frontier π-electrons of the fluorobenzene and argues against epoxide intermediates as important precursors of the phenol metabolites. To what extent the method will be applicable for the prediction of the regioselectivities of the cytochrome P-450 catalyzed hydroxylations of chloro- and bromobenzenes or even of polyaromatic molecules remains a topic for future research.

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