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Metabolism of the insecticide teflubenzuron in rats

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1. The metabolic fate of the insecticide teflubenzuron, orally dosed to the male Wistar rat, was investigated. Particular attention was paid to the metabolic fate of the benzoyl and aniline moiety after hydrolysis of the urea bridge.

2. The 0–48-h urinary and faecal metabolic patterns and recoveries showed that for a dose range of 4–53 μmol (1·5–20 mg) teflubenzuron, 90% of the dose was excreted in the faeces mainly in unmodified form, approximately 4·6% was absorbed from the lumen and excreted in the urine, and 5·4% was retained in the body. Metabolites excreted in the urine could be identified as benzoate and aniline derivatives originating from the two aromatic rings of teflubenzuron liberated from the parent molecule by hydrolysis of the urea bridge.

3. The amount of urinary benzoate-type metabolites was about eight times the amount of aniline-type metabolites, indicating significant differences in efficiency of urinary excretion of the benzoate moiety as compared with the aniline ring.

4. To investigate further the possible reason underlying this difference in urinary excretion efficiency between the two aromatic derivatives formed from teflubenzuron, dose–recovery studies of these aniline- and benzoate-type metabolites were performed. These studies confirmed the discrepancy observed between the urinary recovery of the benzoyl and the aniline moiety of teflubenzuron.

5. Additional results of the present study indicate that the above discrepancy can be explained by the fact that the benzoate derivative is excreted mainly in its unmetabolized form, whereas the aniline derivative needs additional phase I and II modifications before it can be excreted from the body, the former being a relatively slow reaction. Furthermore, conversion of the halogenated aniline derivative in phase I metabolism might result in a reactive benzoquinone-type or *N*-oxidized primary metabolite, which can be retained in the body due to reaction with cellular macromolecules.

Introduction

Over the past 20 years the development of fluorinated agrochemicals has increased considerably and an even further increase in fluorinated agrochemicals is foreseen for the years ahead (Banks 1994). Replacement of an atom by a fluorine can change the properties of a chemical and affect its metabolism, chemical reactivity and lipophilicity (Luteijn and Tipker 1986, Banks 1994, Park and Kitteringham 1994).

An example of such a series of fluorinated agrochemicals consists of the benzoylphenyl urea insect growth regulants which act by inhibiting the enzyme responsible for the chitin synthesis in larves (Verloop and Ferell 1977). Diflubenzuron, which is the active component in the product named Dimilin (figure 1), was introduced in 1975 and is used as a larvicide for fruit, cotton and soyabeans, and it was the first benzoylphenyl urea insect growth regulant commercialized. Teflubenzuron, the active component in the insecticide commercialized as Nomolt (figure 1), was introduced in 1984, and is another benzoylphenyl urea larvicide used for the protection of fruit and vegetables.

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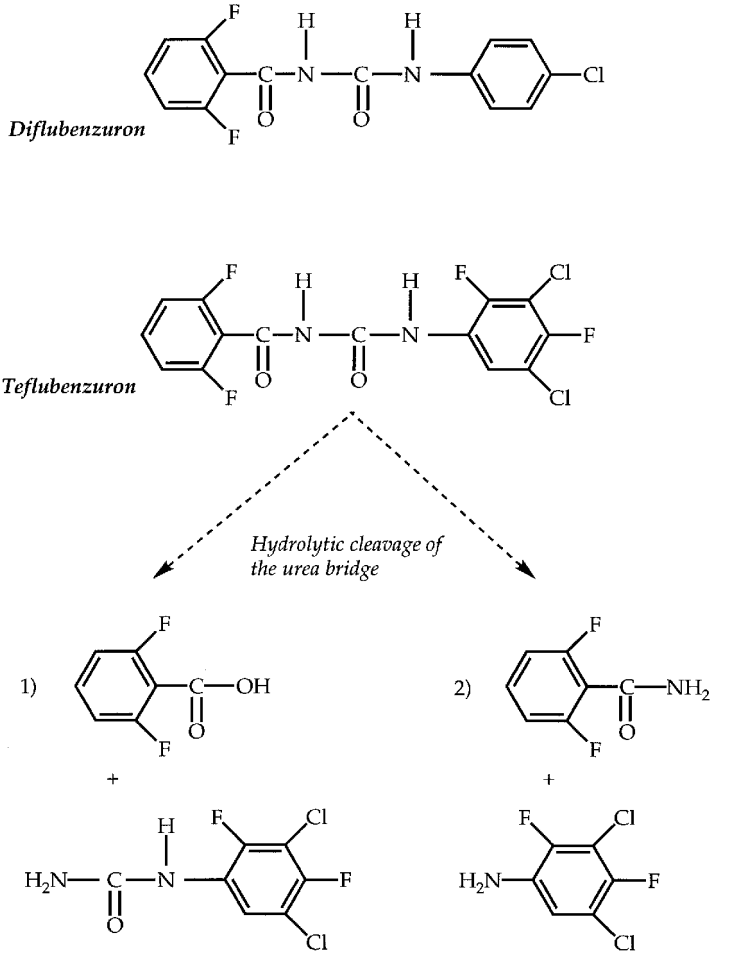

Figure 1. Molecular structure of the insecticides diflubenzuron and teflubenzuron, and the proposed scission products formed by hydrolytic cleavage of the phenylurea bridge of teflubenzuron, pathway 1 being the major route (Verloop and Ferell 1977).

Upon development of an agrochemical it is important not only to investigate they metabolic fate and disposition of the compound in target organisms (Verloop and Ferell 1977, Van Laecke and Degheele 1991) but also in non-target organisms (Metcalf *et al*. 1975, Verloop and Ferell 1977, Ivie 1978, Willems *et al*. 1980, Opdycke *et al*. 1982a). Non-target organisms, including man, are not only exposed to these chemicals upon the manufacture of these compounds but also when these chemicals are released into the environment or are present as a residue in food following crop treatment.

Previous studies on the bioavailability and metabolism of diflubenzuron in various non-target species, i.e. rat, sheep, cattle, swine and chicken, showed that upon a 4–10 mg/kg (13–32 μmol/kg) oral dose of diflubenzuron, > 50% of the dose was excreted unchanged in the faeces (Ivie 1978, Willems *et al*. 1980, Opdycke *et al*. 1982a, b). The remainder of the dose was absorbed from the gastrointestinal tract. Part of the diflubenzuron absorbed was metabolized to conjugated aromatic ring hydroxylated diflubenzuron derivatives and excreted in the bile. The remainder of

the dose absorbed was excreted in the urine mainly as derivatives from products resulting from hydrolysis of the urea bridge, which were identified especially as 2,6-difluorobenzoic acid derived metabolites. However, there were relatively many unidentified metabolites in the urine and a lot of uncertainty remained about the recovery and metabolic fate of the aniline moiety which must also have been formed upon the hydrolytic cleavage of the parent compound. Metabolites of this second scission product, 4-chlorophenylurea containing the aniline ring, could not be detected in the urine or bile although unidentified metabolites observed in the urine were taken as being suggestive of further metabolism of the 4-chlorophenylurea moiety (Willems *et al*. 1980).

Altogether the metabolic fate of benzoylphenylurea insecticides is not well described and especially the fate of the aniline part of the insecticide upon hydrolysis of the urea bridge has not been well investigated. Therefore, the aim of the present study was to investigate the differences in the recovery and metabolic fate of the two aromatic scission products of the benzoylphenyl urea insecticides formed upon hydrolysis of the urea bridge. To achieve this, the benzoylphenyl urea larvicide teflubenzuron was taken as the model compound not only because the presence of fluorine substituents in both the benzoate but also the aniline moiety (figure 1) provides a way actually to detect the metabolic fate of both parts of the molecule, since the fluorine-containing metabolites can be identified and quantified by the use of ^{19}F nmr (Malet-Martino and Martino 1992).

Materials and methods

Chemicals

Teflubenzuron, the active compound in Nomolt (purchased from Shell BV, The Netherlands), was obtained by extracting Nomolt with chloroform in a 1:2 (v/v) ratio. The chloroform phase was evaporated and white powder obtained (7% purity) was dissolved in dimethyl sulphoxide. (3,5-Dichloro-2,4-difluorophenyl)urea (99%) and 3,5-dichloro-2,4-difluoroaniline (99·6%) were a generous gift from Shell Forschung (Schwabenheim, Germany). 2,6-Difluorobenzamide (97%) was purchased from Aldrich Chemie (Steinheim, Germany). 2,6-difluorobenzamide (> 97%) was obtained from Fluorochem (UK). Synthesis of 2,6-difluorobenzoylglycine from 2,6-difluorobenzoic acid was performed according to Vogel (1989). Synthesis of 2-amino-3,5-difluoro-4,6-dichlorophenylsulphate was carried out according to a method for synthesis of *O*-aminophenyl sulphates described by Boyland *et al*. (1953).

In vivo *metabolism*

Male Wistar rats (350–400 g) were exposed to 1–53 μmol (as indicated) teflubenzuron, 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline or (3,5-dichloro-2,4-difluorophenyl)urea, all administered in olive oil with 20% dimethyl sulphoxide by oral gavage. After dosing, 0–24, 24–48-h urine and 0–48-h faeces samples were collected.

For ^{19}F nmr measurement, urine samples were either diluted once in 0·2 M potassium phosphate pH 7·6 or, when necessary, concentrated by freeze-drying and subsequent dissolving in 0·1 M potassium phosphate pH 7·6.

Faeces samples were homogenized by extensive (overnight) stirring in 25 ml ethyl acetate using a magnetic stirrer. The homogenate obtained was filtered over glass mineral wool and the residue washed three times with 25 ml ethyl acetate. The ethyl acetate filtrate was evaporated to dryness. The residue was dissolved in 4 ml ethyl acetate and analysed by ^{19}F nmr.

Acid and enzyme hydrolysis of urine samples

In order to identify phenolic metabolites excreted in the urine in their *N*-acetylated, glucuronidated or sulphated form, urine samples were treated with acid, with β-glucuronidase (from *Escherichia coli* K12 [Boehringer, Mannheim, Germany] [free of arylsulphatase]) or with arylsulphatase/β-glucuronidase (from *Helix pomatia* [Boehringer, Mannheim, Germany]). Enzyme treatments were carried out essentially as described before by Vervoort *et al*. (1990).

For acid hydrolysis of the urine samples 1·0 ml urine sample was incubated with 0·1 ml 37% (12N) HCl for 1 h in a boiling water bath. After acid hydrolysis the mixture was neutralized with 6 N NaOH and the sample diluted once in 0·2 M potassium phosphate pH 7·6 for analysis by ^{19}F nmr.

Microsomal preparations

Preparation of microsomes was carried out essentially as described previously (Vervoort *et al.* 1990). Microsomes were obtained from perfused livers of male Wistar rats (300–400 g), which were exposed to 150 mg/kg body weight of the cytochrome P450 inducer isosafrole (Janssen Chimica, Beerse, Belgium) by i.p. injection daily for 3 days using a stock solution of 100 mg/ml in olive oil. The amount of cytochrome P450 in the microsomes was measured according to the method described by Omura and Sato (1964).

In vitro *microsomal incubations*

In vitro microsomal incubations contained (final concentrations): 0·1 M potassium phosphate pH 7·6, 0–5 mM 3,5-dichloro-2,4-difluoroaniline, added from a 100 times concentrated stock solution in dimethyl sulphoxide and 2 μM microsomal cytochrome P450. Microsomes from the isosafrole-pretreated rat were used to allow comparison of the results to those of microsomal metabolism of aniline derivatives reported in a previous study (Cnubben *et al.* 1994) which also demonstrated that these microsomes are most effective in metabolism of aniline derivatives (Cnubben *et al.* 1994, Koerts *et al.* 1996). The total sample volume was 1 ml. The reaction was started by the addition of NADPH (1 mM final concentration) and the mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 0·3 ml 20% (w/v) trichloroacetic acid after which the mixture was centrifuged for 3 min at 13000 *g*.

Chemical assay for the detection of 4-hydroxy-3,5-dichloro-2-fluoroaniline

4-Hydroxy-3,5-dichloro-2-fluoroaniline present in the urine and microsomal incubations was determined using the method of Brodie and Axelrod (1948) for chemical determination of 4-aminophenol derivatives. Arylsulphatase/β-glucuronidase treated urine samples were extracted with ethyl acetate. To 40 μl of this ethyl acetate fraction, 760 μl demineralized water and 240 μl trichloroacetic acid (20% w/v) were added. To 800 μl of this mixture 80 μl phenol reagent (5% w/v phenol in 2·5 M NaOH) and 160 μl 2·5 M Na_2CO_3 were added. The absorbance at 630 nm was measured after 1 h at room temperature. Ethyl acetate extract from non-enzyme treated urine was used as a blank.

For the *in vitro* microsomal incubations 100 μl phenol reagent and 200 μl Na_2CO_3 were added to 1 ml of the trichloroacetic acid mixture. A microsomal incubation with water instead of substrate was used as blank.

ε_{630} of the 4-hydroxy-3,5-dichloro-2-fluoroaniline derived indophenol is not known. Therefore, based on a previous study on the cytochrome P450-catalysed 4-hydroxylation of halogenated aniline derivatives in which the ε_{630} of various halogenated aminophenol-derived indophenols were determined (Cnubben *et al.* 1994), the ε_{630} of the 4-hydroxy-3,5-dichloro-2-fluoroaniline-derived indophenol was estimated to be 20 $mM^{-1}\ cm^{-1}$.

Data analysis

The apparent Michaelis–Menten constant K_m (mM) and the apparent maximum reaction rate V_{max} (nmol 4-hydroxy-3,5-dichloro-2-fluoroaniline/min/nmol cytochrome P450) for the *in vitro* microsomal cytochrome P450-catalysed 4-hydroxylation of 3,5-dichloro-2,4-difluoroaniline were calculated by fitting the obtained data of the *in vitro* incubations to the Michaelis–Menten equation $v = V_{max} \cdot [S]/(K_m + [S])$. Fitting the experimental data to the Michaelis–Menten equation resulted in correlation coefficients > 0·97.

^{19}F nmr measurements

^{19}F nmr measurements were performed on a Bruker AMX 300 nmr spectrometer at 280 K. Proton-decoupling was achieved with the Waltz-16 pulse sequence at −20 dB from 50 W.

Samples contained 100 μl ^2H_2O to lock the magnetic field and 10 μl 8·4 mM 4-fluorobenzoic acid added as an internal standard. The total sample volume was 1·71 ml. Enzyme and acid treated urine samples were made anaerobic by evacuating and filling with argon four times before analysis. This was done to prevent autoxidation of the phenolic metabolites. Quantification of the different metabolites was done by comparison of the integral of the ^{19}F nmr resonance of the added internal standard 4-fluorobenzoic acid to the integrals of the ^{19}F nmr peaks of the metabolites. The ethyl acetate extract of the faeces was analysed with a coaxial insert containing 4-fluorobenzoic acid dissolved in ^2H_2O. The total sample volume was 1·6 ml. Chemical shifts are reported relative to $CFCl_3$.

Molecular orbital calculations

Semi-empirical molecular orbital calculations were performed on a Silicon Graphics Iris 4D/85 computer with the AM1 (Austin Method 1) Hamiltonian from the AMPAC program (Quantum Chemistry Program Exchange, program no. 506, Indiana University, Bloomington, IN, USA). Calculations were carried out with PRECISE criteria and self-consistent field was achieved for all

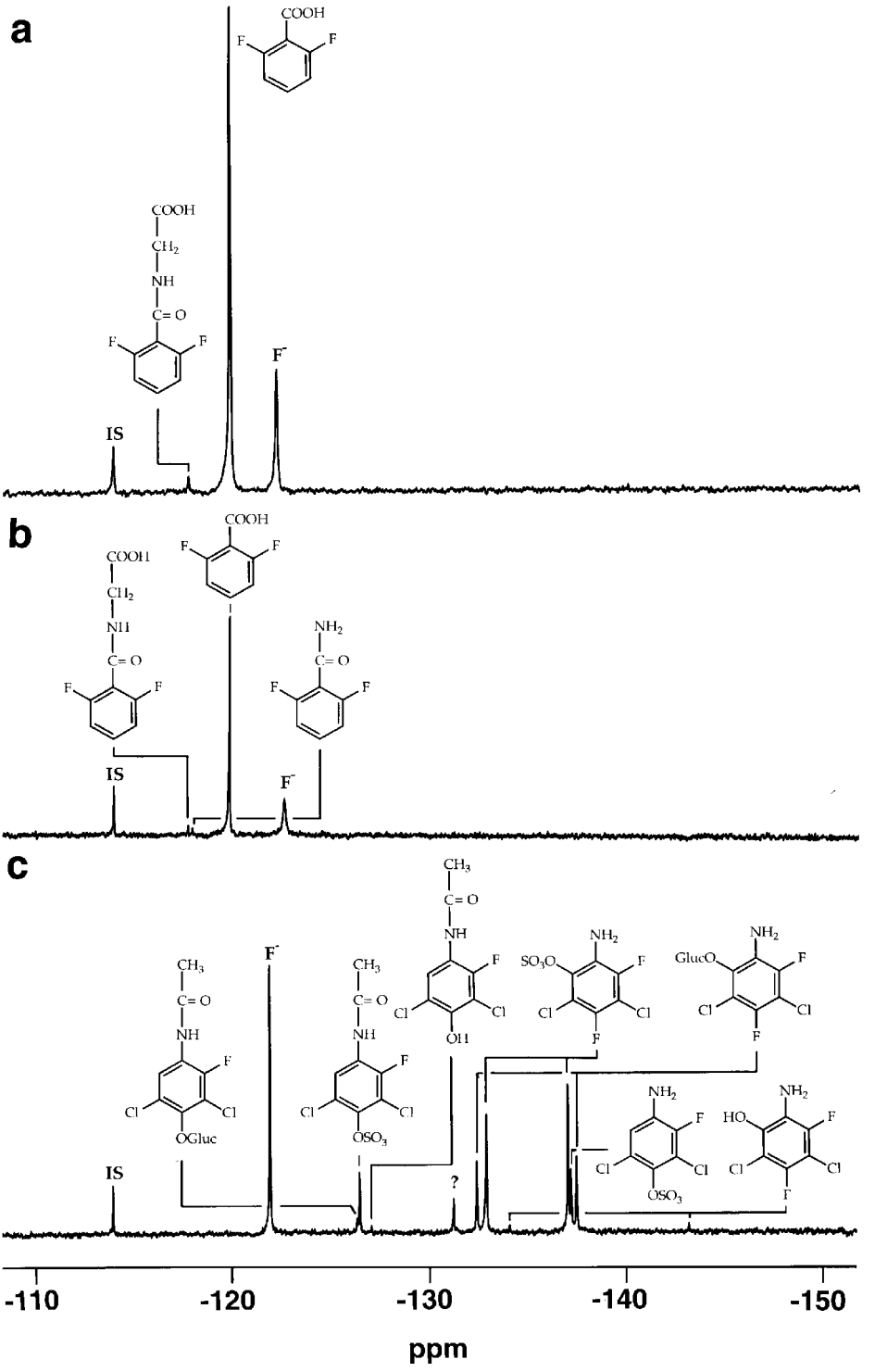

Figure 2. For legend see page 807.

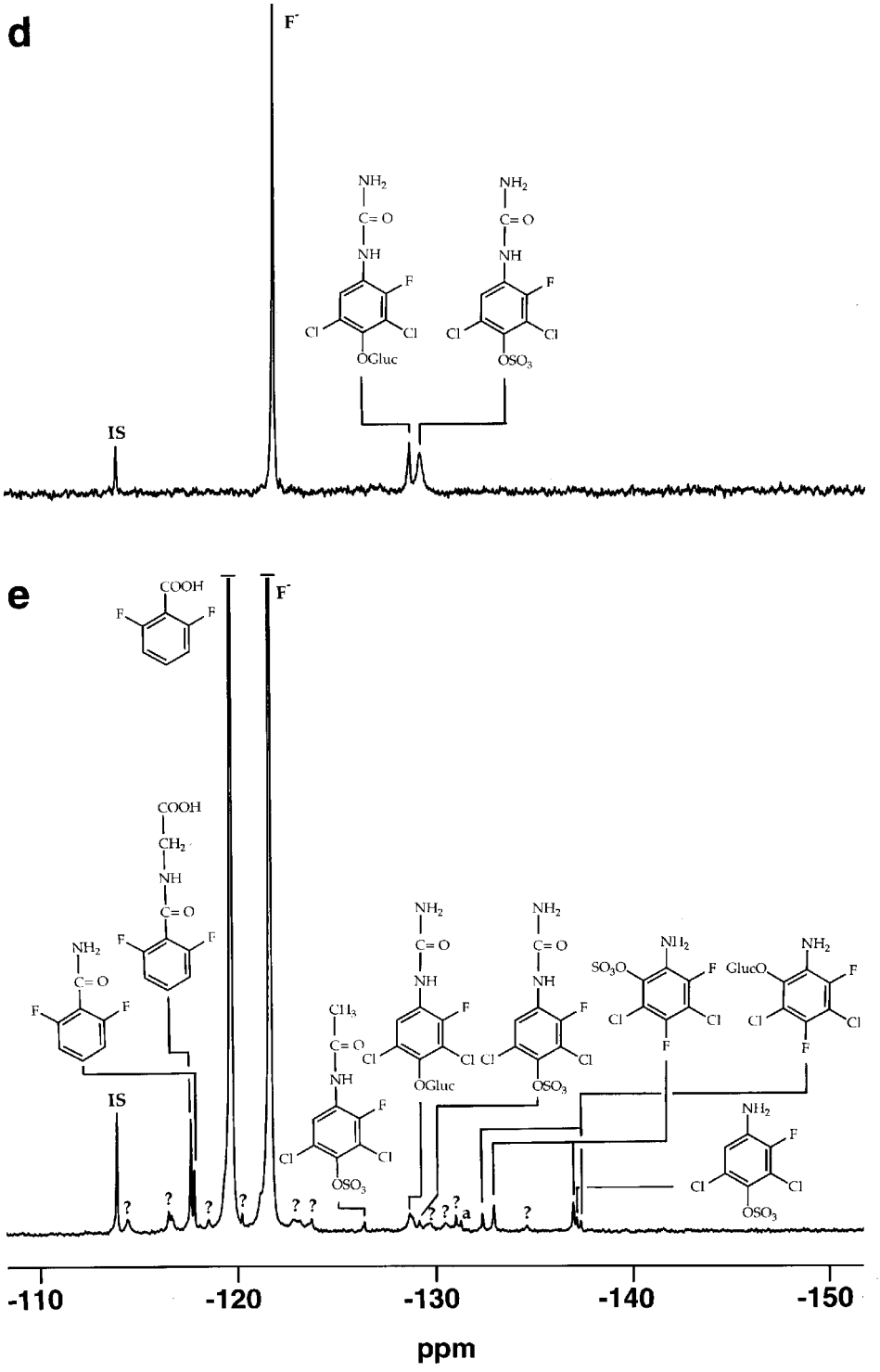

Figure 2. For legend see opposite.

calculations. Geometries were optimised for all bond lengths, bond angles and torsion angles using the Fletcher–Powell criteria.

Results

Urine and faeces metabolite patterns of 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline (3,5-dichloro-2,4-difluorophenyl)urea and teflubenzuron

The *in vivo* metabolism of 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline and (3,5-dichloro-2,4-difluorophenyl)urea was investigated in order to identify the metabolites from teflubenzuron that result from hydrolysis of the phenylurea bridge (figure 1) and subsequent metabolism of the scission products.

2,6-Difluorobenzoic acid. Figure 2a shows the ^{19}F nmr spectrum of the 0–24-h urine of a rat exposed to 10 μmol 2,6-difluorobenzoic acid. Metabolites were identified on the basis of commercially available 2,6-difluorobenzoic acid and its synthesized glycine conjugate. The ^{19}F nmr spectrum shows that urinary excretion of 2,6-difluorobenzoic acid occurs mainly in the form of the unmodified parent compound 2,6-difluorobenzoic acid and to a minor extent as 2,6-difluorobenzoylglycine (see table 1 for quantitative data). The amount of F^- observed could be fully accounted for the by the F^- present in blank urine samples from the unexposed rat (data not shown). The total urinary recovery was 86%.

The ^{19}F nmr spectra of the ethyl acetate extracts of the 0–48-h faeces of the 2,6-difluorobenzoic acid exposed rat show no resonance peaks (detection limit 1 μM for an o/n run) (^{19}F nmr spectrum not shown).

2,6-Difluorobenzamide. Figure 2b shows the ^{19}F nmr spectrum of the 0–24-h urine of a rat exposed to 10 μmol 2,6-difluorobenzamide. Metabolites were identified on the basis of commercially available or synthesized reference compounds. The ^{19}F nmr spectrum shows that urinary excretion of 2,6-difluorobenzamide mainly occurs in the form of 2,6-difluorobenzoic acid and a minor amount was excreted as 2,6-difluorobenzoylglycine and unmodified 2,6-difluorobenzamide (see table 1 for qualitative data). The amount of F^- observed could be fully accounted for by the F^- present in blank urine samples from unexposed rats (data not shown). The 24-h urinary recovery was 100%.

The ^{19}F nmr spectra of the ethyl acetate extracts of the 0–48-h faeces of the 2,6-difluorobenzamide-exposed rat show no metabolite or substrate resonances (detection limit 1 μM for an overnight run) (^{19}F nmr spectra not shown).

3,5-Dichloro-2,4-difluoroaniline. Figure 2c shows the ^{19}F nmr spectrum of the 0–24-h urine of a rat exposed to 10 μmol 3,5-dichloro-2,4-difluoroaniline. ^{19}F nmr

Figure 2. ^{19}F nmr spectra of the 0–24-h urine samples of rats exposed to (a) 2,6-difluorobenzoic acid, (b) 2,6-difluorobenzamide, (c) 3,5-dichloro-2,4-difluoroaniline, (d) (3,5-dichloro-2,4-difluorophenyl)urea and (e) teflubenzuron. The resonance peak marked 'a' in (e) belongs to a metabolite derived from biotransformation of 3,5-dichloro-2,4-difluoroaniline, which is one of the scission products of teflubenzuron after hydrolysis of the urea bridge. The fluorine resonances in the spectra (a–d) are not similar in size due to the different volumes of the 0–24-h urine samples produced by the rats. The resonance peak 'IS' is from the internal standard 4-fluorobenzoic acid. Chemical shifts are reported relative to $CFCl_3$.

Table 1. 24-h Urinary metabolic profile of 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline, (3,5-dichloro-2,4-difluorophenyl)urea and teflubenzuron.[a]

Substrate/metabolite	% total metabolites
2,6-Difluorobenzoic acid	
2,6-Difluorobenzoylglycine	5·4 ± 2·5
Parent compound	94·6 ± 2·5
2,6-Difluorobenzamide	
2,6-Difluorobenzoic acid	97·1 ± 2·4
2,6-Difluorobenzoylglycine	2·3 ± 1·9
Parent compound	0·6 ± 1·0
3,5-Dichloro-2,4-difluoroaniline	
2-Amino-3,5-difluoro-4,6-dichlorophenol	0·4 ± 0·8
2-Amino-3,5-difluoro-4,6-dichlorophenylsulphate	41·3 ± 5·7
2-Amino-3,5-difluoro-4,6-dichlorophenylglucuronide	16·7 ± 5·1
4-Amino-2,6-dichloro-3-fluorophenylsulphate	15·2 ± 2·1
4-Acetamido-2,6-dichloro-3-fluorophenol	0·2 ± 0·4
4-Acetamido-2,6-dichloro-3-fluorophenylsulphate/glucuronide	16·0 ± 2·1
Unidentified	10·2 ± 2·1
(3,5-Dichloro-2,4-difluorophenyl)urea	
(3,5-Dichloro-2-fluoro-4-phenylsulphatephenyl)urea	71·9 ± 8·2
(3,5-Dichloro-2-fluoro-4-phenylglucuronidephenyl)urea	28·1 ± 8·2
Teflubenzuron	
2,6-Difluorobenzoic acid	81·4 ± 3·8
2,6-Difluorobenzoylglycine	4·0 ± 0·5
2,6-Difluorobenzamide	2·0 ± 0·3
2-Amino-3,5-difluoro-4,6-dichlorophenylsulphate	2·8 ± 0·5
2-Amino-3,5-difluoro-4,6-dichlorophenylglucuronide	1·2 ± 0·3
4-Amino-2,6-dichloro-3-fluorophenylsulphate	1·2 ± 0·4
4-Acetamido-2,6-dichloro-3-fluorophenylsulphate	0·6 ± 0·5
(3,5-Dichloro-2-fluoro-4-phenylsulphatephenyl)urea	0·3 ± 0·6
(3,5-Dichloro-2-fluoro-4-phenylglucuronidephenyl)urea	5·2 ± 2·4
Unidentified[b]	1·3 ± 0·7

[a] Results present the average metabolic profile of the indicated dose range as similar metabolic profiles were obtained for the different tested doses.

[b] Similar ppm as the unidentified metabolite observed for 3,5-dichloro-2,4-difluoroaniline.

resonances of conjugated metabolites, i.e. sulphated, glucuronidated or acetylated products, were identified on the basis of the shift of their ^{19}F nmr resonance in the spectrum upon arylsulphatase and/or β-glucuronidase, or acid treatment of the urine. The aromatic ring-hydroxylated product 2-amino-3,5-difluoro-4,6-dichlorophenylsulphate was identified on the basis of its synthesized reference compound. Identification of 4-amino-2,6-dichloro-3-fluorophenol, formed from its *N*-acetylated, sulphated and/or glucuronidated conjugate upon acid, arylsulphatase and/or β-glucuronidase treatment of the urine, was achieved on the basis of the chemical assay for detection of 4-aminophenol derivatives. The amount of 4-amino-2,6-dichloro-3-fluorophenol detected by this chemical assay correlated with the amount of the metabolite giving rise to the ^{19}F nmr resonance at $-136{\cdot}5$ ppm, which could thus be identified. This latter result was supported by the following observation. In a previous study it was demonstrated that the introduction of a hydroxyl group *meta* with respect to a fluorine in fluorinated benzene derivatives results in a ^{19}F nmr chemical shift of $1{\cdot}8 \pm 1{\cdot}2$ ppm (Koerts *et al.* 1996). Based on this result, the ^{19}F nmr chemical shift of 4-amino-2,6-dichloro-3-fluorophenol was predicted to be $-136{\cdot}3 \pm 1{\cdot}2$ ppm clearly supporting the identification of the ^{19}F nmr resonance at $-136{\cdot}5$ ppm as 4-hydroxy-3,5-dichloro-2-fluoroaniline.

The results in Figure 2c show that the urinary excretion of 3,5-dichloro-2,4-difluoroaniline is mainly in the form of the *N*-acetylated, sulphated and/or glucuronidated conjugates of its *ortho* and *para*-hydroxylated metabolites (see table 1 for quantitative data). Only one of the metabolite peaks in the ^{19}F nmr spectrum could not be further identified. However, its presence in the urine spectrum of the 3,5-dichloro-2,4-difluoroaniline-exposed rats clearly identifies this metabolite as derived from the aniline moiety of teflubenzuron. The total 24-h urinary recovery was 59·5% of the administered dose.

The ^{19}F nmr spectra of the ethyl acetate extracts of the 0–48-h collected faeces reveal the presence of some parent aniline compound excreted in the faeces to about 2% of the dose administered (^{19}F nmr spectrum not shown).

(3,5-Dichloro-2,4-difluorophenyl)urea. Figure 2d shows the ^{19}F nmr spectrum of the 0–24-h urine of a rat exposed to 10 μmol (3,5-dichloro-2,4-difluorophenyl)urea. Metabolites were identified in a similar way as described for 3,5-dichloro-2,4-difluoroaniline. The results in figure 2d demonstrate that (3,5-dichloro-2,4-difluorophenyl)urea is excreted in the urine as the sulphated and glucuronidated conjugates of (4-hydroxy-3,5-dichloro-2-fluorophenyl)urea (see table 1 for quantitative data).The total 24-h urinary recovery was 54·5% of the administered dose.

The ^{19}F nmr spectra of the ethyl acetate extracts of the 0–48-h faeces sample reveal the presence of some parent compound excreted in the faeces to about 6% of the dose administered (^{19}F nmr spectrum not shown).

Teflubenzuron. Figure 2e shows the ^{19}F nmr spectrum of the 0–24-h urine of a rat exposed to 53 μmol (20 mg) teflubenzuron. Based on the *in vivo* urinary metabolic patterns of 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline and (3,5-dichloro-2,4-difluorophenyl)urea (figure 2a–d), metabolites resulting from hydrolysis of the urea bridge of teflubenzuron and further metabolism of the aromatic scission products could be identified. The metabolic profile thus identified shows that the benzoate part of teflubenzuron is mainly excreted as 2,6-difluorobenzoic acid, and, to a minor extent, as 2,6-difluorobenzoylglycine and 2,6-difluorobenzamide. The aniline ring of teflubenzuron was excreted as the sulphated and glucuronidated conjugates of (4-hydroxy-3,5-dichloro-2-fluorophenyl)urea and 2-amino-3,5-difluoro-4,6-dichlorophenol, the sulphated conjugate of 4-amino-2,6-dichloro-3-fluorophenol and the glucuronidated conjugate of 4-acetamido-2,6-dichloro-3-fluorophenol (see table 1 for quantitative data). In addition to all these identified metabolites, the ^{19}F nmr spectrum of the urine of the teflubenzuron-exposed rat shows several unidentified resonance peaks, one of which has the same chemical shift value as the unidentified resonance peak observed in the urine of rats exposed to 3,5-dichloro-2,4-difluoroaniline indicating that this metabolite can also be ascribed to a metabolite derived from the aniline part of teflubenzuron. The percentage of unidentified peaks amounts to only 12% of the total metabolic pattern. These unidentified metabolites might be ascribed to metabolites with the teflubenzuron structure intact as these kind of metabolites were also observed in the urine of diflubenzuron exposed rats, cattle and sheep (Willems *et al.* 1980, Ivie 1978). It is also possible that the unidentified metabolites can be ascribed to impurities and metabolites derived from these impurities present in the teflubenzuron dosed to the animals. The ^{19}F nmr spectrum of the administered teflubenzuron dose indicates a purity of about 97%. The ^{19}F nmr resonances of

these impurities were not observed in the faeces and urine of the teflubenzuron exposed rats, indicating that these unknown compounds might be absorbed from the lumen, metabolized in the liver and excreted in the urine and/or via the bile in the faeces. Since the actual structure and nature of these impurities are unknown, suggestions for the nature of their possible urine metabolites can not be given.

Although a similar amount of benzoate and aniline metabolites should originate from the hydrolytic cleavage of teflubenzuron, the amount of benzoate derived metabolites identified in the urine was 87% of the total identified metabolic pattern whereas the amount of aniline derivatives was 13%, indicating that the amount of benzoate-derived metabolites was about seven times the amount of aniline derived metabolites. Thus, the 12% of unidentified minor metabolites does not alter the conclusion that there is a substantial difference in urinary recovery of the two aromatic moieties derived from teflubenzuron. The 24–48-h urine sample of the rat exposed to 53 μmol teflubenzuron was also analysed in order to investigate whether due to retarded excretion the remainder of the aniline moiety was excreted in the 24–48-h urine. However, the amount of metabolites in this 24–48-h urine was only 1·5% of the amount excreted in the 0–24-h urine. Altogether, these data show that part of the aniline moiety is retained in the body. Since total recovery in 0–24-h urine, 24–48-h urine and 0–48-h faeces amounted to 4·5, 0·1 and 90% respectively, approximately 5·4% of the dose of teflubenzuron could not be accounted for. Taking into account that recovery of the dose of teflubenzuron administered as benzoate and aniline-derived urinary metabolites amounts to approximately 7·5, and 0·9% respectively, this indicates that a significant amount of the dose retained in the body in the first 48 h may indeed be ascribed to aniline moiety containing metabolites.

The ^{19}F nmr spectra of the ethyl acetate extracts of the 0–48-h faeces sample of the teflubenzuron-exposed rat showed mainly (91%) unchanged teflubenzuron. Scission products of teflubenzuron or metabolites derived from them were not observed. The recovery of teflubenzuron in the faeces was about 90% of the dose.

*Dose–recovery studies of teflubenzuron, 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-*difluoroaniline and *(3,5-dichloro-2,4-difluorophenyl)urea*

To investigate whether the amount of teflubenzuron administered and of the scission products derived from it would affect the extent of absorption and excretion via urinary and faecal excretion routes, dose–recovery studies of teflubenzuron and its possible scission products were performed. Analysis of the 0–48-h urinary samples showed that over the whole dose-range tested, i.e. 4–53 μmol for teflubenzuron (n = 1–2) and 1–10 μmol for the scission products (n = 1), their urinary recoveries did not vary significantly. For the scission products this dose range of 1–10 μmol was chosen because the results obtained from the dose–recovery study of teflubenzuron showed that about 0·5–4·0 μmol teflubenzuron was absorbed from the gastrointestinal tract and excreted in the 0–24-h urine indicating that the range of 1–10 μmol of aromatic scission products would be in the range expected to be formed in the rats exposed to 4–53 μmol teflubenzuron.

At all dose-levels of teflubenzuron, about 4·5% of the dose is excreted in the 0–24-h urine mainly as metabolites derived from the scission products resulting from hydrolysis of the urea bridge of teflubenzuron, whereas about 90% of the dose

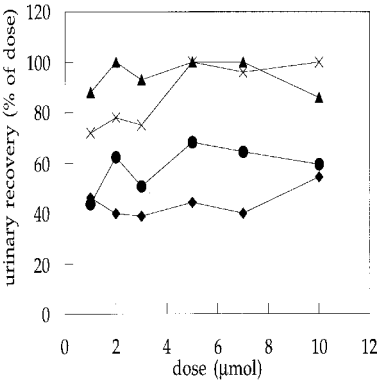

Figure 3. Dose–recovery curves of (▲) 2,6-difluorobenzoic acid, (×) 2,6-difluorobenzamide, (●) 3,5-dichloro-2,4-difluoroaniline and (◆) (3,5-dichloro-2,4-difluorophenyl)urea. The recovery is presented as the percentage of the dose excreted in the urine 24 h after an oral dose of the chemical (n = 1).

is excreted in the faeces mainly as unmodified teflubenzuron. Furthermore, the urinary recovery of the aniline moiety was only about 12% of the excreted benzoate moiety over the entire 4–53 μmol dose range.

The dose–recovery studies of the benzoate and aniline scission products of teflubenzuron (figure 3) show that, for a dose range of 1–10 μmol, the 0–24-h urinary recovery of 2,6-difluorobenzoic acid and of 2,6-difluorobenzamide was always significantly ($p < 0{\cdot}001$) higher and about twice the recovery observed for similar doses of 3,5-dichloro-2,4-difluoroaniline or (3,5-dichloro-2,4-difluorophenyl)urea. This two-fold difference thus explains only part of the 8-fold difference in recovery of the benzoate and aniline moiety in case of their formation from teflubenzuron. This discrepancy might be due to the fact that at the doses tested the rate-limiting phase I metabolism of the aniline-type scission products is still operating at almost saturating aniline derivative concentrations, whereas upon their formation *in situ* by hydrolysis of teflubenzuron their steady-state concentration for phase I metabolism is below V_{max} conditions. Because dose–recovery studies at lower doses of the aniline derivatives were not feasible due to the detection limit of the ^{19}F nmr urine analysis, further experiments were performed to determine the kinetic values for this phase I aromatic hydroxylation of the aniline derivative.

In vitro *microsomal incubations*

Figure 4 presents the Michaelis–Menten plot for the microsomal cytochrome P450-catalysed 4-hydroxylation of 3,5-dichloro-2,4-difluoroaniline. The K_m and V_{max} derived from these data were $0{\cdot}58 \pm 0{\cdot}06$ mM and $0{\cdot}24 \pm 0{\cdot}01$ nmol/min/nmol cytochrome P450 respectively.

Molecular orbital computer calculations

Since the microsomal conversion of (3,5-dichloro-2,4-difluorophenyl)urea could not be followed by the sensitive chemical assay available for the aniline derivatives, support for its equally low V_{max} was derived from theoretical studies. Molecular orbital computer calculations on 3,5-dichloro-2,4-difluoroaniline and (3,5-dichloro-2,4-difluorophenyl)urea were performed to find theoretical support for the low rate of phase I aromatic hydroxylation of these teflubenzuron-derived splitting products.

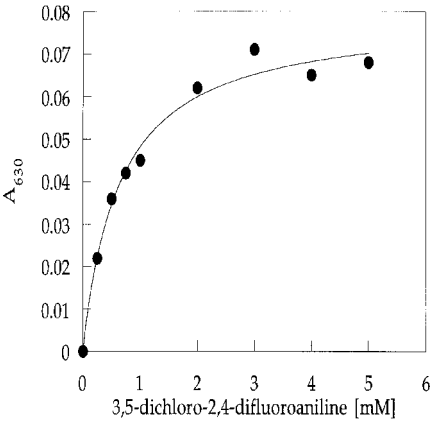

Figure 4. Michaelis–Menten plot for the microsomal cytochrome P450-catalysed 4-hydroxylation of 3,5-dichloro-2,4-difluoroaniline (n = 2). From these data the K_m and V_{max} were derived.

The energy of the HOMO (highest occupied molecular orbital) of 3,5-dichloro-2,4-difluoroaniline and (3,5-dichloro-2,4-difluorophenyl)urea is −9·0 and −9·3 eV respectively, indicative of a V_{max} for (3,5-dichloro-2,4-difluorophenyl)urea that can not be higher than that obtained for the aniline derivative (Cnubben *et al.* 1994) since the lower E(HOMO) indicates an even lower reactivity of this compound for an electrophilic attack by the activated $(FeO)^{3+}$ species of cytochrome P450.

Discussion

The aim of the present study was to investigate the biotransformation of the insecticide teflubenzuron in rat. Particular attention was focused on the metabolic fate of the aniline part of teflubenzuron because in previous studies on the biotransformation of diflubenzuron (an analogue of teflubenzuron) a lot of uncertainty remained about the recovery and metabolic fate of the aniline derivative formed after hydrolysis of the urea bridge (Verloop and Ferell 1977, Ivie 1978, Willems *et al.* 1980, Opdycke *et al.* 1982a, b).

Male Wistar rats were orally exposed to different dose levels of teflubenzuron and urine samples as well as faecal extracts were analysed by ^{19}F nmr. The ^{19}F nmr spectra of urine samples of rat orally exposed to teflubenzuron (figure 2e) mainly showed metabolites derived from products resulting from hydrolysis of the urea bridge. Analysis of faecal extracts of the teflubenzuron-exposed rat revealed the presence of mainly unmodified teflubenzuron. Within 48 h almost the total dose of teflubenzuron was recovered, partly as metabolites in the urine (4·6% of the dose administered) and mainly in unmodified form in the faeces extract (90% of the dose administered). Independent of the dose of teflubenzuron, the amount of urinary aniline derived metabolites observed amounted to only 12% of the amount of benzoate-derived metabolites, in spite of the fact that hydrolytic cleavage of the urea bridge in teflubenzuron will generate equal amounts of benzoate and aniline derivatives. This observation of inefficient excretion of the aniline part of teflubenzuron, formed upon hydrolytic cleavage of the urea bridge in the benzoylphenyl urea agrochemical, is in agreement with the qualitative results described previously for diflubenzuron (Verloop and Ferell 1977, Ivie 1978, Willems *et al.* 1980, Opdycke *et al.* 1982a, b).

Further experiments described in the present study were performed to find a rationale for this significant difference between the urinary recovery of the benzoate- and aniline-type scission products.

First, it was investigated to what extent the observed discrepancy might be ascribed to a difference in excretion efficiency of the benzoate and aniline derivatives themselves. Dose–recovery studies were performed for the four possible scission products, i.e. 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 3,5-dichloro-2,4-difluoroaniline and (3,5-dichloro-2,4-difluorophenyl)urea. The results obtained showed a difference in the excretion efficiency between the benzoate-type derivatives on the one hand, which were, at all dose levels investigated, almost fully excreted within the first 24 h following administration, and the aniline-type derivatives on the other hand, which were excreted, also independent of the dose, to about 50% of the dose administered. Based on these data, excretion efficiencies of the aniline-type compounds are demonstrated to be at least two-fold lower than that of the benzoates. However, the difference observed in the excretion efficiency of the benzoate- and aniline-type compounds when compounds are formed *in situ* from teflubenzuron is 8-fold. This discrepancy between the excretion efficiency of the benzoate- and aniline-type compounds of teflubenzuron compared with the experiments when the compounds were dosed as such might be explained on the basis of the following additional observations of the present study.

First, is of importance to note that the benzoate moiety is almost exclusively excreted in its unmodified form and to a minor extent as its glycine conjugate, suggesting that after hydrolysis no further metabolism is required to obtain efficient excretion of the 2,6-difluorobenzoate or the 2,6-difluorobenzamide as was shown before for the metabolism of diflubenzuron (Willems *et al*. 1980) and a series of fluorinated benzoic acids (Ghauri *et al*. 1992). In contrast, the aniline part of teflubenzuron, i.e. 3,5-dichloro-2,4-difluoroaniline or (3,5-dichloro-2,4-difluorophenyl)urea require further metabolism in both phase I and II metabolism before they can be excreted from the body. The aromatic hydroxylation of such aniline derivatives catalysed by cytochromes P450 to give the aminophenol metabolites suitable for phase II conjugation and formation of the products that can be efficiently excreted, could be a relatively slow reaction. It is likely that the rate of this cytochrome P450-catalysed aromatic ring hydroxylation is relatively low because cytochrome P450-catalysed aromatic hydroxylation is often the rate-limiting step in biotransformation and generally much slower than Phase II conjugation reactions (Cnubben *et al*. 1994, Soffers *et al*. 1994).

If the rate of cytochrome P450 conversion of the aniline derivative would indeed be the rate-limiting factor for its excretion, the actual concentrations of the aniline derivative in relation to the K_m for the reaction might be a crucial factor influencing the actual efficiency of the conversion to a large extent. Especially when the concentration is around or below the K_m, variation in the actual concentration of the compound will affect the efficiency of its conversion and excretion. This may then be the explanation for the fact that upon direct administration of 1–10 μmol of the aniline derivatives their excretion is more efficient, then when a similar dose is formed over a long time interval upon the hydrolytic cleavage of teflubenzuron. The latter might result in a lower time averaged concentration of the compound.

Comparison of the actual K_m and V_{max} determined for the cytochrome P450-catalysed C4-hydroxylation of 3,5-dichloro-2,4-difluoroaniline in the present study, to an estimate of the concentrations expected in the liver, supports this latter

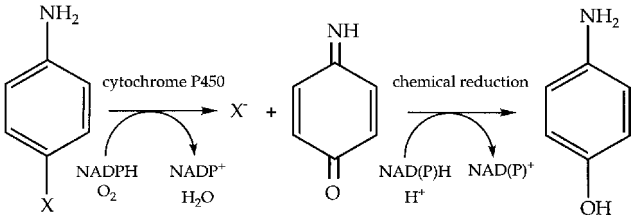

Figure 5. Proposed reaction pathway for the cytochrome P450-catalysed dehalogenation of 4-halogenated anilines (Cnubben *et al*. 1995, Riefjens and Vervoort 1991).

hypothesis. Assuming a liver volume of 5–10 ml, and full absorption and transport of a 1–10 μmol aniline dose from the gastrointestinal tract to the liver, a maximum concentration of 3,5-dichloro-2,4-difluoroaniline in the liver of about 0·1–2·0 mM can be foreseen. This is around the K_m for the cytochrome P450-catalysed oxidative C4-dehalogenation determined to be 0·58 ± 0·06 mM. In the teflubenzuron-exposed animals, the same amount of aniline is liberated from the parent compound over a time interval that is, for example, four times longer than the time interval required for the efficient uptake of the single dose of the aniline, and therefore will result in lower liver concentrations that will be below the K_m resulting in lower rates of conversion. Such an effect could explain that in the teflubenzuron-exposed rat the efficiency for excretion of the aniline-type metabolites is 8-fold lower than for the benzoate-type of metabolites whereas in the direct exposure experiments this difference is only 2-fold.

Although this difference in excretion efficiency of the benzoate and aniline-type compounds can explain the observed 8-fold difference in their urinary recovery when formed from teflubenzuron, one can not neglect the fact that even upon a single dose of the aniline derivative about 50% of the dose can not be recovered in the 0–48-h urine or faeces. This means that part of the aniline derivative remains in the body. An explanation for this observation might be related to the mechanism by which the cytochrome P450-catalysed oxidative dehalogenation of these halogenated anilines to 4-aminophenol derivatives occurs. This oxidative dehalogenation represents the first step required for formation of the 4-hydroxylated urinary metabolites observed. Figure 5 presents the reaction pathway suggested for this reaction. The primary product formed in such a cytochrome P450-catalysed oxidative dehalogenation of a C4-halogenated aniline derivative is the reactive benzoquinoneimine and not its two-electron reduced aminophenol form (Cnubben *et al*. 1995, Rietjens and Vervoort 1991). Rapid binding of this reactive benzoquinoneimine to tissue macromolecules instead of its chemical and/or enzymatic reduction to the non-toxic aminophenol, might cause the compound to be retained in the body. In addition, the cytochrome P450-catalysed N-oxidation of the aniline derivative might be another explanation for the observed low urinary recovery of this compound. Results of previous studies (Kiese 1963, Eadsforth *et al*. 1984, Cnubben *et al*. 1996) showed that methaemoglobinaemia is induced in rats which were orally exposed to C4-substituted aniline derivatives. In one of these studies (Cnubben *et al*. 1996) the extent of methaemoglobinaemia was shown to correlate with the rate of *N*-oxidation of the aniline derivatives *in vitro*. *In vivo* these *N*-oxidized metabolites were not observed probably due to their high reactivity and abundant possibilities to react with components in the body. The extent of the

cytochrome P450-catalysed N-oxidation of the 3,5-dichloro-2,4-difluoroaniline derivative of importance in the present study can be estimated based on previous results for the *in vitro* cytochrome P450-catalysed conversion of C4-halogenated anilines (Cnubben *et al*. 1995). The metabolic data on the total *in vitro* cytochrome P450-catalysed conversion of aniline, 4F-, 4Cl-, 4Br- and 4I-aniline show that the sum of aromatic ring and *N*-hydroxylation of the tested halogenated anilines was similar but that the ratio between *N*- and *para* or *ortho*-hydroxylation varied with the substituent patterns. When a halogen substituent hampers mono-oxygenation at C4, as can also be expected for the 3,5-dichloro-2,4-difluoroaniline derived from teflubenzuron, the extent of *N*-hydroxylation equals 1–11 times the extent of C4-oxidative dehalogenation. This means that lower possibilities for aromatic ring hydroxylation result in a relatively higher extent of N-oxidized products. This means that the extent of cytochrome P450-catalysed *N*-oxidation of the aniline cleavage product of teflubenzuron, 3,5-dichloro-2,4-difluoroaniline, can be expected to result in a significant extent of *N*-oxidized products that at least equals the amount of aromatic C2 and/or C4 hydroxylated metabolites observed in urine. Since binding of the reactive *N*-oxidized metabolites to tissue molecules hampers their excretion, the extent of *N*-oxidation of 3,5-dichloro-2,4-difluoroaniline can be an additional factor in causing the lower urinary recoveries. However, whether the formation of these *N*-oxidized metabolites will result in measurable levels of methaemoglobinaemia is uncertain as the amount of the absorbed teflubenzuron from the lumen is only 10% of the dose given and likely to result in concentrations of *N*-oxidized aniline metabolites that are below the doses required for induction of methaemoglobinaemia (Cnubben *et al*. 1996).

The possibility that reaction pathways leading to reactive benzoquinoneimine and *N*-oxidized metabolites cause covalent binding of the aniline part of teflubenzuron to tissue macromolecules seems to be supported by several other observations. First, about 5·4% of the dose of teflubenzuron could not be recovered in urine or faeces. At least part of this 5·4% was expected to be excreted in the form of urinary aniline-type of metabolites since the urinary recovery in the form of benzoate-type metabolites was 3·9% compared with only 0·7% in the form of aniline-type of metabolites. Furthermore, the amount of the fluorine in the 24-h urine of (3,5-dichloro-2,4-difluorophenyl)urea-exposed rat was above the amount that could be accounted for by the fluorine originating from food and/or drinking water and from the amount of dehalogenated products observed. This observation suggests that part of the aniline moiety is dehalogenated but not excreted from the body. Finally, the absence of full excretion of the urea agrochemical from the body would be in line with the results obtained in a study with rats orally exposed to 5 mg/kg diflubenzuron for which 4% of the dose was eventually recovered in the carcass (Willems *et al*. 1980). Finally, the observation of the present study that the 24–48-h urine recovery of teflubenzuron was significantly below the 0–24-h recovery to some extent argues against the slow cytochrome P450-catalysed conversion of the aniline moiety as the main reason for its relatively low recovery. Slow metabolism of a circulating amount of 3,5-dichloro-2,4-difluoroaniline would imply that even after 24 h, metabolism and excretion would continue at a level comparable with that observed in 0–24-h sample. Since this is not what is observed, the explanation for low aniline recovery due to covalent interaction of a reactive metabolite derived from it with tissue macromolecules provides the best explanation for this phenomenon. Altogether, the mechanism depicted in figure 5 and/or *N*-oxidation might provide

the best explanations for the low recovery of the C4-halogenated aniline part of the molecule, both in case of teflubenzuron, but also in case of diflubenzuron, where the C4 position in the aniline moiety is chlorinated. Clearly such a process resulting in formation of a reactive benzoquinoneimine or *N*-oxidized metabolite may also be of importance from a toxicological point of view, especially when taking into account that diflubenzuron was reported to produce hepatocellular changes in mouse (Young *et al*. 1986).

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References

Banks, R. E. (ed.), 1994, Fluorine in Agriculture 9–11 January 1995. In *Conference Papers University of Manchester 1995* (Fluorochem).

Boyland, E., Manson, D. and Sims, P., 1953, The preparation of *O*-aminophenyl sulphates. *Journal of the Chemical Society*, 3623–3628.

Brodie, B. B. and Axelrod, J., 1948, The estimation of acetanilide and its metabolic products, aniline, *N*-acetyl *p*-aminophenol and *p*-aminophenol (free and conjugated) in biological fluids and tissues. *Journal of Pharmacology and Experimental Therapeutics*, **94**, 22–28.

Cnubben, N. H. P., Peelen, S., Borst, J.-W., Vervoort, J., Veeger, C. and Rietjens, I. M. C. M., 1994, Molecular orbital-based quantitative structure-activity relationship for the cytochrome P450-catalyzed 4-hydroxylation of halogenated anilines. *Chemical Research in Toxicology*, **7**, 590–598.

Cnubben, N. H. P., Vervoort, J., Boersma, M. G. and Rietjens, I. M. C. M., 1995, The effect of varying halogen substituents on the cytochrome P450-catalyzed dehalogenation of 4-halogenated anilines to aminophenol metabolites. *Biochemical Pharmacology*, **49**, 1235–1248.

Cnubben, N. H. P., Van den Berg, C. L. and Rietjens, I. M. C. M., 1996, Relationships between the regioselectivity of the hydroxylation of C4-substituted 2-fluoroaniline derivatives and their toxic endpoints. *Toxicology and Applied Pharmacology*, **141**, 403–415.

Eadsforth, C. V., Logan, C. J., Morrison, B. J. and Warburton, P. A., 1984, 2,4-Difluoroaniline and 4-fluoroaniline exposure: monitoring by methaemoglobin and urine analysis. *International Archives of Occupational Environmental Health*, **54**, 223–232.

Ghauri, F. Y., Blackledge, C. A., Glen, R. C., Sweatman, B. C., Lindon, J. C., Beddell, C. R., Wilson, I. D. and Nicholson, J. K., 1992, Quantitative structure-metabolism relationships for substituted benzoic acids in the rat. *Biochemical Pharmacology*, **44**, 1935–1946.

Ivie, G. W., 1978, Fate of diflubenzuron in cattle and sheep. *Journal of Agriculture and Food Chemistry*, **26**, 81–89.

Kiese, M., 1963, The effect of certain substituents upon the *N*-oxidation of aniline *in vivo*. *Naunyn-Schmiedeberg's Arch. exp. Pathologie und Pharmakologie*, **244**, 387–404.

Koerts, J., Boeren, S., Vervoort, J., Weiss, R., Veeger, C. and Rietjens, I. M. C. M., 1996, Interaction between the substrate and the high-valent iron-oxo porphyrin cofactor as a possible factor influencing the regioselectivity of cytochrome P450-catalysed aromatic ring hydroxylation of 3-fluoro(methyl)anilines. *Chemico-Biological Interactions*, **99**, 129–146.

Luteijn, J. M. and Tipker, J., 1986, A note on the effects of fluorine substituents on the biological activity and environmental chemistry of benzoylphenylureas. *Pesticide Sciences*, **17**, 456–458.

Malet-Martino, M. C. and Martino, R., 1992, Magnetic resonance spectroscopy: a powerful tool for drug metabolism studies. *Biochimie*, **74**, 785–800.

Metcalf, R. L., Lu, P. and Bowlus, S., 1975, Degradation and environmental fate of (2,6-difluorobenzyl)-3-(4-chlorophenyl)urea. *Journal of Agriculture and Food Chemistry*, **23**, 359–364.

Omura, T. and Sato, R., 1964, The carbon monoxide pigment of liver microsomes, I. Evidence for its haemprotein nature. *Journal of Biological Chemistry*, **239**, 2370–2378.

Opdycke, J. C., Miller, R. W. and Menzer, R. E., 1982a, Metabolism and fate of diflubenzuron in swine. *Journal of Agriculture and Food Chemistry*, **30**, 1223–1227.

Opdycke, J. C., Miller, R. W. and Menzer, R. E., 1982b, *In vivo* and liver microsomal metabolism of diflubenzuron by two breeds of chickens. *Journal of Agriculture and Food Chemistry*, **30**, 1227–1233.

Park, B. K. and Kitteringham, N. R., 1994, Effects of fluorine substitution on drug metabolism: pharmacological and toxicological implications. *Drug Metabolism Reviews*, **26**, 605–643.

RIEFJENS, I. M. C. M. and VERVOORT, J., 1991, Bioactivation of 4-fluorinated anilines to benzoquinoneimines as primary reaction products. *Chemico-Biological Interactions*, **22**, 263–281.

SOFFERS, A. E. M. F., VEEGER, C. and RIEFJENS, I. M. C. M., 1994, Influence of the type of halogen substituent on *in vivo* and *in vitro* phase II metabolism of 2-fluoro-4-halophenol metabolites formed from 3-halo-fluorobenzenes. *Xenobiotica*, **24**, 759–774.

VAN LAECKE, K. and DEGHEELE, D., 1991, Detoxification of diflubenzuron and teflubenzuron in the larvae of beet armyworm (*Spodoptera exigua*) (Lepidoptera: Noctuidae). *Pesticide and Biochemical Physiology*, **40**, 181–190.

VERLOOP, A. and FERELL, C. D., 1977, Benzoylphenyl ureas—a new group of larvicides interfering with chitin deposition. In *Pesticide Chemistry in the 20th Century*, vol. 37, edited by J. R. Plimmer, pp. 237–270.

VERVOORT, J., DE JAGER, P. A., STEENBERGEN, J. and RIEFJENS, I. M. C. M., 1990, Development of a ^{19}F-nmr method for studies on the *in vivo* and *in vitro* metabolism of 2-fluoroaniline. *Xenobiotica*, **20**, 657–670.

VOGEL, A. J., 1989, In *Vogel's Textbook of Practical Organic Chemistry*, edited by B. S. Furniss, A. J. Hannaford, P. W. G. Smith and A. R. Tatchell (London: Longman Scientific and Technical), pp. 1074, 1274.

WILLEMS, A. G. M., OVERMARS, H., SCHERPENISSE, P., DE LANGE, N. and POST, L. C., 1980, Diflubenzuron: intestinal absorption and metabolism in the rat. *Xenobiotica*, **10**, 103–112.

YOUNG, M. F., TROMBETTA, L. D. and CARSON, S., 1986, Effects of diflubenzuron on the mouse liver. *Journal of Applied Toxicology*, **6**, 343–348.