Nonylphenol (NP) is a mixture of isomeric compounds with a varied and undefined degree of branching of the attached nonyl chain. This side chain is either para- or ortho-positioned in relation to the phenol group. Commercially produced NP is predominantly 4-NP. NP is widely used for the synthesis of the nonionic surfactants NP-polyethoxylates (NP-PEs). Smaller quantities of NP are used as stabilizers and antioxidants in the manufacture of various plastics such as polystyrene and polyvinyl chloride, including those used as food contact plastics. In the environment, NP contamination predominantly results from the degradation of NP-PEs, but part of it is also associated with a direct release of NP from plastics. NP residues were reported in river water and sediment; traces have also been detected in drinking water (Bennie, 1999). In addition NP has been found to bioconcentrate in fish (Ekelund et al., 1990; Ahel et al., 1993). Therefore, the main source of human exposure is expected to be food (Inoue et al., 2001; Guenther et al., 2002) and, to a lesser extent, drinking water.

The estrogenic activity of NP has been demonstrated both in vitro and in vivo. It was shown that 4-NP can stimulate the growth of human MCF7 cell lines (Soto et al., 1991) and the production of vitellogenin by rainbow trout hepatocytes (Jobling and Sumpter, 1993; Flouriot et al., 1995). Following exposure to NP, reproductive disorders have been evidenced in aquatic invertebrates as well as in fish (Jobling et al., 1996). In rainbow trout, NP exposure leads to male feminization with an increase of vitellogenin synthesis (Harries et al., 1997). In this species, metabolic studies have demonstrated the existence of two major biotransformation pathways for 4-NP, the linear side chain para-isomer of NP: 1) the conjugation of the phenol group by glucuronic acid and 2) the oxidation of the alkyl side chain (Thibaut et al., 1998; Thibaut et al., 1999). To our knowledge, only a few studies have been reported concerning the metabolic fate of NP in mammalian species. Extensive studies by Lee et al. (1996a,b, 1998) using rat or human liver microsomes have been published. However, these studies did not aim to identify the metabolites of NP formed in vitro but focused on the interactions of NP and hepatic cytochrome P450. Recently, Doerge et al. (2002) dosed rats orally with a mixture of branched 4-NP and demonstrated the formation of two metabolites, namely NP glucuronide and catechol-NP glucuronide.

The biological activity of NP is weak when compared with that of estradiol (1/10,000th to 1/1,000th). Still, experimental results indicate that this compound may act as an endocrine disruptor in vertebrates. Given the fact that human exposure to NP very likely occurs following repeated direct or indirect contact with this xenobiotic, one hypothesis explaining NP endocrine disruption is the bioaccumulation of lipophilic residues. This activity could also be related to the formation of metabolites exhibiting a greater estrogenic activity than the parent compound. Although NP was shown to bind per se to human estradiol receptor (Safe et al., 2001), it has been suggested that the hydroxylation of the aliphatic side chain might result in a bioactivation of the parent compound. To acquire basic knowledge about the metabolic fate of alkylphenols in mammals, we choose to perform several experiments using Wistar rats. In this species, we studied the
biotransformations of radiolabeled 4-n-NP, at both low and high oral dosages. The low dose (1 μg/kg) was used to investigate the metabololism of 4-n-NP at a level corresponding to a possible daily oral intake for humans. The high dose was used to investigate possible modifications of 4-n-NP metabolic routes and to obtain enough material for metabolites structural identification. Additional experiments were run in pregnant rats to better understand the distribution of 4-n-NP during gestation and the possible changes occurring in the metabolic pathways of this xenobiotic.

Materials and Methods

Chemicals. Ring-2,6-[^3]H-labeled nonylphenol ([^3]H-4-n-NP) and ring-[^14]C-labeled nonylphenol ([^14]C-4-n-NP) were purchased from Isotec (Ganagobie-Peyrus, France) and had specific activities of 1.72 GBq/μmol and 2 MBq/μmol, respectively; radiopurity was >96% based on radio-chromatographic analyses. Unlabeled 4-n-NP was obtained from Riedel-de-Haën (Seelze, Germany). Solvents used for extraction and high performance liquid chromatography (HPLC) analyses were of the highest commercial grade available from Scharlab Chemie S.A. (Barcelona, Spain) or Merck (Briare-Le-Canal, France). Ultrapure water from Milli-Q system (Millipore, Saint Quentin-en-Yvelines, France) was used for HPLC mobile phases preparation.

Apparatus. Radioactivity in urine and all other liquid samples was determined by direct counting on a Packard scintillation analyzer (model Tricarb 2200CA; PerkinElmer Life Sciences, Boston, MA) with automatic quench correction for an external standard method. Packard Ultima Gold was used as the scintillation cocktail (PerkinElmer Life Sciences). Radioactivity in rat carcasses, tissues, feces, as well as extraction pellets, was determined by complete combustion using a Packard oxidizer 306 (PerkinElmer Life Sciences). For [^13]C-labeled samples, the resultant [^14]CO2 was trapped in Carbosorb and mixed automatically with Pernaffluor E+ (PerkinElmer Life Sciences) whereas for tritiated samples, [^3]H2O was collected and mixed with Packard Mononase S and Packard Permafluer E+ prior to radioactivity quantification on the Packard scintillation counter. Combustion efficiency was greater than 95% throughout the experimental period for both radiochemicals. Three replicates were analyzed for each sample.

HPLC and radio-HPLC Samples were analyzed by HPLC on a HP1050 apparatus (Hewlett Packard, Waldbronn, France) with a reverse-phase column (250 × 4.6 mm, 5 μm; Interchim, Montluçon, France) used for metabolic profiling. HPLC system consisted of a Capcell Pak C18 column (250 × 4.6 mm, 5 μm; Interchim). The mobile phases were A, formic acid solution, pH 2.7; B, acetonitrile/formic acid solution, pH 2.7 (90:10, v/v) and were delivered according to the following gradient: 0- to 4 min 100% A; 4- to 6-min linear gradient from 100% A to A/B 90:10 v/v; 6- to 28-min A/B 90:10 v/v; 28- to 30-min linear gradient from 100% B to 40% B; 30- to 45-min A/B 60:40 v/v; 45- to 47-min linear gradient leading to 100% B; 47- to 63-min 100% B. The flow rate was 1 ml/min, and the temperature was controlled at 35°C.

The structure of most NP metabolites was established using a Finnigan LCQ ion trap spectrometer (Thermo Finnigan, Les Ulis, France) equipped with an electrospray ionization source. Typical conditions for recording mass spectra were as follows: needle voltage, 4.5 kV; capillary voltage, −32V; capillary temperature, 230°C. All mass spectra were acquired using automatic gain control conditions, and the helium buffer gas was used as collision gas for MSn studies. Sample solutions [5–10 ng/μl in methanol/water (1:1, v/v)] were infused at a flow rate of 3 μl/min into the ion source. Liquid chromatography coupled to mass spectrometry (LC-MS) was also used for the analysis of some metabolites. LC-MS experiments were carried out using the HPLC system described above with a postcolumn split of 25%, allowing the introduction of 0.25 ml/min of mobile phase into the ion source. All samples were stored and processed in single-use glass flasks or clean glassware.

In Vivo Metabolism Studies. Metabolic balance After an adaptation period of 5 days, 7-week-old Wistar rats (six males, six females) were individually housed in stainless steel metabolic cages with free access to water and to a standard diet (UAR 210; U.A.R., Villemoissoin-Orge, France) under a 12-h light/dark cycle. After 5 additional days, three males and three females were randomly assigned to group A, and the remaining rats were assigned to group B. Male rats had a mean body weight of 190 g with no significant difference between lots A and B (A, 190.3 ± 3.1 g; B, 189.7 ± 3.5 g). Female rats had a mean body weight of 160 g, with no significant difference between lots A and B (A, 160.3 ± 2.1 g; B, 160 ± 2.6 g). At 0, 24, 48, and 72 h, animals were gavaged with 0.8 MBq [^3]H-4-n-NP fortified with the appropriate amount of unlabeled 4-n-NP prior dissolution in 0.5 ml of corn oil. Doses were respectively adjusted to 1 μg/kg/day (group A, “low-dose”) or 10 mg/kg/day (group B, “high-dose”). The material used for intragastric gavage was washed with ethanol. The radioactivity recovered with ethanol was counted, thus allowing the calculation of the actual dose administered daily to each animal. Urine and feces were collected daily, at time points 24, 48, 72, and 96 h (day 1 to day 4). Urine was collected in glass flasks to which sodium azide (0.02%) and sodium ascorbate (0.015%) were added. At 96 h, rats were anesthetized with ether and killed by exsanguination by cardiac puncture. Blood samples were centrifuged at 2000g, 7°C, 10 min; plasma were separated from the red cell pellet, and the radioactivity within these samples was evaluated, by direct counting and combustion in the oxidizer, respectively. Brain, liver, kidney, testis (or ovaries + uteri), muscle, perirenal fat, the rest of viscera and carcasses were collected. These samples were homogenized in a Dangoumeau ball-grinder (Prolabo, Strasbourg, France). Representative triplicate aliquots of 200 to 300 μl of these tissues were precisely weighed for combustion prior to radioactivity determination. At the end of the experiment, metabolic cages were washed with 200 ml of ethanol from which aliquots were taken for residual radioactivity determination. All samples were stored at −20°C if not used immediately.

Two additional male rats were handled as described above and were gavaged with a single oral nominal 1 μg/kg [^14]C-4-n-NP dose. These animals, as described previously, were sacrificed to determine the results obtained with [^3]H-4-n-NP, were kept 24 h in metabolic cages and were later killed as indicated above. Similar samples were taken for radioactivity determination.

Biliary excretion. Six adult Wistar rats (3 males, 3 females) were gavaged with a single oral dose of 1 μg/kg [^3]H-4-n-NP. Radioactivity losses were determined as detailed for the metabolic balance studies. Immediately after gavage, rats were anesthetized using a urethane solution (2 g/kg, intraperitoneal injection). For each animal, the biliary duct was cannulated following abdominal laparotomy using a polyethylene catheter (i.d. 0.3 mm; o.d. 0.7 mm; Biotrol Pharma, Paris, France). Bile collection started 30 min after 4-n-NP administration. Bile was collected in glass flasks at time points 1 (0.5–1 h), 2, 3, 4, 5, and 6 h. Prior to radio-HPLC analyses, bile samples were mixed with methanol (1:3 v/v) and centrifuged at 4000g, 4°C, 5 min. The supernatant was concentrated under a nitrogen stream then diluted in mobile phase A before analysis.

Metabolism in pregnant rats. Two-day pregnant Wistar female rats (n = 3) were purchased from Iffa Credo (Saint-Germain l’Arbresle, France) and were housed individually in stainless steel metabolic cages with free access to water. From day 3 to day 19 of gestation, animals were dosed with 2.05 MBq [^3]H-4-n-NP. The dose was adjusted to 1 μg/kg/day with unlabeled 4-n-NP and was mixed to the semisynthetic diet (U.A.R. 210). Prior to commencement of dosing, the 24-h stability of [^3]H-4-n-NP, when incorporated to the diet, had been successfully verified by HPLC following ethanol extraction (yield, 100%; only unchanged 4-n-NP detected). To determine the daily ingestion of [^3]H-4-n-NP each 24 h the food left by rats was collected and extracted with ethanol, and aliquots were taken for radioactivity quantification. At day 20 of gestation, animals were sacrificed as described for the metabolic balance studies. In addition to the tissues sampled in the other experiments, placentas, fetuses, and amniotic fluid were collected for radioactivity measurement.

Sample Processing and Metabolite Isolation for MS Studies. Urinary radio-chromatographic profiles were acquired for all rats from the metabolic balance studies using radio-HPLC. Day 1 to day 4 urine samples were analyzed separately for each animal. Metabolite isolation for structural analysis was carried out using day 1 and day 2 urine samples from rats dosed with 10 mg/kg 4-n-NP (group B). Pooled urine was filtered using 0.45- then 0.22-μm Millex
**Results**

**Metabolic Balance and Radioactivity Distribution.** Group A rats (nominal dosage, 1 \( \mu g/kg \)) were dosed with 0.93 ± 0.01 \( \mu g/kg \) 4-\( n \)-NP. Group B rats (nominal dosage, 10 mg/kg) were dosed with 9.02 ± 1.08 mg/kg 4-\( n \)-NP. No significant difference was recorded, respectively, for A and B males versus females.

Over the 4-day study, most of the administered radioactivity was excreted in urine. Urinary excretion was significantly higher in male rats (57%) than in females (40%). These values were similar for groups A and B (Table 1). Fecal radioactivity excretion was found to be higher in females, for which it represented more than 20% of the dose. A significant difference between sexes was observed only for group A animals (Table 1). The cumulated amounts of radioactivity excreted in urine and feces over the 4-day period showed no significant difference between males and females, even though the values...
recorded for males were slightly higher for both dosage levels (Fig. 1).
Residual radioactivity levels measured in tissues and in the digestive tract are summarized in Table 1. For rats receiving a 1 μg/kg dose, liver and kidney contained the highest concentrations of residues (0.20–0.22 ppb). These levels were only twice higher than those measured in all other tissues, as well as in carcasses. Radioactivity repartition and residual concentrations of radioactivity calculated for samples from group B rats (dosed 10 mg/kg) were about 10,000-fold higher than those recorded for group A rats (dosed 1 μg/kg). No significant difference was observed between males and females.

The overall amount of radioactivity recovered during the 1H-4-n-NP metabolic balance study corresponded respectively for males and females to 78.5 ± 0.5% and 68.4 ± 0.3% of the administered dose. In a complementary 24-h metabolic balance experiment using two male rats dosed with 1 μg/kg 14C-4-n-NP, the total amount of radioactivity recovered was very close (73.85 ± 2.1%), and again most of the radioactivity was excreted in urine and to a lesser extent in feces.

**Urinary Metabolites Isolation and Identification.** A typical radiochromatogram corresponding to the analysis of urine from rats dosed with labeled 4-n-NP is displayed in Fig. 2. Urine was found to contain at least nine different metabolites of 4-n-NP. As expected due to the low solubility of 4-n-NP in water, the unchanged parent compound was not detected in this matrix. Metabolite purification was achieved combining C8 and Oasis extraction cartridges, followed by HPLC separation. Only 56% of the radioactivity put on C8 cartridges was retained and recovered after elution with methanol (fraction I, see Materials and Methods for details). This fraction mainly consisted of metabolites 5 to 8 and was further purified on Oasis cartridges with a recovery of 96%. The polar fraction (fraction II), corresponding to 44% of the radioactivity put on C8 cartridges, was better retained on Oasis cartridges (recovery: 86%). Chromatographic separation using the analytical HPLC system allowed to prepare enough material for the MS studies.

The structure of most of the urinary metabolites of 4-n-NP (>90% of the urinary radioactivity) was elucidated, using negative electrospray ionization mode (ESI-MS), either following direct injection of the purified metabolites or liquid chromatography coupled to MS. With the exception of metabolite 5, which was identified as the para-hydroxy benzoic acid, all 4-n-NP metabolites were characterized as conjugates (Table 2). For all metabolites, the side chain of the molecule, initially of nine-carbon length, had been shortened by successive oxidations resulting in 1-carbon length (metabolites 1, 5, 7) and 3-carbon length (all other metabolites) side-chains. Glucurono-conjugates (metabolites 1 to 4) were the most polar compounds in our HPLC conditions; their MS/MS spectra exhibited the characteristic m/z 175 ion. Metabolite 1 was the glucuronic acid conjugate of metabolite 5. Its fragmentation pathways and that of all other characterized metabolites are summarized in Table 2. Metabolites 3 and 4 were characterized as the glucuronic acid conjugates of 3-(4-hydroxyphenyl)-2-propenoic acid and 3-(4-hydroxyphenyl)-2-propionic acid, respectively. The LC-MS2 analysis of metabolite 2 is presented in Fig. 3. The MS2 spectrum of the quasi-molecular [M – H]− m/z 327 ion showed the presence of the m/z 175 ion characteristic of a glucuronic acid moiety, as well as the loss of CH3CHO (m/z 283) and that of a molecule of water (m/z 309), indicating the existence of an alcohol function. This was the only urinary metabolite of 4-n-NP that was not a carboxylic acid.

Metabolites 6 to 9′ were sulfate conjugates, with their mass spectra displaying a loss of a 80 atomic mass units neutral group diagnostic of a sulfate group. Metabolite 7 was the sulfate conjugation product of the para-hydroxy benzoic acid. Again, both the saturated (8) and the unsaturated (9, 9′) three-carbon side chain metabolites were identified. The latter metabolite was characterized from peaks collected at retention times (Rf) of 34 min (9) and 37 min (9′). Both purified metabolites (either 9 or 9′), when submitted to HPLC analysis, were found to elute at 34 and 37 min, thus allowing to hypothesize that these two peaks actually corresponded to the Z and E isomers of the sulfo-conjugate of the 3-(4-hydroxyphenyl)-2-propenoic acid. Indeed, these two isomers displayed similar MS/MS spectra, as detailed in Table 2. Metabolite 6, another three-carbon side chain compound, was identified following MS2 analysis. The successive fragmentations of the quasi-molecular [M – H]− m/z 261 ion in MS2, MS3, and MS4 experiments (Fig. 4), respectively, showed the loss of a sulfate group (m/z 181), the loss of CO2 (m/z 137), and ultimately, the loss of an ethyl group leading to the observation of the fragment ion at m/z 109 and allowing the characterization of a doubly hydroxylated aromatic ring. Thus, metabolite 6 was identified as the ring hydroxylation product of the sulfate conjugate of the 3-(4-hydroxyphenyl)-2-propenoic acid.

**Urinary Metabolites Quantification.** Four radio-HPLC analyses were performed for each rat belonging to the A and B groups and corresponding to the four days of experiment. Radioactive peak areas were integrated. To highlight possible differences between the two dosage levels, results were expressed for both groups in nanograms of 4-n-NP administered per microgram of 4-n-NP (mean % ± S.D., n = 3 animals for each time point). No significant differences were observed between animals of the same sex dosed 1 μg/kg or 10 mg/kg, except for metabolite 7, which was predominant in males in the low-dose group. In contrast, several significant differences existed between

Fig. 1. Four-day urinary and fecal cumulative radioactivity excretion in Wistar rats dosed daily with 1 μg/kg (group A) or 10 mg/kg (group B) 3H-labeled 4-n-NP (mean % ± S.D., n = 3 animals for each time point).
males and females for both dosage levels, as detailed in Fig. 5. In brief, the amount of 1-carbon side chain metabolites (1, 5, and 7) and sulfate conjugates (7, 8, 9, 9′) was generally higher in males regardless of the exposure level.

**Fecal Metabolites.** For the twelve rats in the metabolic balance study, mean radioactivity recovery from feces after 3 extractions was 73.4%. The nonextractable radioactivity accounted for 12.3%. No significant difference was observed between groups. Feces extracts were analyzed for each rat separately. As detailed in Table 3, most of the fecal radioactivity was associated with unchanged 4-n-NP. Only one other major metabolite was detected. This metabolite was found to coelute ($R_f$, 50 min) with ω and ω-1 hydroxylated 4-n-NP, purified from fish (Arukwe et al., 2000; Thibaut et al., 2000). Due to the low amount of material available, no further analysis was performed on this metabolite. No significant difference was observed between the different rat lots for the amounts of hydroxylated 4-n-NP excreted in feces. The rest of the radioactivity was eluted at different $R_f$, most of which corresponded to metabolites already characterized from urine. However, each compound was only associated with a very low amount of radioactivity.

**Hepatic Residues.** About 76% of the radioactivity in liver could be extracted, and 12% were recovered from the extraction pellets. The only significant difference observed was for the amount of nonextractable radioactivity in pellets from male and female rats dosed 10 mg/kg (Table 2).

![Fig. 2. Typical radio-chromatographic analysis of urine in rats dosed with $^{3}$H-labeled 4-n-NP (displayed radio-chromatogram, 0–24 h urine from a male rat dosed orally with 1 μg/kg 4-n-NP).](image)

**TABLE 2**

*In vivo urinary metabolites of 4-n-NP in rat*

<table>
<thead>
<tr>
<th>$R_f$ (min)</th>
<th>Metabolite</th>
<th>Structure</th>
<th>[M - H]+</th>
<th>Fragmentation Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.6</td>
<td>para-Hydroxy benzoic acid glucuronide</td>
<td><img src="structure1" alt="" /></td>
<td>313</td>
<td>175 (a)</td>
</tr>
<tr>
<td>13.3</td>
<td>3-(4-Hydroxyphenyl) propanol glucuronide</td>
<td><img src="structure2" alt="" /></td>
<td>327</td>
<td>175 (a) 283 (b) 309 (-H2O)</td>
</tr>
<tr>
<td>15.9</td>
<td>3-(4-Hydroxy-phenyl)-2-propenoic acid glucuronide</td>
<td><img src="structure3" alt="" /></td>
<td>339</td>
<td>175 (a)</td>
</tr>
<tr>
<td>17.6</td>
<td>3-(4-Hydroxy-phenyl)-2-propionic acid glucuronide</td>
<td><img src="structure4" alt="" /></td>
<td>341</td>
<td>175 (a)</td>
</tr>
<tr>
<td>18</td>
<td>para-Hydroxy benzoic acid sulfate</td>
<td><img src="structure5" alt="" /></td>
<td>MS² analysis (see Fig. 4)</td>
<td></td>
</tr>
<tr>
<td>22.4</td>
<td>Ring-hydroxylated 3-(4-hydroxyphenyl)-2-propionic acid sulfate</td>
<td><img src="structure6" alt="" /></td>
<td>261</td>
<td></td>
</tr>
<tr>
<td>24.2</td>
<td>para-Hydroxy benzoic acid sulfate</td>
<td><img src="structure7" alt="" /></td>
<td>217</td>
<td>137 (a) 173 (b)</td>
</tr>
<tr>
<td>26.8</td>
<td>3-(4-Hydroxy-phenyl)-2-propionic acid sulfate</td>
<td><img src="structure8" alt="" /></td>
<td>245</td>
<td>165 (a)</td>
</tr>
<tr>
<td>34</td>
<td>3-(4-Hydroxy-phenyl)-2-propenoic acid sulfate</td>
<td><img src="structure9" alt="" /></td>
<td>243</td>
<td>163 (a) 199 (b)</td>
</tr>
</tbody>
</table>
4). Whatever the sample, the radioactivity contained in the first extract accounted for more than 75% of the total extractable radioactivity. However, it was impossible to achieve an HPLC-profiling, since most of it was lost during the concentration step, probably in relation with the presence of volatile compounds. This possibility was supported by further observations; when a large volume injection loop was used to shorten the concentration procedure, 100% of the detected radioactivity was eluted at 3 min, which corresponded to the dead volume of the HPLC system. Moreover, in rats dosed with \(^{14}\)C-4-\(\alpha\)-NP, similar results were established; the extractable and nonextractable fractions, respectively, accounted for about 80 and 10% of the hepatic radioactivity. Again, the extractable radioactivity appeared to be associated with volatile compounds that could not be retained on the HPLC column (nor on anion exchange cartridges, data not shown).

The extraction protocol developed for liver was applied to other tissues (brain, kidney, gonads, adipose tissue). The extractable radioactivity always accounted for at least 75% of the radioactivity present in the tissue but was always lost during the concentration step.

**Biliary Excretion.** Corrected dosages received by male and female rats in the biliary excretion experiment were respectively 0.73 ± 0.04 µg/kg and 0.82 ± 0.05 µg/kg. Over the 6-h study, cumulated radioactivity excretion was higher in males (9.5% of the dose; Fig. 6) than in females (5.9%). The difference was statistically significant at all time points with the exception of the first sample (0.5–1 h). Radiochromatographic profiles were carried out for all 1- and 6-h samples, showing a similar pattern for males and females. Biliary metabolites were identified on the basis of their respective \(R_f\) compared with that of previously identified urinary metabolites. All biliary conjugates were collected and submitted to enzymatic digestion with *Helix pomatia* juice, before an additional radio-HPLC analysis to confirm the \(R_f\) of the corresponding aglycone.

Three major compounds were detected; unchanged nonylphenol accounted for 43.6 and 40.5% of the radioactivity, in males and females, respectively. The \(\alpha\)-hydroxy benzoic acid glucuronide (\(R_f\), 39 min) was better represented in males (21.2%) than in females (11.7%, \(p < 0.05\)). A metabolite with a \(R_f\) of 39 min accounted for 12.4 and 22.6% in males and females, respectively. It was identified as the glucuronic acid conjugate of hydroxylated 4-\(\alpha\)-NP, on the basis of \(R_f\) comparison with an authenticated metabolite isolated from rainbow trout (Thibaut et al., 2000). When this compound was submitted to enzymatic digestion, the associated radioactivity shifted to 50 min, thus supporting the hypothesis that the
hydroxylated 4-n-NP found in feces was actually excreted in bile as a glucurono-conjugate.

**Nonylphenol Residue Distribution in Pregnant Rats.** Pregnant rats were dosed from day 3 to 19 of gestation with a mean oral dosage of 0.649 μg/kg/day 4-n-NP. Radioactivity losses (food left by rats and radioactivity washed from cages) accounted for only 1.4% of the dose. Radioactivity levels in tissues (Table 5) were higher than the levels measured for the low-dose group rats in the metabolic balance study.
the highest levels being measured in liver, kidney, and fat. However, given the difference between the two experimental protocols, no direct comparison can be attempted between the two groups. The amount of radioactivity recovered from fetuses, uteri, and placenta was relatively low and corresponded to an average residue concentration of 0.12 ppb.

**Fig. 5.** Quantitative analysis of urinary radioactivity in rats dosed orally with 1 μg/g (group A) or 10 mg/g (group B) 4-n-NP over a 4-day study (mean of 3 animals ± S.D.).

DV, radioactivity eluted at the dead volume of the HPLC system.
A four-step extraction of maternal livers allowed to recover 81 ± 8% of the hepatic radioactivity, extraction pellets accounting for an additional 12.1 ± 0.9%. These results were in the same range as those established for rats in the metabolic balance studies, but in the case of pregnant rats, only 90% of the radioactivity was lost during the extract concentration step. The remaining 10% were analyzed by radio-HPLC. Based on chromatographic behavior, the main hepatic metabolites were tentatively identified as hydroxylated 4-n-NP, the glucuronide conjugate of 4-n-NP and unchanged 4-n-NP. Peaks corresponding to saturated and unsaturated sulfates (metabolites 8 and 9) as well as a glucuronide (metabolite 4) were also detected.

Blood radioactivity was mainly associated with the plasma fraction (74.3%). Methanol extractions allowed to recover 67% of this radioactivity, which was again mainly associated with volatile compounds. Only 3.6% of it could be analyzed by radio-HPLC, showing the presence of peaks eluted at the same R<sub>t</sub> as the glucuronic acid conjugate of 4-n-NP and metabolites 4, 7, and 8. Amniotic fluids contained an average radioactivity level corresponding to 0.31 ppb of 4-n-NP equivalent and were extracted as indicated for plasma. The radioactivity fraction that was not lost during the extract concentration step eluted at the dead volume of the HPLC system. Radioactivity contained in fetuses was extracted using the protocol applied to liver. Respectively 97.9 and 97.6% of male and female fetuses radioactivity was extractable, but again all of it was lost during the concentration step.

### Discussion

Metabolic balance experiments were carried out using two different 4-n-NP dosages. Whatever the dosage, the ingested radioactivity was mainly excreted in urine and feces. There was a significant difference between males and females in the proportion of radioactivity excreted in urine, which accounted for 57% of the dose in males but only 40% in females, regardless of the dose administered. However, cumulated urinary and fecal excretion of radioactivity was not significantly higher in males over the 4-day study. Moreover, no differences were observed between males and females in the proportion of radioactivity excreted in urine and feces. There was a significant difference in females, regardless of the dose administered. However, cumulated urinary and fecal excretion of radioactivity was not significantly higher in males over the 4-day study. Moreover, no differences were observed between males and females in the proportion of radioactivity excreted in urine.

Precautions were taken to quantify radioactivity losses during the experiments, but radioactivity recovery only reached 80% for all metabolic balance studies. Most of the experiments were carried out using tritiated 4-n-NP to achieve enough sensitivity for radio-chromatographic profiling. Consequently part of the radioactivity may...
have been lost because of isotopic exchanges between the labeled molecule and water. Radioactivity losses may also have occurred because of the formation of low molecular weight volatile metabolites. The latter hypothesis was supported by the fact that identical results were obtained when using 14C-labeled 4-n-NP. Moreover, most tissue extractions led to the conclusion that the radioactivity present in tissues was mainly associated with volatile compounds, thus supporting the hypothesis of a complete breakdown of 4-n-NP into very small molecules.

Metabolite identification and radio-HPLC profiling showed that in vivo, 4-n-NP is extensively metabolized in rat. About 10 different metabolites were characterized. Most of them were formed by the ω or β-oxidation of the 9-carbon side chain of 4-n-NP. In rat, unlike in fish (Thibaut et al., 1998), no 5-carbon and 7-carbon side chain metabolites were detected. The main part of urinary radioactivity was indeed associated with 1- and 3-carbon side chain metabolites, most of which were identified as conjugates. The proportion of sulfo-conjugates was notably higher than reported in rainbow trout and salmon, for which glucuronides have been shown to prevail (Thibaut et al., 1998; Arukwe et al., 2000). In addition, it appeared that sulfo-conjugation was more pronounced in male than in female rats. The metabolic pathways of 4-n-NP in rat mainly led to the formation of para-hydroxy benzoic acid and of the corresponding sulfate, the two major biotransformation products of this alkylphenol. However, it cannot be excluded that the para-hydroxy benzoic acid may be further metabolized into smaller molecules, thus explaining the formation of volatile residues.

Based on radio-chromatographic profiles, feces and bile were shown to contain several 4-n-NP metabolites very likely resulting from the β-oxidation of the parent compound, but these metabolites were only detected in very low amounts. In these samples the major metabolite was characterized as hydroxylated 4-n-NP, resulting from the ω (and/or the ω-1) hydroxylation of 4-n-NP. The relative contribution of the β-oxidative metabolic pathway (mainly urinary metabolites) and of the hydroxylation metabolic pathway was not dose-dependent. Fecal hydroxy-4-n-NP was excreted in bile as a glucuronic acid conjugate, which was very likely deconjugated by the intestinal flora into the corresponding aglycone, a common process in mammalian species (Scheline, 1973). It is worth noting that although female rats excreted more radioactivity in feces, biliary excretion was significantly more important in males. Thus, the intestinal reabsorption of 4-n-NP residues and their possible entero-hepatic cycling could be different in male and female rats.

Based on these results, it is concluded that in Wistar rat, 4-n-NP is mainly metabolized, very likely through β-oxidation of the alkyl side chain (prior or after the conjugation of the phenol group). Two carbons are lost following each loop of the β-oxidation cycle, giving rise to metabolites bearing a side chain with an odd number of carbon atoms. The first two compounds, that should be formed by successive β-oxidation cycles (C7 and C5), have not been detected in Wistar rat. Two C3 metabolites have been characterized: saturated C3 [3-(4-hydroxyphenyl)-2-propionic acid] and unsaturated C3 [3-(4-hydroxyphenyl)-2-propenoic acid], ultimately giving rise to a one-carbon side chain metabolite, the para-hydroxy benzoic acid. All these metabolites are submitted to extensive conjugation on the phenol moiety and have been characterized as the corresponding sulfates and glucuronides. The second major metabolic pathway of 4-n-NP is the hydroxylation of the alkyl side chain, followed by the glucuronidation of the phenol moiety. The corresponding metabolite is mainly excreted by fecal route.

Regardless of the possible estrogenic activity of 4-n-NP metabolites, which remain to be assayed, several hypothesis can be drawn on the basis of the characterized structures. The reduced analog of the 3-(4-hydroxyphenyl)-2-propionic acid (metabolite 4, when conjugated to glucuronic acid, namely the corresponding alcohol, was characterized from urine. Since this type of reduction is rarely achieved by mammalian biotransformation enzymes (Keneth et al., 1990), this metabolite is very likely formed in the gut by bacterial reductases, then reabsorbed by the digestive tract mucosa and glucuronidated in liver prior to urinary excretion. Another interesting finding is the characterization of metabolite 6. Due to the low amount of isolated material, it was not possible to determine unequivocally the hydroxylation site for this metabolite. However, mass spectra clearly indicated that the hydroxylation took place on the aromatic ring. Thus, there is a very high probability that metabolite 6 is a catechol. This metabolic pathway has also recently been evidenced for branched 4-n-NP (Doerge et al., 2002). Given the known reactivity of such kind of structure, as already demonstrated for natural estrogens (Bolton et al., 1998), the biological significance of these results should be further investigated for alkylphenols.

Experiments in pregnant rats showed that no tissue-specific distribution of 4-n-NP residue occurred following a 2-week oral course of treatment. Radio-chromatographic profiles were obtained from pregnant rat liver and plasma, demonstrating the presence of extractable nonvolatile metabolites. However, it was impossible to detect any 4-n-NP metabolite in fetuses or in amniotic fluids. Since 4-n-NP (or its metabolites) are not detected in fetuses, we conclude that no significant amounts of this xenobiotic cross the placental barrier in Wistar rat. 4-n-NP metabolism by fetal enzymes is very unlikely, and the fact that a very high proportion (98%) of the fetal radioactivity is extractable indicates that only traces of 4-n-NP residues should be present in fetuses as bound residues.

These conclusions apply only for 4-n-NP, and the situation may be different for branched 4-NP (Doerge et al., 2002). About 15 NP-PE isomers are found in commercial NP mixtures (Gundersen, 2001). The resulting branched side chain 4-NP isomers will not undergo a complete breakdown of the alkyl side chain because β-oxidation will only proceed on the linear terminal part of this side chain. Consequently, it is expected that the β-oxidation pathway will be less extensive for multibranched isomers than it is for 4-n-NP. Hydroxylation of the side chain of 4-n-NP was demonstrated in rat, as it previously was in rainbow trout (Thibaut et al., 1998). It is important to investigate the biological significance of this biotransformation, because the presence of two hydroxy groups on the NP molecule may imply the enhancement of the estrogenicity of these xenobiotics, given the known importance of these chemical functions when considering natural estrogens. The alkyl side chain hydroxylation metabolic pathway is expected to be better represented for branched molecules, as the β-oxidation route will be partly blocked. The current experiments should be helpful to workout and understand the metabolic fate of these complex mixtures, in which some isomers may produce residues of longer half-life and/or higher biological activity than the parental compounds.

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References


