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Regioselectivity and Quantitative Structure–Activity Relationships for the Conjugation of a Series of Fluoronitrobenzenes by Purified Glutathione S-Transferase Enzymes from Rat and Man

Ans E. M. F. Soffers,*,† Jan H. T. M. Ploemen,‡ Mariëlle J. H. Moonen,† Theo Wobbes,§ Ben van Ommen,‡ Jacques Vervoort,† Peter J. van Bladeren, and Ivonne M. C. M. Rietjens

Department of Biochemistry, Agricultural University, Wageningen, The Netherlands, Department of Surgical Oncology, University Hospital, Nijmegen, The Netherlands, and Division of Toxicology, TNO Nutrition, Zeist, The Netherlands

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Quantitative structure–activity relationships (QSAR's) are described for the rate of conjugation of a series of fluoronitrobenzenes with cytosolic as well as with two major alpha and mu class enzymes of rat and human liver, viz., glutathione *S*-transferases (GST) 1-1, 3-3, A1-1, and M1a-1a. For all purified enzymes studied, the natural logarithm of the rate of conversion of the fluoronitrobenzenes correlates with both the calculated reactivity of the fluoronitrobenzenes for an electrophilic attack (i.e., *E*(LUMO)) and the calculated relative heat of formation for formation of the respective Meisenheimer complex intermediate (ΔΔHF). In addition, the regioselectivity of the reaction was determined and compared. The results obtained strongly support the conclusion that chemical reactivity of the fluoronitrobenzenes is the main factor determining the outcomes of their conversion by all glutathione *S*-transferase enzymes. The regioselectivities vary only a few percent from one enzyme to another, whereas QSAR lines for all purified enzymes are in the same region and run parallel. This indicates that in the overall reaction the nucleophilic attack of the thiolate anion on the fluoronitrobenzenes, leading to formation of the Meisenheimer complex, is the rate-limiting step in the overall catalysis. The fact that chemical reactivity of the fluoronitrobenzenes is the main factor in setting the outcomes of the overall conversion by the different glutathione *S*-transferase enzymes implies that extrapolation from rat to results of other species including man, and also from one individual to another, must be feasible. That this is actually the case is clearly demonstrated by the results of the present study.

Introduction

Upon mammalian exposure to halogenated nitrobenzene derivatives, these compounds are known to be metabolized by glutathione *S*-transferases before they can be excreted as mercapturic acids from the body. The glutathione *S*-transferase catalyzed conversion of halogenated nitrobenzenes is known to proceed by an aromatic nucleophilic substitution reaction which proceeds through formation of a so-called σ or Meisenheimer complex intermediate (*1*–*3*). Studies with substituted 1-chloro-2-nitrobenzenes have demonstrated that formation of this σ complex might be the rate-limiting step in overall catalysis (*1*). A previous study (*4*) described quantitative structure–activity relationships (QSAR's)[1] for the overall rate of conjugation of a series of fluoronitrobenzenes catalyzed by rat cytosolic glutathione *S*-transferases. The natural logarithm of the rate of conjugation of the series of fluoronitrobenzenes correlated with the calculated energy (*E*) of their lowest unoccupied molecular orbital (LUMO) ($r = -0.986$) and also with the calculated relative heat of formation (ΔΔHF) for formation of the Meisenheimer complex of the fluoronitrobenzenes with a MeS^- model nucleophile ($r = -0.987$). These QSAR's for the enzyme catalyzed glutathione conjugation of the fluoronitrobenzenes indicated that the interaction between the thiolate anion of glutathione and the fluoronitrobenzene, leading to the Meisenheimer reaction intermediate, is the rate-limiting step in the overall enzymatic conversion of these substrates. The QSAR's also indicated that in the enzymatic reaction the chemical reactivity of the substrates is of major importance. Furthermore, the fact that the QSAR's for the chemical reaction between GS^- and the fluorinated nitrobenzenes were parallel to the QSAR for the enzymatic conversion, and the observation that regioselectivities in the enzyme catalyzed and the chemical reactions only varied by a few percent, further supported the overall importance of the role of chemical reactivity of the fluoronitrobenzenes in their glutathione *S*-transferase catalyzed conversion.

If chemical reactivity of the fluoronitrobenzenes is indeed a main factor in setting the outcomes of their conversion by glutathione *S*-transferases, it can be argued that results obtained in studies with Wistar rats will be relevant for other species, including man.

* Address correspondence to this author at the Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands. Phone: 31-317-482868; Fax: 31-317-484801.
† Agricultural University, Wageningen.
‡ TNO Nutrition, Zeist.
§ University Hospital, Nijmegen.
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[1] Abbrevations: QSAR's, quantitative structure activity relationships; *LUMO*, lowest unoccupied molecular orbital; *HOMO*, highest occupied molecular orbital; ΔΔHF, calculated relative heat of formation; CDNB, 1-chloro-2,4-dinitrobenzene.

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Table 1. Some Characteristics of the Patients of Which Liver Specimens Were Obtained

batch	gender	age yr	histology liver	alcohol (U/day)	smoking	medication
A	female	65	normal	no	no	asparine monohydrate
B	male	63	steatosis ±	2	no	no
C	male	61	normal	1	no	no
D	female	66	steatosis ± fibrosis +	no	no	no
E	male	67	normal	4	no	no
F	female	60	steatosis +	no	no	no

The objective of the present study was to investigate this hypothesis. This was done by studying the in vitro metabolism of the series of fluoronitrobenzenes, using cytosolic preparations as well as purified enzymes from both rat and human origin.

Materials and Methods

Chemicals. 2-Fluoronitrobenzene was obtained from Aldrich Chemie (Steinheim, Germany). 2,4-Difluoronitrobenzene, 2,4,6-trifluoronitrobenzene, and 2,3,4,6-tetrafluoronitrobenzene were purchased from Fluorochem (Derbyshire, U.K.).

NMR Measurements. Proton decoupled ^{19}F-NMR measurements of cytosolic incubations were performed on a Bruker AMX 300 spectrometer as described before (*5*, *6*). Between 500 and 1000 scans were recorded. The sample volume was 1.725 mL containing 100 μL of $^{2}H_2O$ for locking the magnetic field and 25 μL of a 8.4 mM 4-fluorobenzoic acid solution, added as internal standard. 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.

Preparation of Cytosol. Rat cytosol was prepared from the perfused livers of male Wistar rats (400 g) as described before (*7*). Cytosol from human livers (labeled A, B, C, D, E, and F) were prepared in a similar way from specimens from patients who underwent resection of one or two liver segments for metachronous liver metastases of colorectal carcinoma, or for direct involvement of the liver of a renal cell carcinoma (C). In all patients, preoperative liver function tests were normal. Table 1 presents data on gender, age, histology of the liver, alcohol consumption, smoking behavior, and drug intake of the patients of which liver specimens were obtained. Immediately after resection, a representative specimen of normal liver tissue was excised from the resected segment and cooled. All patients had epidural anesthesia, and after introduction (fentanyl citrate, thiopental, vecuroniumbromide), nitrous oxide and isoflurane as anesthetic agents were administered continuously during the operation. All patients gave permission preoperatively for the use of some liver tissue for scientific purposes. Protein content was determined by the method of Lowry et al. (*8*), using bovine serum albumin as the standard. The activity of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation by glutathione *S*-transferases was determined as described by Habig et al. (*9*).

Purification of Glutathione *S*-Transferase Enzymes. Glutathione *S*-transferase enzymes were purified from the liver (rat GST 1-1, 2-2, 3-3, 4-4, human GST A1-1, A2-2, and M1a-1a), kidney (rat GST 7-7), and placenta (human GST P1-1), using *S*-hexylglutathione affinity chromatography, as described previously (*10*). The separation of the different enzymes was achieved by chromatofocusing on polybuffer exchangers (Pharmacia, Upsala, Sweden) as described previously (*11*). The rat recombinant GST 5-5 was purified from a culture of *Escherichia coli* JM 105 containing the pKK233-2 plasmid with the GST construct (kindly provided by Prof. B. Ketterer, Research Campaign Molecular Toxicology Group, London, U.K.), as previously described (*12*). Purity of all enzymes was confirmed by HPLC analysis according to Ostlund Farrants et al. (*13*), using the modified method of Bogaards et al. (*14*) and isoelectric focusing (*15*).

Determination of Cytosolic Glutathione *S*-Transferase Subunits. After purification of the cytosolic glutathione *S*-transferases on an *S*-hexylglutathione affinity column, the separation of the glutathione *S*-transferase subunits was carried out by reversed-phase high-pressure liquid chromatography performed as described by Bogaards et al. (*14*). It is of importance to notice that, in contrast to the rat liver sample, for the human samples the recovery of the glutathione *S*-transferases after isolation on the S-hexyl affinity column was 64−84% instead of 95%. Most likely this is due to the fact that the presence of hemoglobin in the unperfused human hepatic samples inhibits the binding of the glutathione *S*-transferases on the *S*-hexylglutathione affinity column and causes the relatively high loses of glutathione *S*-transferase activity in the human samples.

For HPLC experiments on separation of the subunits, a Vydac 201 TP 5 (200 × 3 mm; 5 μm) RP-18 column was used. The sample volume was 50 μL. Elution of the glutathione *S*-transferase subunits was obtained by a gradient of 0.1% trifluoroacetic acid in nanopure (eluent A) and 0.1% trifluoroacetic acid in acetonitrile (eluent B). From 0 to 18 min a linear gradient of 35−45% eluent B was applied, followed by a linear gradient from 45% to 55% eluent B from 18 to 23 min and isocratic elution at 55% eluent B from 23 to 30 min, and the flow rate was 0.6 mL/min. For identification and quantification, a reference mix containing 0.2 mg of each purified enzyme/mL was used. Detection was at 214 nm.

In Vitro Incubations. Glutathione *S*-transferase catalyzed conversion was studied in incubations containing (final concentrations) 0.1 M potassium phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM glutathione (reduced form) (Sigma St. Louis, MO, USA), 0.1−5.0 mg of cytosolic protein/mL or 10 μg−2.0 mg of enzyme/mL (depending on the activity with the respective substrate), and 0.1−20 mM of the fluoronitrobenzene (depending on the apparent K_m), added as 1% of a 100 times concentrated stock solution in dimethyl sulfoxide. The reaction was started by the addition of the fluoronitrobenzene and carried out at 37 °C for 40 min for 2-fluoronitrobenzene, 25 min for 2,4-difluoronitrobenzene, 10 min for 2,4,6-trifluoronitrobenzene, and 2 min for 2,3,4,6-tetrafluoronitrobenzene. At respectively 4, 2, and 1 min and 10 s time intervals, depending on the activity observed, 100 μL samples were taken from the incubation mixture, mixed with 15 μL of 33% trichloroacetic acid to stop the reaction, and assayed for GSH content using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) (Boehringer Mannheim, FRG) (*1*). Time intervals were chosen in such a way that 10 samples were taken over the time needed for use of about 20% of the glutathione present. From the linear decrease in time of the GSH content, the glutathione *S*-transferase catalyzed activity was calculated. The chemical reaction rate between glutathione and the fluoronitrobenzenes at pH 6.5 was <0.1% of the overall rate of conversion. Cytosolic incubations for ^{19}F-NMR analysis were similar, containing 1 mg/mL cytosolic protein or 1−10 ng/mL purified enzyme, 3 mM fluoronitrobenzene, and 2 mM GSH. Incubation was at 37 °C, but the incubation time varied with the fluoronitrobenzene derivative, being 1 min for 2,3,4,6-tetrafluoronitrobenzene, 5 min for 2,4,6-trifluoronitrobenzene, and 25 min for 2,4-difluoronitrobenzene for the cytosolic incubations. For the enzyme incubations with the same substrates the incubation times were 3, 30, and 240 min, respectively. The reaction was stopped by freezing the sample into liquid nitrogen. Samples for ^{19}F-NMR analysis were stored at −20 °C until analysis. Identification of the fluoroglutathionylnitrobenzene metabolites was based on previous investigations (*4*).

Molecular Orbital Calculations. Molecular orbital calculations were carried out on a Silicon Graphics Indigo^2 using

Insight II (Biosym, San Diego, CA, USA). The semiempirical molecular orbital method was used, applying the AM1 or the PM3 Hamiltonian from the MOPAC program. Because the results obtained with the PM3 Hamiltonian were in all cases similar to those obtained with the AM1, only the results of the AM1 calculations are presented. 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 BFGS criteria. Electrophilic reactivity of the fluoronitrobenzenes was characterized by the calculated energy of their LUMO (*17, 18*). In the present study, the outcomes of the semiempirical calculations on molecules in vacuum are related to the electronic characteristics of the substrates in the active site of the glutathione *S*-transferases. Due to solvation effects and a different dielectric constant, the intrinsic properties of the compounds might be influenced upon binding to this active site. However, it is assumed that this phenomenon will not influence the relative differences of parameters between a series of closely related compounds or between similar centers within one molecule, to a significant extent. The outcomes of the in vacuum computer calculations can thus be used as an approach to study relative differences within a series of related compounds (*4, 19, 20*) or within one molecule (*6*).

Relative heats of formation, i.e., ΔΔHF values, were calculated as follows. Using the AM1 (or PM3) Hamiltonian, the heats of formation for the various fluoronitrobenzenes as well as for the various MeS^- Meisenheimer complexes were calculated. The heat of formation of a fluoronitrobenzene was then subtracted from the heat of formation for its Meisenheimer MeS^- complex, leading to a ΔHF value. The ΔHF values thus obtained were set to a relative scale in which the lowest ΔHF was set to zero, thus providing the relative ΔHF, i.e., the ΔΔHF values. The ΔΔHF values, calculated from the computer programs in kcal/mol, were converted to eV to provide values in the same unit as used for the *E*(LUMO). For 2,4-difluoro-, 2,4,6-trifluoro-, and 2,3,4,6-tetrafluoronitrobenzene, more than one Meisenheimer complex can be formed. For these compounds a weighted average ΔΔHF was calculated, using a weight factor for the various Meisenheimer complexes that was based on the regioselectivity observed for the glutathione conjugation.

Calculation of Log $P_{octanol}$. Log $P_{octanol}$ values were calculated as described by Rekker and De Kort (*21*).

Results

Characterization of Glutathione *S*-Transferase Subunits in Rat and Human Hepatic Cytosol. Figure 1 shows the HPLC patterns that characterize the glutathione *S*-transferase subunit composition of the human cytosolic samples as well as of the rat cytosolic sample used in the present study. The subunit composition of rat glutathione S-tranferase enzymes was as follows (expressed as μg/mg of cytosolic protein), 5.2 μg/mg subunit 4, 7.6 μg/mg subunit 3, 8.9 μg/mg subunit 2, and 13.1 μg/mg subunit 1. The specific activity toward CDNB of this sample was 1.8 $\mu mol \cdot min^{-1} \cdot (mg\ of\ cytosolic\ protein)^{-1}$. These values are in the same range as published previously (*14,22*). Also similar to previously reported results (*24, 25*) is the fact that the human liver specimens show a large variation in glutathione *S*-transferase subunit composition. The most striking differences between the subunit composition of the various samples used in this study are the absence of the subunit A2 and the presence of subunit M1b in sample D (3.1 μg/mg of cytosolic protein). The mean level of glutathione *S*-transferase subunit M1a in the four individuals with the GST M1 positive genotype was 6.3 μg/mg of cytosolic protein. The average levels of glutathione *S*-transferase subunits A1 and A2 were 9.7 and 12.0 μg/mg of cytosolic protein, respectively. Significant amounts of subunit P1 were only found in two individuals, A and C (1.2 and 0.5 μg/mg of cytosolic protein, respectively). The expression levels were in the same range as previous published data (*24–25*). Additional experiments demonstrated that the CDNB activity of the human samples varied in the range from 1.31 to 2.00 $\mu mol \cdot min^{-1} \cdot (mg\ of\ cytosolic\ protein)^{-1}$, which is in accordance to a previous study (*25*), in which a range from 0.39–2.17 $\mu mol \cdot min^{-1} \cdot (mg\ of\ cytosolic\ protein)^{-1}$ was reported for the human hepatic samples of 20 individuals.

In *Vitro* Regioselectivity of the Glutathione Conjugation of Fluoronitrobenzenes by Human Cytosolic Glutathione *S*-Transferases. Figure 2 presents the ^{19}F-NMR spectra of human cytosolic incubations (batch A) of fluorinated nitrobenzenes in the presence of glutathione. For 2,4-difluoronitrobenzene (Figure 2a) formation of two metabolites is observed, which can be ascribed to 4-fluoro-2-glutathionylnitrobenzene and 2-fluoro-4-glutathionylnitrobenzene, based on their splitting patterns in ^{19}F- and ^1H-NMR as previously described (*4*). The two metabolites, resulting from glutathione conjugation at C2 and C4 of 2,4-difluoronitrobenzene, are formed in a ratio of 96:4 for this human cytosolic incubation, the reaction at C2 being favored over a reaction at C4. The formation of the two glutathione adducts is accompanied by formation of an amount of fluoride anions that equals the sum of formation of the two fluoroglutathionylnitrobenzenes. Elimination of the nitro moiety, resulting in 2,4-difluorophenylglutathione, is not observed. This can be derived from the observation that, in addition to the parent and fluoride anion peak and the two resonances of the glutathione conjugates, no other fluorine resonances are observed in this spectrum.

Conversion of 2,4,6-trifluoronitrobenzene (Figure 2b) results in formation of 4,6-difluoro-2-glutathionylnitrobenzene and 2,6-difluoro-4-glutathionylnitrobenzene at a ratio of 98:2 for this human cytosolic incubation. Apparently, as for 2,4-difluoronitrobenzene, formation of the metabolite resulting from conjugation at one of the two positions ortho with respect to the nitro moiety is favored over the one resulting from conjugation at the fluorinated para position. The formation of the two glutathione adducts is accompanied by formation of an amount of fluoride anions that equals the sum of formation of the two fluorinated glutathione conjugates.

Figure 2c presents the ^{19}F-NMR spectrum of a human cytosolic incubation with 2,3,4,6-tetrafluoronitrobenzene, where formation of 3,4,6-trifluoro-2-glutathionylnitrobenzene and 2,3,6-trifluoro-4-glutathionylnitrobenzene can be observed at a ratio of 91:9, accompanied by formation of a corresponding amount of fluoride anions. Formation of 2,3,4-trifluoro-6-glutathionylnitrobenzene and 2,4,6-trifluoro-3-glutathionylnitrobenzene is not observed. Table 2 summarizes the regioselectivities thus obtained for the glutathione conjugation of the various fluoronitrobenzenes by the human liver cytosolic samples of six individuals. In spite of the relatively large differences in the enzyme pattern (Figure 1), the actual regioselectivity of the glutathione conjugation does not vary significantly for the various cytosolic preparations, i.e., at most a few percent.

Regioselectivity of the Glutathione Conjugation of Fluoronitrobenzenes by Purified Glutathione *S*-Transferase Enzymes. Results for the regioselectivity of glutathione conjugation by cytosolic preparations showed limited variation between the different cytosolic preparations, in spite of differences in their enzyme

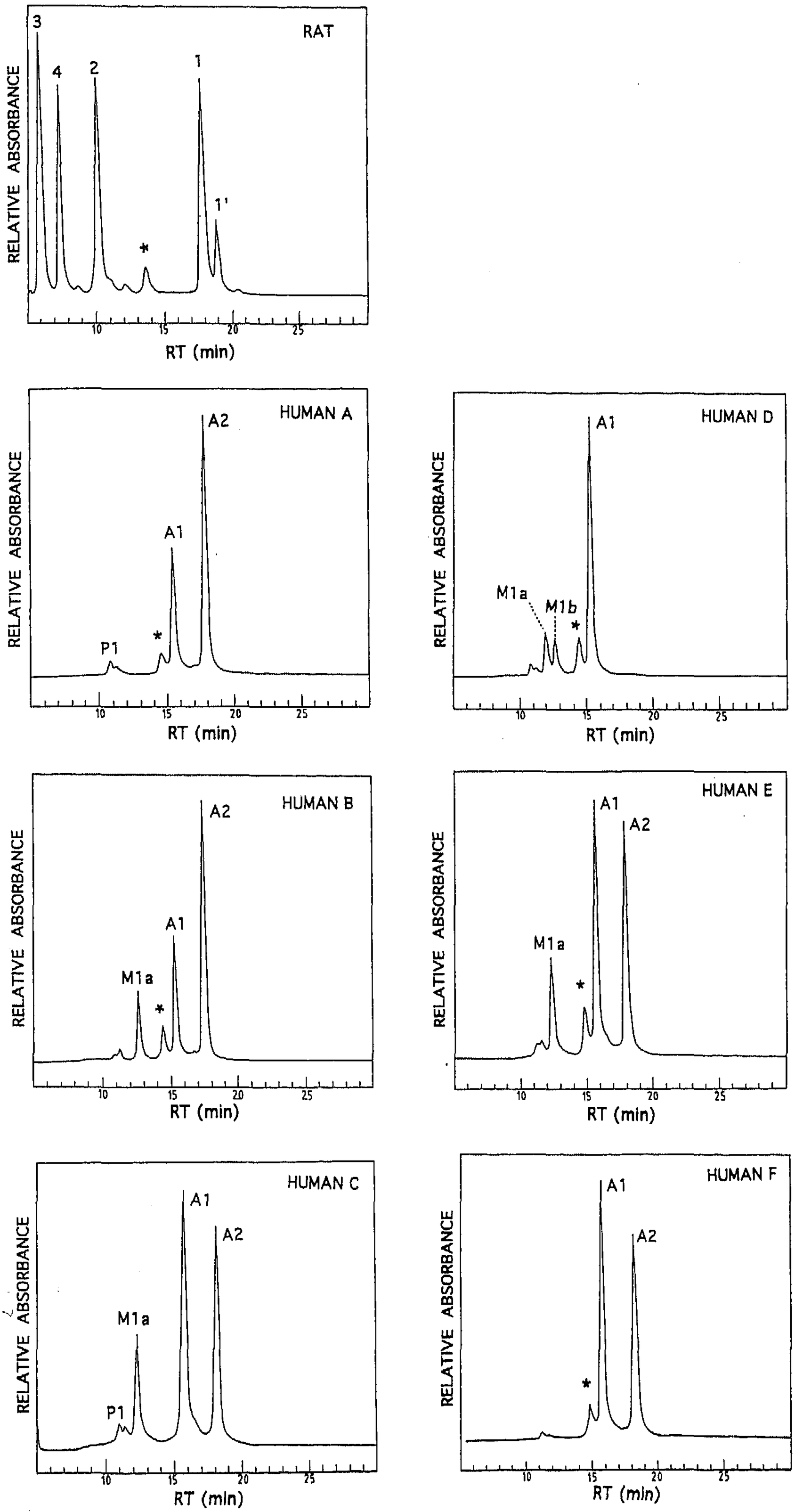

Figure 1. HPLC chromatograms of glutathione enzyme subunit composition of rat and six human cytosolic samples. The nomenclature of the human glutathione *S*-transferases is according to Mannervik et al. (*23*). The peaks marked with an asterisk remain unidentified. The detection was at 214 nm.

pattern. This indicates that either one enzyme, that is relatively equally important in the various cytosolic samples, is responsible for the major part of the conversion, or that various enzymes are catalyzing the conjugation, all with an almost similar regioselectivity. To investigate this in more detail, the regioselectivity of the glutathione conjugation was determined using purified human and rat glutathione *S*-transferase enzymes. The

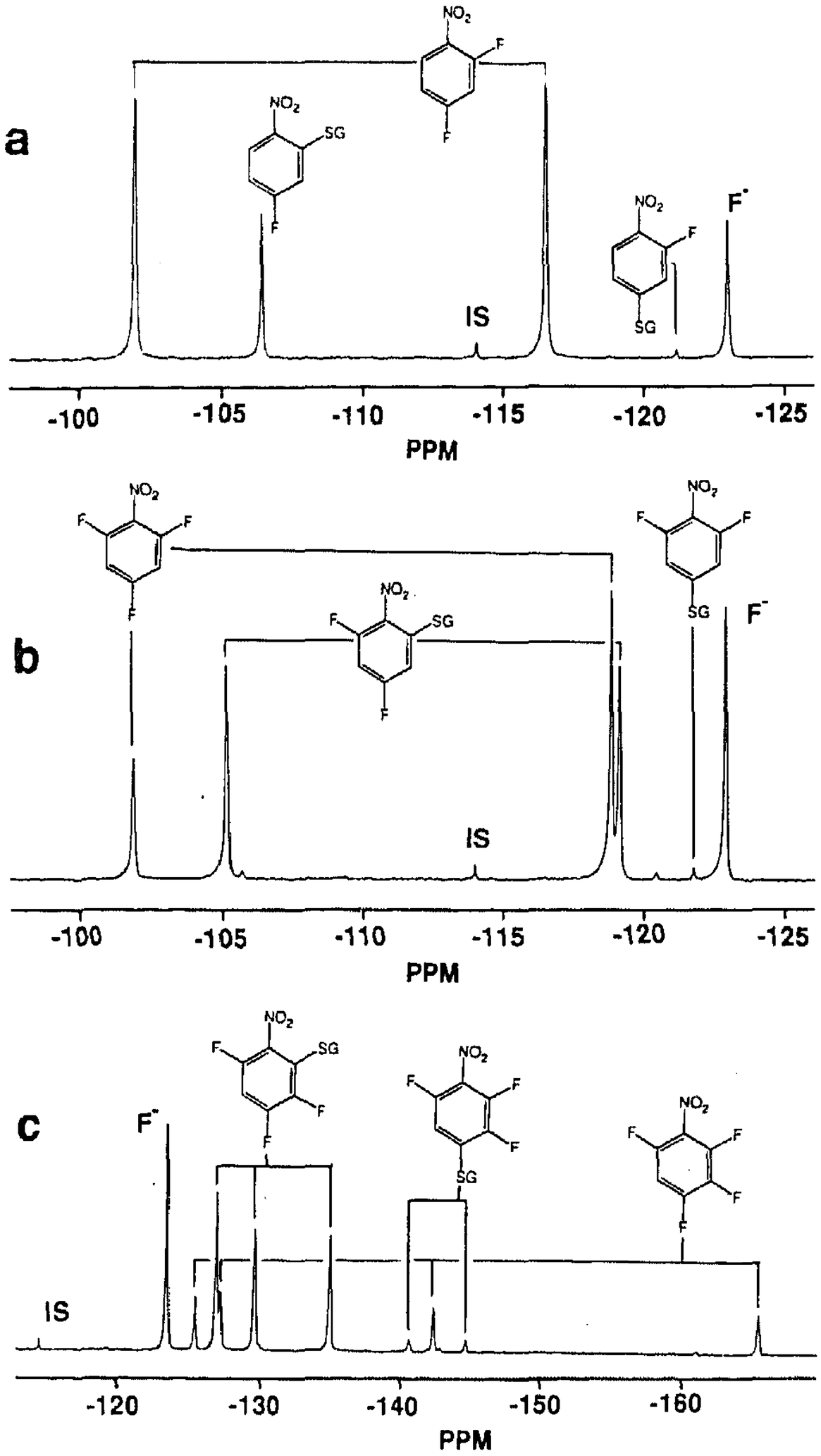

Figure 2. ^{19}F-NMR spectra of glutathione (GSH) containing human cytosolic incubations with (a) 2,4-difluoronitrobenzene, (b) 2,4,6-trifluoronitrobenzene, and (c) 2,3,4,6-tetrafluoronitrobenzene. The resonance marked with IS is from the internal standard 4-fluorobenzoic acid. The third resonance of 2,3,6-trifluoro-4-glutathionylnitrobenzene at −126.8 ppm (panel c) is masked by one of the resonances of the other fluorinated compounds. The minor peaks with the resonances −105.8, −109.4, and -120.6 ppm (panel b) and −119.0, −142.6, and −160.6 ppm (panel c) were not identified.

results obtained, derived from ^{19}F-NMR analysis of the incubations, are summarized in the Tables 2 and 3. As for the cytosolic preparations, all human enzymes show a preferential glutathione conjugation at the position ortho with respect to the nitro moiety. The main difference between the various human enzymes is that human enzyme P1-1 shows a slightly decreased ratio in ortho over para glutathione conjugation for all nitrobenzenes. However, because P1-1 is present in a relatively low concentration in the cytosolic preparations (Figure 1), this does not influence the cytosolic ortho over para conjugation in a significant way. No remarkable differences are observed between the ortho over para glutathione conjugation between the purified enzymes A1-1, A2-2, and M1a-1a. Because of the high contribution of these three enzymes to the composition of the human cytosolic samples (Figure 1), the overall regioselectivity of the human cytosolic samples reflects the regioselectivity of these enzymes.

Table 2. Regioselectivity of Glutathione *S*-Transferase Catalyzed Glutathione Conjugation of Fluorinated Nitrobenzenes in Vitro by Human Liver Cytosol of Six Individuals and Purified Glutathione *S*-Transferase Enzymes from Human Tissue at pH 6.5[a]

nitrobenzene	conjugation at center	human liver cytosol		human enzyme	
2,4-difluoro-	C2:C4	A	96:4	A1-1	90:10
		B	100:0	A2-2	99:1
		C	95:5	M1a-1a	98:2
		D	92:8	P1-1	81:19
		E	96:4		
		F	96:4		
2,4,6-trifluoro-	C(2+6):C4	A	98:2	A1-1	91:9
		B	99:1	A2-2	98:2
		C	97:3	M1a-1a	98:2
		D	96:4	P1-1	85:15
		E	98:2		
		F	95:5		
2,3,4,6-tetrafluoro-	C2:C4	A	91:9	A1-1	92:8
		B	91:9	A2-2	95:5
		C	90:10	M1a-1a	93:7
		D	88:12	P1-1	83:17
		E	93:7		
		F	93:7		

[a] ^{19}F-NMR spectroscopy was used for identification and quantification of the glutathione conjugates ($n = 1-2$).

Table 3. Regioselectivity of Glutathione *S*-Transferase Catalyzed Glutathione Conjugation of Fluorinated Nitrobenzenes in Vitro by Rat Liver Cytosol and Purified Glutathione *S*-Transferase Enzymes from Rats at pH 6.5[a]

nitrobenzene	conjugation at center	rat liver cytosol	rat enzyme	
2,4-difluoro-	C2:C4	66:34	1-1	66:34
			2-2	96:4
			3-3	82:18
			4-4	80:20
			5-5	89:11
			7-7	84:16
2,4,6-trifluoro-	C(2+6):C4	82:18	1-1	84:16
			2-2	94:6
			3-3	91:9
			4-4	87:13
			5-5	93:7
			7-7	77:23
2,3,4,6-tetrafluoro-	C2:C4	77:23	1-1	77:23
			2-2	90:10
			3-3	88:12
			4-4	89:11
			5-5	94:6
			7-7	85:15

[a] ^{19}F-NMR spectroscopy was used for identification and quantification of the glutathione conjugates ($n = 1-2$).

Table 3 also summarizes the regioselectivities obtained for the conjugation of the fluoronitrobenzenes by purified glutathione *S*-transferases as compared to rat liver cytosol. From the data obtained, it follows that the preferential site of glutathione conjugations is again at the positions ortho with respect to the nitro moiety for all fluorinated nitrobenzenes. From the data obtained, it can be concluded that the enzymes 1-1, 4-4, and 7-7 have a somewhat increased para glutathione conjugation. Forty percent of the glutathione *S*-transferase subunits of the cytosolic population consists of subunit 1 and 15% of subunit 4, which explains that the regioselectivities observed for the incubations with cytosol are approximately the same as observed for the incubations with these enzymes. Comparison of the results obtained from rat enzymes to those for human enzymes shows a

Table 4. Apparent V_{max} and K_m Values for the Overall Conversion of Fluoronitrobenzenes by Cytosolic and Purified Human Glutathione S-Transferases at 1 mM GSH (pH 6.5)

protein	V_{max} (nmol·mg^{-1}·min^{-1})	K_m (mM)
	2-Fluoronitrobenzene	
human A	2.5	4.5
human D	2.5	3.1
human F	1.8	4.9
enzyme A1-1	194	12.9
enzyme M1a-1a	83	13.5
	2,4-Difluoronitrobenzene	
human A	40	5.3
human D	38	5.3
human F	35	5.5
enzyme A1-1	861	5.9
enzyme M1a-1a	487	7.2
	2,4,6-Trifluoronitrobenzene	
human A	200	2.9
human D	102	3.0
human F	186	3.4
enzyme A1-1	4353	6.1
enzyme M1a-1a	1706	7.5
	2,3,4,6-Tetrafluoronitrobenzene	
human A	6866	0.8
human D	2288	2.2
human F	3574	0.4
enzyme A1-1	38621	1.8
enzyme M1a-1a	21852	2.1

somewhat larger extent of para conjugation for the rat than for the human enzymes. However, in all cases a reaction at the ortho position is highly favored over a reaction at the para position for all enzymes studied, suggesting a preferential influence of factors other than a regioselective orientation of the substrate by the active site of the glutathione *S*-transferases.

Kinetic Characteristics of the Conversion of the Series of Fluoronitrobenzenes by Cytosolic Glutathione S-Transferases and Purified Glutathione S-Transferase Enzymes. In addition to the regioselectivities, the kinetic characteristics of the conversion of the fluoronitrobenzenes by the various glutathione *S*-transferases were investigated. Table 4 presents the results from the kinetic experiments characterizing the apparent V_{max} and K_m for the series of fluoronitrobenzenes in human cytosolic and purified human hepatic glutathione *S*-transferase enzyme incubations with GSH. From the data obtained, it can be concluded that generally the apparent K_m decreases whereas the apparent V_{max} rises substantially with increasing number of fluorine substituents. Figure 3a shows that the change in the mean apparent K_m ($n = 5$) values for human cytosolic and enzyme incubations correlates quantitatively ($r = -0.984$) with the log $P_{octanol}$ for the fluoronitrobenzenes. Table 5 presents the kinetic data obtained in incubations with cytosolic and purified glutathione *S*-transferase enzymes from rat hepatic tissue. In analogy to the results obtained with human enzyme incubations, the apparent K_m decreases whereas the apparent V_{max} rises substantially with increasing number of fluorine substituents. The correlation between the mean apparent K_m values ($n = 3$) for rat material and the log $P_{octanol}$ for the nitrobenzenes is presented in Figure 3b ($r = -0.980$). The negative correlations between the log $P_{octanol}$ of the fluoronitrobenzenes and the apparent K_m values obtained indicates that the apparent K_m values decrease upon increased hydrophobicity (increased log $P_{octanol}$) of the fluoronitrobenzene substrates and it confirms our previously reported QSAR between hydrophobicity of the substrates, and apparent K_m values obtained with rat cytosolic incubations at pH 7.6 (*4*).

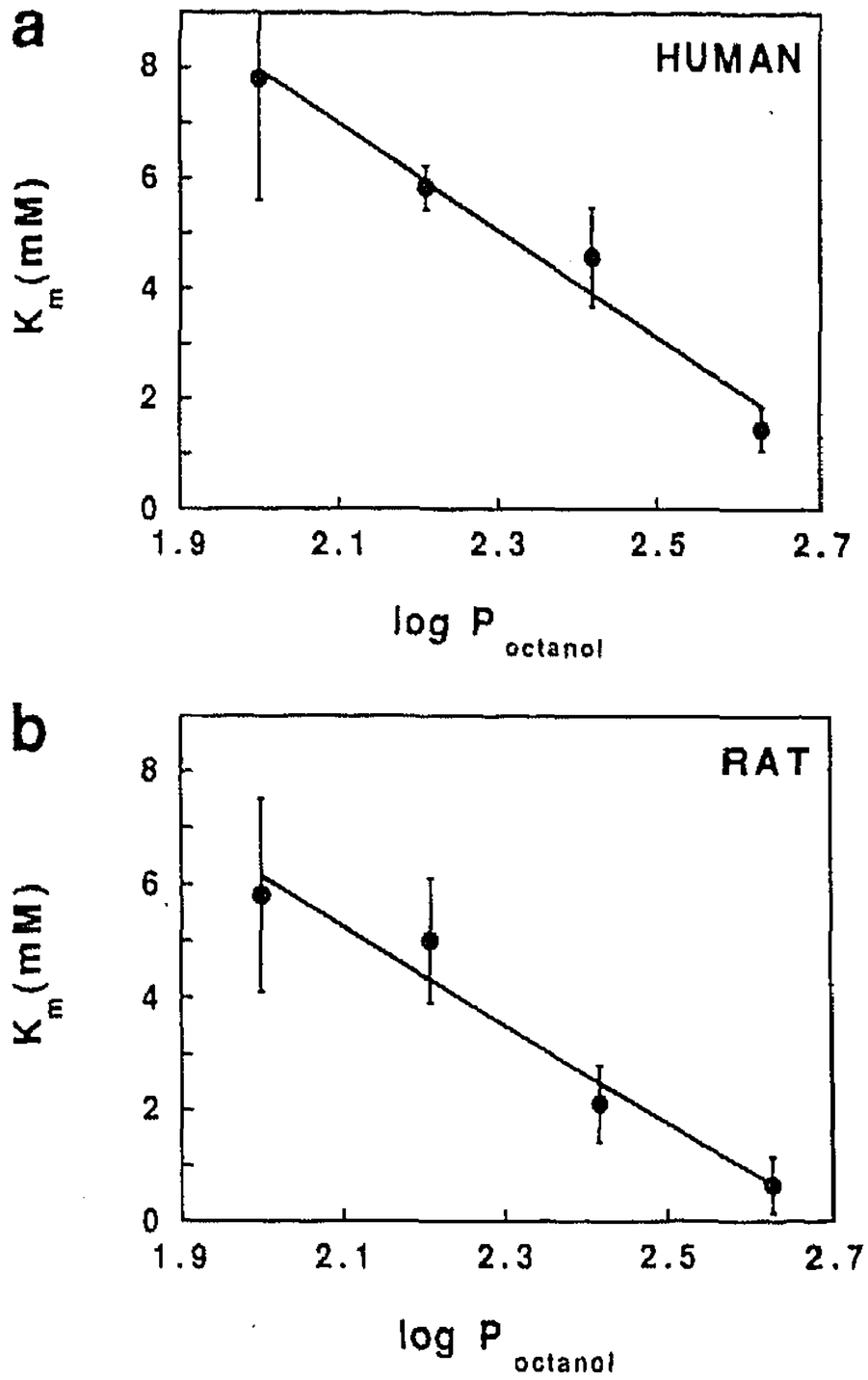

Figure 3. QSAR's describing the quantitative structure–activity relationships between the mean apparent $K_m \pm$ SEM for conversion of a series of fluorinated nitrobenzenes by cytosolic glutathione *S*-transferases and purified glutathione *S*-transferase enzymes of human ($n = 5$) and rat ($n = 3$) origin and their log $P_{octanol}$. The correlation coefficients of the QSAR's are respectively −0.984 and −0.980.

Table 5. Apparent V_{max} and K_m Values for the Overall Conversion of Fluoronitrobenzenes by Cytosolic and Purified Rat Glutathione S-Transferase at 1 mM GSH (pH 6.5)

protein	V_{max} (nmol·mg^{-1}·min^{-1})	K_m (mM)
	2-Fluoronitrobenzene	
rat cytosol	2.0	2.9
enzyme 1-1	114	5.7
enzyme 3-3	79	8.7
	2,4-Difluoronitrobenzene	
rat cytosol	5	5.0
enzyme 1-1	846	4.8
enzyme 3-3	1869	1.95
	2,4,6-Trifluoronitrobenzene	
rat cytosol	156	1.8
enzyme 1-1	3786	3.4
enzyme 3-3	5078	1.2
	2,3,4,6-Tetrafluoronitrobenzene	
rat cytosol	1076	0.16
enzyme 1-1	35722	1.6
enzyme 3-3	42292	0.2

Molecular Orbital Substrate Characteristics for Electrophilic Attack; Description of MO-QSAR's. Our previous investigations demonstrated a clear correlation between the natural logarithm of the V_{max} and the calculated energy of the LUMO of the various fluoronitrobenzenes. The LUMO is the substrate parameter characteristic for its electrophilic reactivity because it is the orbital that will interact with the most reactive electrons in the highest occupied molecular

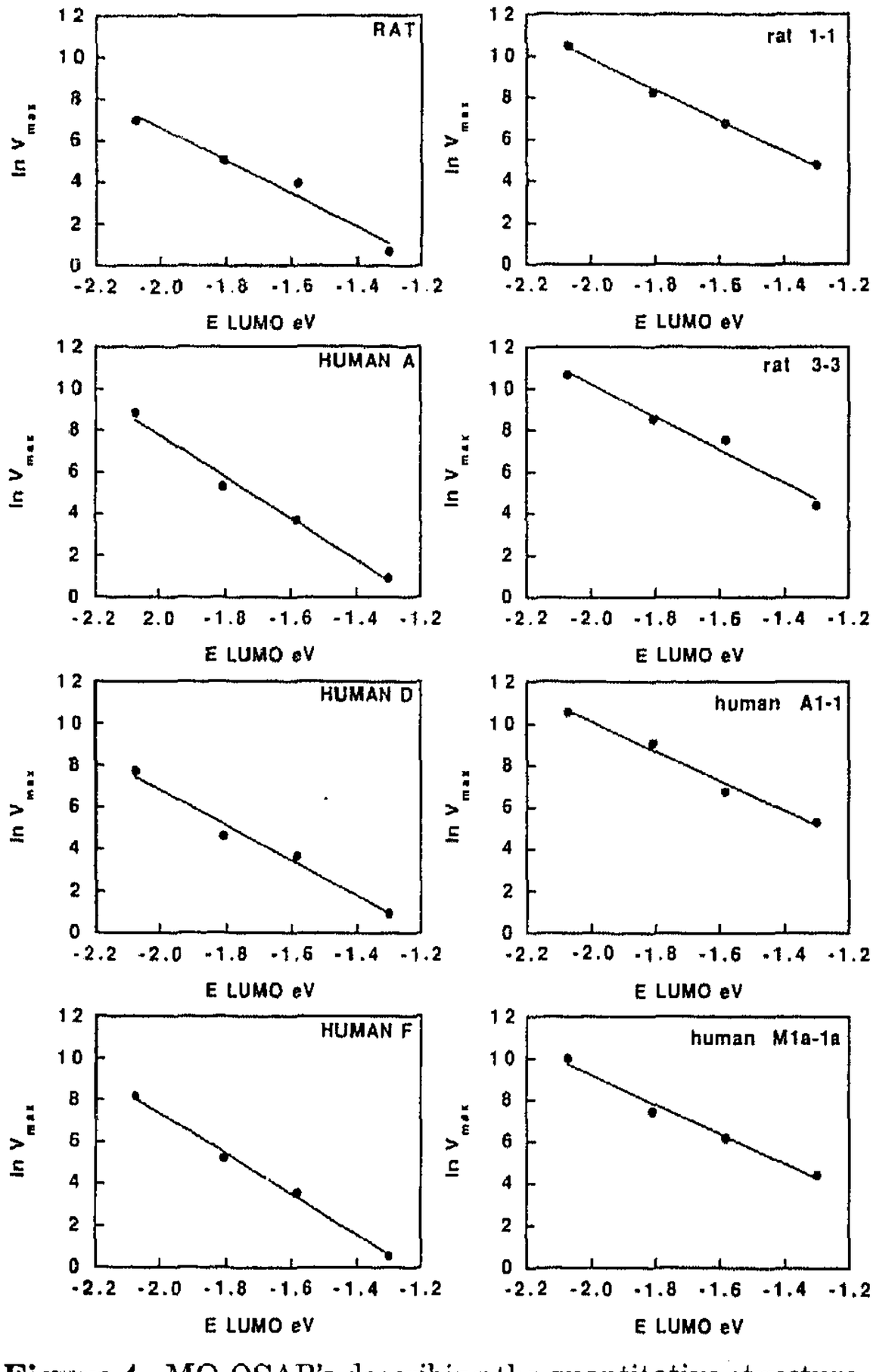

Figure 4. MO-QSAR's describing the quantitative structure–activity relationships between the natural logarithm of the apparent V_{max} for glutathione *S*-transferase catalyzed conjugation of a series of fluorinated nitrobenzenes and the *E*(LUMO) in eV of the compounds. The correlation coefficients of the MO-QSAR's vary from −0.985 to −0.999.

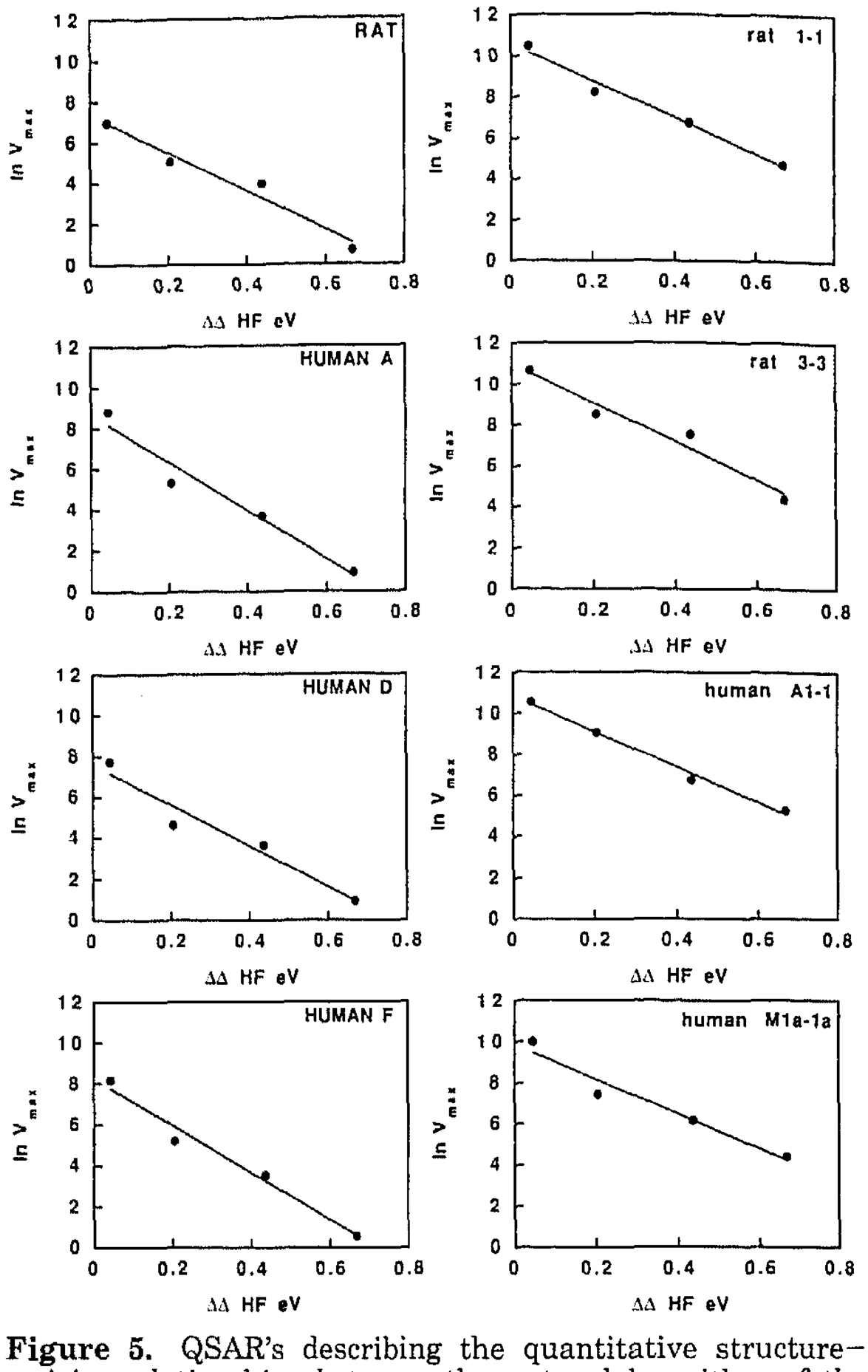

Figure 5. QSAR's describing the quantitative structure–activity relationships between the natural logarithm of the apparent V_{max} for glutathione *S*-transferase catalyzed conjugation of a series of fluorinated nitrobenzenes and the calculated relative ΔΔHF for formation of the MeS^- Meisenheimer complex taken as a model representative for the transition state.

orbital (HOMO) of the glutathione thiolate anion. The results from the calculations of the energy of the LUMO orbitals of the substrates were respectively −1.35, −1.58, −1.81, and −2.07 eV for 2-fluoro-, 2,4-difluoro-, 2,4,6-trifluoro-, and 2,3,4,6-tetrafluoronitrobenzene, demonstrating a decrease in energy of the LUMO with increasing number of fluorine substituents, pointing at increased electrophilic reactivity of the fluoronitrobenzenes with increased number of fluorine substituents. Figure 4 shows the linear correlations between the calculated energy of the LUMO from the various fluoronitrobenzenes and the natural logarithm of V_{max} for incubations with rat and human hepatic cytosolic protein, as well as for incubations with purified rat and human glutathione *S*-transferase enzymes. The correlation coefficients of the QSAR's vary from −0.985 to −0.999, and the lines are, within experimental errors, all parallel.

Relative Heats of Formation ΔΔHF of the Meisenheimer Complexes. Figure 5 presents the QSAR's obtained when the natural logarithm of the V_{max} for the rat and human cytosolic samples and purified enzymes is plotted against the weighted ΔΔHF values calculated for the formation of the Meisenheimer complex. The weighted average ΔΔHF values were calculated as previously described (*4*), using the results for the regioselectivity of the glutathione conjugation presented in the Tables 2 and 3. The linear correlation coefficients of the ΔΔHF-QSAR's vary from −0.977 to −0.996. As for the MO-based QSAR's of Figure 4, the lines of all ΔΔHF-QSAR's presented in Figure 5 run parallel to one another. The fact that the intercepts of the QSAR's for cytosolic samples differ from those obtained for the purified enzymes relates to the fact that the protein in the cytosolic preparations consists of only 2−5% of glutathione *S*-transferase, leading to lower V_{max} values when expressed per milligram of protein.

Discussion

In the present study it was investigated whether QSAR's which correlate the characteristics of a biotransformation reaction to calculated chemical parameters of the compounds converted can be used as a basis to predict the outcomes of the biotransformation in other species or by other enzymes than the one(s) for which the QSAR is described.

To this purpose, it was investigated whether the QSAR's previously described for the conversion of a series of fluoronitrobenzenes by a preparation of rat cytosolic glutathione *S*-transferases (*4*) can be extrapolated to systems in which the same fluoronitrobenzenes are converted by different purified glutathione *S*-transferase enzymes from rat but also from man. It is demonstrated that, for all enzymes studied, the natural logarithm of the rate of conversion of the fluoronitrobenzenes correlates with both the calculated reactivity of the fluoro-

nitrobenzenes for an electrophilic attack (i.e., E(LUMO)) and the calculated relative heat of formation for formation of the respective Meisenheimer complex intermediate ($\Delta\Delta$HF). Furthermore, the QSAR lines for all purified enzymes run parallel to one another as well as to the QSAR's previously reported for the chemical reaction of glutathione with the various fluoronitrobenzenes at different pH values (*4*). This indicates that in the overall reaction the nucleophilic attack of the thiolate anion formed from glutathione in the active site of glutathione *S*-transferases (*26*–*28*), on the fluoronitrobenzenes, leading to formation of the Meisenheimer complex, is the rate-limiting step in the overall catalysis. Differences between the enzymes studied, for example, in (i) the distance between the sulfur atom of the thiolate anion of glutathione and the reaction center in the fluoronitrobenzene, or in (ii) the extent of deprotonation of the –SH moiety of glutathione, or in (iii) the local dielectric constant, influenced by the various either hydrophobic or hydrophilic amino acid residues surrounding the active site, are of relatively smaller influence on the overall rate of catalysis by the various rat and human enzymes than the actual reactivity of the fluoronitrobenzene substrate. As a result, the difference in V_{max} for conversion of a specific fluoronitrobenzene by the different enzymes studied (rat 1-1, rat 3-3, human A1-1, human M1a-1a) varies by a factor of at most 2-fold, leading to QSAR's which run parallel and over a range of similar V_{max} values. The observation that in the crystal structures of human A1-1 (*29*) and rat 3-3 (*30*) the distance between the oxygen of the tyrosine, assumed to be involved in the deprotonation of the glutathione SH moiety, and the sulfur atom of GSH is almost identical (3.26 Å as compared to 3.23 Å), supports that for instance the extent of deprotonation and, thus, the reactivity of the thiolate anion may not be substantially different between the two enzymes.

In addition, in the present study the regioselectivity of the glutathione conjugation of the fluoronitrobenzenes by the different enzymes was determined and compared. Again, the results obtained strongly support that the chemical reactivity of the fluoronitrobenzenes is the main factor determining the regioselectivity of their conversion by all the different glutathione *S*-transferase enzymes. The regioselectivities vary only a few percent from one enzyme to another, as well as from the regioselectivities observed for the chemical reaction between glutathione and the fluoronitrobenzenes at various pH values (*4*).

Furthermore, it is relevant to outline that previous studies with 1-chloro-2-nitrobenzenes have demonstrated that the formation of the Meisenheimer complex might indeed be the rate-limiting step for the overall catalysis (*1*), but that for 1-chloro-2,4-dinitrobenzene, converted by glutathione *S*-transferase 3-3 from rat liver, product release has been reported to be rate limiting (*2, 31*). Obviously, for the fluoronitrobenzenes of the present study, with only one nitro moiety, their chemical reactivity for a nucleophilic attack by the glutathione thiolate anion in the active site of enzyme 3-3 and not the release of their product from the enzyme 3-3 appears to be the rate-limiting step in overall catalysis.

The use of molecular orbital computer calculations for characterizing a QSAR for the rate of conversion of a series of substrates by glutathione *S*-transferases provides another approach than the description of QSAR's on the basis of Hammett substituent constants, which is the approach mostly described up to now for the glutathione *S*-transferase catalyzed reactions (*32*).

Finally, it is of importance to notice that the results of the present study demonstrate that the description of QSAR's for the biotransformation of compounds on the basis of calculated chemical reactivity characteristics provides a means to extrapolate data obtained with enzymes from rat to results to be expected for enzymes obtained from other species, including man. It also implies that, although the glutathione *S*-transferase enzyme subunit composition can vary largely from one individual to another (Figure 1) (*24,25*), the outcomes of the conversion of the series of the fluoronitrobenzenes of the present study are mainly determined by the chemical characteristics of the substrate and not by the enzyme characteristics and, thus, are similar for all individuals.

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