IDENTIFICATION OF URINARY METABOLITES OF ISOPRENE IN RATS AND COMPARISON WITH MOUSE URINARY METABOLITES

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ABSTRACT:

Isoprene, a major commodity chemical used in production of polyisoprene elastomers, has been shown to be carcinogenic in rodents. Similar to findings for the structurally related compound butadiene, mice are more susceptible than rats to isoprene-induced toxicity and carcinogenicity. Although differences in uptake, and disposition of isoprene in rats and mice have been described, its in vivo biotransformation products have not been characterized in either species. The purpose of these studies was to identify the urinary metabolites of isoprene in Fischer 344 rats and compare these metabolites with those formed in male B6C3F1 mice. After i.p. administration of 64 mg [14C]isoprene/kg to rats and mice, isoprene was excreted unchanged in breath (~50%) or as urinary metabolites (~32%). In rats isoprene was primarily excreted in urine as 2-hydroxy-2-methyl-3-butenonic acid (53%), 2-methyl-3-buten-1,2-diol (23%), and the C-1 glucuronide conjugate of 2-methyl-3-buten-1,2-diol (13%). These metabolites are consistent with preferential oxidation of isoprene’s methyl-substituted vinyl group. No oxidation of the unsubstituted vinyl group was observed. In addition to the isoprene metabolites found in rat urine, mouse urine contained numerous other isoprene metabolites with a larger percentage (25%) of total urinary radioactivity associated with an unidentified, polar fraction than in the rat (7%). Unlike butadiene, there was no evidence that glutathione conjugation played a significant role in the metabolism of isoprene in rats. Because of the unidentified metabolites in mouse urine, involvement of glutathione in the metabolism of isoprene in mice cannot be delineated.

Isoprene (2-methyl-1,3-butadiene) is a major commodity chemical that is used principally in the preparation of cis-1,4-polyisoprene for the manufacture of rubber tires, automotive parts, footwear, adhesives, and flooring (Lybarger, 1995; U.S. International Trade Commission, 1995). Workers involved in the manufacturing, processing, or handling of products made with isoprene are at the greatest potential health risk due to their exposure to this compound. Toxicological interest in isoprene stems from the potential for significant human exposure and because of its structural similarity to 1,3-butadiene, a potent rodent carcinogen and probable human carcinogen (Huff et al., 1985; Owen et al., 1987; Melnick et al., 1990a; IARC, 1992; NTP, 1995; Melnick and Kohn, 1995; also see Bond et al., 1995). Studies with isoprene in rodents have resulted in a spectrum of toxic and carcinogenic effects that are strikingly similar to those observed with butadiene (Melnick et al., 1990b, 1994, 1996; Placke et al., 1996). As with butadiene, short-term inhalation exposure to isoprene elicited hematological effects, olfactory degenerative changes, testicular atrophy, and forestomach hyperplasia in mice with no significant effects in rats (Melnick et al., 1990b). After 26-week inhalation exposure of rats and mice to isoprene or butadiene, multiple organ neoplasia was observed in mice whereas a marginal increase in the incidence of benign interstitial cell testicular tumors was the only tumorigenic response in rats (Melnick et al., 1994, 1996).

The observed species differences in sensitivity and target organ toxicity may be related in part to quantitative and/or qualitative differences in chemical disposition or biotransformation. Differences in uptake and disposition of isoprene between rats and mice have been reported. For example, metabolism of isoprene appeared saturable at lower concentrations in rats compared with mice and the rate of isoprene metabolism in the mouse was 2 to 3 times that in the rat (Peter et al., 1990; Bond et al., 1991). Both isoprene and butadiene are metabolized in vitro to monooxepines and diepoxides in rats and mice (Del Monte et al., 1985; Gervasi and Longo, 1990; Wistuba et al., 1994). Butadiene metabolism was further characterized by the formation of glutathione conjugates, and quantitative differences in metabolism have been demonstrated among mice, rats, and humans (Bechtold et al., 1994).

Urinary metabolites of isoprene have not been identified. The primary objectives of the present studies were to identify the major urinary metabolites of isoprene in rats and to compare these metabolites with those formed in mice. Characterization of metabolic pathways may lead to further insight into species differences in sensitivity to isoprene toxicity and carcinogenicity and thus aid in assessing the risk to humans of inhaled isoprene.

Materials and Methods

Chemicals. Unlabeled isoprene was obtained from Aldrich Chemical Co. (Milwaukee, WI, >98% pure) and the chemical identity was verified by gas
chromatography/mass spectroscopy (GC/MS; see below). Isoprene was obtained from DuPont-NEN (Boston, MA) at a specific activity of 0.79 mCi/mmol. Radiolabeled isoprene was transferred into an ethanol solution containing 0.1% 4-methoxyphenol (to inhibit polymerization) using vacuum line techniques. Radiochemical purity (>98%) was verified using HPLC System 1 (see below). Labeled and unlabeled isoprene were stored at −20°C in tightly capped containers in the dark.

Synthesis of 2-hydroxy-2-methyl-3-butenolic acid was adapted from the procedure of Kitahara et al. (1988). Pyruvic acid (Aldrich, 4.4 g, 50 mmol) was dissolved in 44 ml of tetrahydrofuran, and the solution was cooled to −20°C under nitrogen. Vinyl magnesium bromide (Aldrich, 100 mmol in 100 ml of tetrahydrofuran) was added dropwise with stirring, maintaining the temperature between −10 and −5°C. After addition, the temperature was allowed to rise to 20°C, and 15 ml of 6 N hydrochloric acid was added. The layers were separated and the organic layer washed with saturated sodium chloride solution and subsequently dried over magnesium sulfate. The solvent was removed under reduced pressure and a portion of the crude product purified by HPLC (C18 column with water mobile phase). 2-Methyl-3-buten-1,2-diol was prepared by acid-catalyzed hydrolysis of 1,2-epoxy-2-methyl-3-buten-1,2-diol (Aldrich) as described by Del Monte et al. (1985).

Animals and Treatment. The species and strains of animals used were chosen based on the chronic bioassays with isoprene (Melnick et al., 1994). Young, adult male B6C3F1 mice and Fischer 344 rats were purchased from Charles River Laboratories (Laleigh, NC or Kingston, NY) and weighed to 25 and 230 to 300 g, respectively, at the time of treatment. Animals were housed individually in glass, Roth-type metabolism chambers, which provided for the separate collection of urine, feces, and exhaled volatiles. [%C]Isoprene in corn oil was administered i.p. at a dose of 64 mg/kg [5–10 ml/kg; 15–30 ml/kg] to rats (Peter et al., 1990). Urine and feces were collected separately over dry ice in timed fractions ending at 3, 6, 12 (mice only), and 24 h post dose. Samples were stored at −20°C in tightly capped containers in the dark until analyzed. Radiolabeled compounds in breath were collected by passing the air from the metabolism cage through a series of traps containing ethanol at 0°C and 20°C in tightly capped containers in the dark until analyzed. Radiolabeled compounds in breath were collected by passing the air from the metabolism cage through a series of traps containing ethanol at 0°C and −60°C followed by a final 1 N sodium hydroxide trap. Traps were changed at 3, 6, 12 (mice only), and 24 h.

At 24 h, the animals were anesthetized (60 mg/kg i.p. pentobarbital, mice; 7.1: ketamine:xyazine, 60 mg/kg i.m., rats) and blood was collected by cardiac puncture. Mice were sacrificed by CO2 asphyxiation and rats by intracardiac injection of pentobarbital. Selected tissues were excised, weighed, and assayed for radiochemical content. The remaining carcass was digested in ethanolic sodium hydroxide and assayed for radiochemical content. Samples were assayed for total radioactivity either directly (after dissolution in scintillation cocktail; Ultima Gold, Packard Instruments) or after digestion in Soluene-350 (Packard Instruments) or in 2 N ethanolic sodium hydroxide.

HPLC. HPLC was performed on systems consisting of two Waters dual piston (6000A or 510) pumps, a system controller, a Rheodyne model 7125 injector, a Kratos model 773 or Applied Biosystems 757 Absorbance Detector set at 220 or 230 nm, and a NUNUS β-RAM or Ramona LS scintillation detector, each equipped with a ca. 100 μl flow-through scintillator cell. All mobile phase systems were prepared by volume and all gradients were linear. Unless otherwise stated, all chromatography was performed at room temperature (−22°C). In system 1, 250 × 4.6 mm Zorbax C-8 column and an isocratic mobile phase consisting of methanol:water (3:1) at a flow rate of 1 ml/min were used. In system 2, the same column was used but with a mobile phase consisting of mixtures of acetonitrile and a 0.05 M ammonium acetate buffer (pH 5) at a flow rate of 1 ml/min. The mobile phase was maintained at 100% buffer for 5 min after injection of the sample, then changed over a 2-min gradient to 90% acetonitrile and then over a 10-min gradient to 100% acetonitrile. System 3 (a-c) was used for the collection of urinary metabolites and consisted of either a 250 × 10 mm (3a,b) or 250 × 21.4 mm (3c) Dynamax C-18 column and the mobile phase described for system 2. In system 3a, the mobile phase was isocratic at 98% buffer. In systems 3b and 3c, the mobile phase was maintained at 100% buffer for 5 min after injection of the sample, then changed over a 20-min gradient to 25% acetonitrile and finally over a 2-min gradient to 100% acetonitrile. Flow rates were 2 ml/min for systems 3a and 3b and 5 ml/min for system 3c. System 4 consisted of a Supelcosil LC-ABZ column (15 × 4.6 mm) and a water:methanol mobile phase containing 0.1% triethylamine. The initial mobile phase composition, 64% methanol, was maintained for 5 min after injection of the sample and then changed over a 5-min gradient to 77.5% methanol and finally over a 10-min gradient to 91% methanol. The flow rate was 1 ml/min. System 5 consisted of an Aminex HPX-87H column (300 × 7.8 mm) maintained at 60°C and a mobile phase of 0.005 M sulfuric acid at a flow rate of 0.6 ml/min. System 6 consisted of a Partisil 10 SAX column (250 × 4.6 mm) and a mobile phase of 0.035 M ammonium acetate buffer, pH 5, at a flow rate of 1 ml/min.

Identification of Urinary Metabolites. Preliminary profiles of radiolabeled urinary metabolites of isoprene were obtained using HPLC system 2. Metabolites were collected using HPLC system 3b and c.

Typical radiochromatograms of rat and mouse urine are shown in Fig. 1. Individual radiolabeled metabolites from rat urine were collected and identified as peaks A through D. Corresponding radiolabeled metabolites from mouse urine were also collected. Individual peak fractions were pooled and lyophilized. There was an insufficient amount of peak D metabolite in mouse urine to be collected for subsequent analyses.

Metabolite peak A, the most polar metabolite peak in the rat urine, was rechromatographed on HPLC system 5. Fractions were neutralized with 0.02 N sodium hydroxide to pH 5 to 6, pooled, and lyophilized to dryness. The residue was reconstituted in methanol, in which sodium sulfate is insoluble, and centrifuged to pellet the salt crystals. The process of evaporation, reconstitution with methanol, and centrifugation was repeated several times to remove any remaining salt before NMR analysis.

The single metabolite in peak B (metabolite B) was reconstituted in isopropanol and purified using HPLC system 6. Collected peak fractions of metabolite B were lyophilized and analyzed by NMR and GC/MS. Purified rat and mouse metabolite B were derivatized using trimethylsilyldiazomethane (TMSCNHN2; Aldrich Chemicals, 2.0 M solution in hexanes) which, in the presence of methanol, converts carboxylic acids to methyl esters. To ensure that metabolite B was in the protonated form, 0.1 M acetic acid in methanol was added (Hashimoto et al., 1981). Excess TMSCNHN2, diluted with tolune, was added dropwise to the metabolite B solutions until the yellow tint of unreagent TMSCNHN2 remained. The reaction mixtures were allowed to mix at room temperature for at least 1 h. Excess TMSCNHN2 was removed by evaporation with a stream of nitrogen gas. HPLC profiles of derivatized and underivatized material were compared.

The residue of the metabolite peak C fraction containing rat urinary me-
Chemical ionization spectra were obtained using methane as the reagent gas. Standard electron impact spectra were acquired at 70 eV. The source and quadrupoles were held at 200°C and ramped at 40°C/min to 210°C for 5 min, then ramped at 40°C/min to 250°C, respectively. The oven temperature was held at 40°C for 1.5 min, ramped at 40°C/min to 170°C for 5 min, and 100°C, respectively. 

**Table 1**

Disposition and time course of excretion of total radioactivity after a single i.p. dose of 64 mg [14C]isoprene/kg (% administered dose)

<table>
<thead>
<tr>
<th>Time Post Dose</th>
<th>Volatile Breath</th>
<th>CO2 Breath</th>
<th>Urine</th>
<th>Feces</th>
<th>Carcass and Tissues</th>
<th>Total % Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.8 ± 0.6</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>3.1 ± 0.6</td>
<td>12.9 ± 2.7</td>
</tr>
<tr>
<td>6</td>
<td>13.7 ± 1.9</td>
<td>1.7 ± 0.3</td>
<td>32.2 ± 2.1</td>
<td>0.2 ± 0.2</td>
<td>3.1 ± 0.6</td>
<td>90.8 ± 0.8</td>
</tr>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>45.6 ± 6.7</td>
<td>1.6 ± 0.2</td>
<td>1.0 ± 1.9</td>
<td>0.9 ± 0.6</td>
<td>6.7 ± 0.6</td>
<td>45.6 ± 6.7</td>
</tr>
<tr>
<td>6</td>
<td>0.8 ± 0.2</td>
<td>0.1 ± d</td>
<td>16.7 ± 10.2</td>
<td>0.7 ± 0.4</td>
<td>6.7 ± 0.6</td>
<td>43.1 ± 1.6</td>
</tr>
<tr>
<td>12</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± d</td>
<td>15.3 ± 11.7</td>
<td>5.6 ± 5.6</td>
<td>1.7 ± 0.4</td>
<td>22.9 ± 10.9</td>
</tr>
<tr>
<td>24</td>
<td>45.6 ± 6.6</td>
<td>9.1 ± 0.2</td>
<td>32.9 ± 3.4</td>
<td>7.2 ± 5.5</td>
<td>1.7 ± 0.4</td>
<td>91.0 ± 4.7</td>
</tr>
</tbody>
</table>

*Mean ± S.D.; n = 3 (rats) or 4 (mice).

**Results**

The disposition and time course of excretion of [14C]isoprene recovered in the excreta, exhaled breath, carcass, and tissues of rats and mice are summarized in Table 1. An average of 91% of the dose was recovered in each study. The largest fraction of the dose (~50%) was exhaled as volatiles. HPLC analysis (system 1) of the 3-h volatile breath traps for mice indicated that at least 94% of the recovered radioactivity was parent [14C]isoprene. Urine was the next major pathway of excretion, accounting for approximately 30% of the dose. Carbon dioxide and feces were relatively minor excretion pathways. Excretion was rapid with ~50% of the recovered dose exhaled within 3 h post dosing.

Little of the dose (2–3%) remained in the carcass and tissues at 24 h (Table 1). The distribution of radioactivity in the rat and mouse tissues is summarized in Table 2. Consistent with urinary excretion being a major route of elimination, the kidney had both the highest tissue concentration and tissue-blood ratio followed by the bladder. Concentrations in the remaining tissues were similar, ranging from 0.5 and 1.2 μg-eq/g in adipose tissue to 1.8 and 3.3 μg-eq/g in liver of mice and rats, respectively.

Representative radiochromatograms (HPLC system 3b) of rat and mouse urine are shown in Fig. 1. No parent isoprene was excreted in the urine and all urinary metabolites were much more polar than isoprene. Four major metabolite peaks (A-D) were consistently present in rat urine at each collection timepoint. There was a shift in excretion to the more polar metabolite B over the 24-h period. In addition to the four major metabolite peaks found in rat urine, mouse urine contained several other minor [14C]metabolites. No metabolite degradation was observed when any of the isolated metabolites were re-assayed by the original methods or in subsequent purification procedures.

In rats the most abundant metabolite peak was B, accounting for ca. 53% of the metabolized isoprene. The single metabolite in peak B (metabolite B) was identified as 2-hydroxy-2-methyl-3-butenoic acid based on NMR and GC/MS analyses. The 1H NMR spectrum of metabolite B (Fig. 2, top) contained signals for the C-5 methyl group at 1.35 ppm (s) and the two C-4 olefinic protons at 5.07 (d, J = 10.5 Hz) and 5.67 (d, J = 10.5 Hz).
Hz) and 5.24 (d, J = 17.3 Hz) ppm, each coupled to the C-3 proton at 6.01 ppm (dd, J = 17.3, 10.5 Hz). Interestingly, smaller, replica peaks that were only slightly up- or down-shifted from the primary peaks were also present; the significance of these “shadow” signals is unclear. The $^{13}$C NMR spectrum of metabolite B contained signals at 24.1 (C-5), 77.7 (C-2), 114.6 (C-4), 142.8 (C-3), and 182.8 (C-1) ppm.

$^1$H and $^{13}$C NMR spectra of synthetic 2-hydroxy-2-methyl-3-butenoic acid were essentially identical with those of metabolite B except that the proton spectrum of the synthetic compound did not contain the “shadow” signals. Data acquired from the GC/MS analyses of metabolite B was consistent with the proposed structure. GC/MS analysis of the metabolite B after derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA) in acetonitrile revealed a molecular ion at m/z 260 amu; the spectrum also contained prominent signals at m/z 245, m/z 217, m/z 143, and m/z 73 (trimethylsilyl; TMS), which could be formed through the fragmentation pathways shown (Fig. 3). Metabolite B was also derivatized with butaneboronic acid in acetonitrile, which resulted in the anticipated ions at m/z 183 amu (M $^+$ 1, due to protonation of the analyte) and signals at m/z 138 (M $^-$ CO$_3^-$), m/z 125 (M $^-$ C$_6$H$_5$O$_2^-$), and m/z 81 ([M $^+$ 1] $^-$ CO$_2$ and C$_6$H$_5$O$_2^-$). The synthetic standard was derivatized with BSTFA and butaneboronic acid in acetonitrile. GC/MS fragmentation results from the two derivatization analyses were practically identical with those obtained from metabolite B (data not shown). The mass spectra of the TMS derivatives of both metabolite B and synthetic 2-hydroxy-2-methyl-3-butenoic acid are identical with that previously reported for this chemical (Steen and Ransnas, 1983).

Metabolite peak D accounted for approximately 23% of the metabolized isoprene. Identification of the single metabolite in this peak (metabolite D) was hampered by difficulties in isolating sufficient quantities of this volatile metabolite from water. Based on preliminary NMR analyses, metabolite D was tentatively identified as 2-methyl-3-buten-1,2-diol, the aconjugate of metabolite C. This diol was synthesized and NMR spectra and GC/MS data from the synthetic 2-methyl-3-buten-1,2-diol were compared with that obtained from metabolite D. The $^1$H NMR spectra of metabolite D (Fig. 2, bottom spectrum) contains signals for the two C-4 olefinic protons at 5.28 ppm (d, J = 17.4 Hz) and 5.21 ppm (d, J = 10.9 Hz), each coupled to the C-3 olefinic proton at 5.94 ppm (dd, J = 17.4 and 10.9 Hz). The C-1 methylene protons at 3.48 ppm (s, 2H) and the C-5 methyl protons at 1.26 ppm (s, 3H) were also present. The spectrum of synthetic 2-methyl-3-buten-1,2-diol was essentially identical with that of the metabolite. The $^{13}$C NMR spectra of metabolite D and the synthetic diol standard contained identical signals at 144.3 ppm and 116.9 ppm (C-3 and C-4), 76.6 ppm (C-2), 71.3 ppm (C-1), and 25.6 ppm (C-5).

Metabolite D and the diol standard were derivatized with butylboronic acid, which specifically reacts with vicinal hydroxyls (Knapp, 1979). GC/MS analyses of the products were compared. The GC

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration</th>
<th>Tissue-Blood Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg-eq/g</td>
<td></td>
</tr>
<tr>
<td>Rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>1.20 ± 0.17$^{a,b}$</td>
<td>0.45 ± 0.16</td>
</tr>
<tr>
<td>Bladder</td>
<td>3.72 ± 0.59</td>
<td>1.38 ± 0.38</td>
</tr>
<tr>
<td>Blood</td>
<td>2.77 ± 0.48$^b$</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.54 ± 1.87$^b$</td>
<td>2.50 ± 1.17</td>
</tr>
<tr>
<td>Liver</td>
<td>3.32 ± 0.47$^b$</td>
<td>1.25 ± 0.42</td>
</tr>
<tr>
<td>Lung</td>
<td>2.50 ± 0.44$^b$</td>
<td>0.94 ± 0.35</td>
</tr>
<tr>
<td>Skin</td>
<td>2.55 ± 0.44$^b$</td>
<td>0.96 ± 0.36</td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>0.48 ± 0.13</td>
<td>0.44 ± 0.18</td>
</tr>
<tr>
<td>Bladder</td>
<td>2.21 ± 1.81</td>
<td>1.81 ± 1.15</td>
</tr>
<tr>
<td>Blood</td>
<td>1.13 ± 0.22</td>
<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.16 ± 0.11</td>
<td>1.96 ± 0.37</td>
</tr>
<tr>
<td>Liver</td>
<td>1.79 ± 0.32</td>
<td>1.65 ± 0.50</td>
</tr>
<tr>
<td>Lung</td>
<td>1.14 ± 0.22</td>
<td>1.01 ± 0.09</td>
</tr>
<tr>
<td>Skin</td>
<td>1.07 ± 0.39</td>
<td>0.95 ± 0.36</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.; n = 3 (rats) or 4 (mice).

$^b$ Concentration in rats significantly higher than in mice ($P < .05$).
correlation techniques, showed three bond correlations between each of the C-1 protons and C-1'. This established the glucuronide conjugation of metabolite C. The $^{13}$C NMR spectrum contained signals at 178.9 (C-6'); 142.7 (C-3); 113.7 (C-4); 103.6 (C-1'); 77.3 (C-1); 73.2 (C-2); 76.3, 75.6, 73.8, and 72.5 (C-2', 3', 4', and 5'); and 24.1 (C-5) ppm. The $^1$H NMR spectrum of metabolite C (Fig. 2, center) contains signals for the C-5 methyl [1.18 ppm (s)], the C-1 methylene [3.78 ppm (d, $J = 10.4$ Hz) and 3.46 ppm (d, $J = 10.4$ Hz)], and the three coupled olefinic protons on C-3 [5.89 ppm (dd, $J = 11.0$ Hz, 17.5 Hz)] and C-4 [5.22 ppm (dd, $J = 1.5$ Hz, 17.5 Hz) and 5.02 ppm (dd, $J = 1.5$ Hz, 11.0 Hz)]. The protons of the glucuronic acid portion of metabolite C are located in the region of 3.27 to 4.40 ppm. The signal for the proton on the anomeric carbon, C-1', which is obscured by suppression of the water peak, was located at 4.32 ppm using COSY. The proton on C-2', adjacent to the anomeric carbon, is at 3.27 ppm (m). The protons on C-3' and C-4' have overlapping signals at 3.42 ppm. The proton on C-5' is at 3.55 ppm (d, $J = 3.55$ Hz).

GC/MS analyses were also consistent with identification of metabolite C as the proposed glucuronide conjugate. Electron impact GC/MS analysis of metabolite C after formation of the TMS derivatives with BSTFA versus deuterated BSTFA indicated the attachment of five TMS groups. Positive chemical ionization of the TMS-$d_9$ derivative gave a prominent ion at $m/z$ 666 that would be formed by the loss of $CD_3$ ion from the molecular ion of MW 684. In support of this interpretation, negative chemical ionization of this derivative yielded an ion at $m/z$ 601 that could be readily formed by the loss of the TMS-$d_9$ group from the proposed glucuronide conjugate.

Metabolite peak A was the least abundant of the urinary metabolite peaks, accounting for ca. 7% of the metabolized isoprene. Rechromatography of metabolite peak A on a modified HPLC system 5 demonstrated the existence of at least four poorly resolved $^{13}$C components, two major and two minor. Preliminary NMR analysis of the metabolites in peak A also indicated the presence of multiple components. An attempt was made to derivatize the metabolites in peak A with TMSCHN$_2$, however, no shift in retention time was observed. Further attempts to develop a chromatographic method that resolved these metabolites using ion exchange and polar stationary phase HPLC columns were unsuccessful and exhausted the limited supply of the metabolites in this fraction.

Chromatographic comparison of mouse urinary metabolites, their conjugation, and their TMS derivatives with the known rat urinary metabolites confirmed that rat metabolites B, D, and C were also present in mouse urine. The major isoprene metabolite in rat urine, 2-hydroxy-2-methyl-3-butenoic acid, accounted for $\sim$15% of the total urinary radioactivity in mouse urine. 2-Methyl-3-buten-1,2-diol and its glucuronide conjugate accounted for $\sim$3.5 and $\sim$2.5%, respectively, of the total urinary radioactivity in mouse urine. Finally, HPLC analyses of mouse metabolite peak A suggested a direct correlation with the metabolites in rat metabolite peak A.

Discussion

The overall disposition of isoprene in rodents was consistent with that previously observed after inhalation exposure (Dahl et al., 1987; 1990; Peter et al., 1990; Bond et al., 1991). After i.p. administration, most of the dose was excreted as unchanged isoprene in exhaled breath or as urinary metabolites. By 24 h post dose, tissue distribution of isoprene-derived radioactivity was fairly uniform. Adipose tissue contained the lowest concentration of isoprene equivalents, suggesting that remaining radioactivity was associated with water-soluble metabolites. The higher tissue concentrations in rats compared with mice suggests that isoprene was more efficiently cleared by mice. A higher
rate of metabolic clearance has been reported previously for mice compared with rats (Peter et al., 1990; Bond et al., 1991).

Based on the urinary metabolites identified in the present study, a proposed scheme for isoprene metabolism in rats is shown in Fig. 4. In previous work, the monoepoxide was tentatively identified in the blood and tissues of isoprene-exposed rats (Dahl et al., 1987). Other metabolites in blood were collectively identified as the diepoxide/ diols (as detected by vacuum-line cryogenic distillation/fractional trapping techniques). Isoprene is metabolized by rat liver microsomes to products arising from the C-1, C-2 epoxide versus the C-3, C-4 epoxide in a ratio of about 3:1 (Gervasi and Longo, 1990). These authors found that the C-3, C-4 epoxide, but not the C-1, C-2 epoxide, could be further oxidized to the mutagenic diepoxide by rat microsomes. In the present study with rats, isoprene was exhaled or metabolized and excreted primarily as 2-hydroxy-2-methyl-3-butenic acid (metabolite B), 2-methyl-3-buten-1,2-diol (metabolite D), and its glucuronide conjugate (metabolite C). These metabolites, accounting for about 90% of the total isoprene metabolized, are all products of the C-1, C-2 epoxide, suggesting that in vivo isoprene is preferentially oxidized at the methyl-substituted vinyl group. Thus, the lack of significant carcinogenic effects of isoprene in rats may be due to the diminished quantity of the C-3, C-4 epoxide formed in vivo by this species.

The metabolite profile of isoprene in the urine of mice contained the same metabolites as were excreted by rats, although the profile for mice was more complex, suggesting other, multiple metabolic pathways in this species. A larger percentage of total urinary radioactivity was associated with the unidentified, polar fraction (metabolite peak A) in the mouse (25%) versus the rat (7%).

For butadiene, it was reported (Sabourin et al., 1992) that treatment of urine with β-glucuronidase produced no change in the urinary metabolite profile. We also observed that there was little change in the isoprene urinary metabolite profile when the urine was treated with β-glucuronidase. However, the isolated metabolite C was readily cleaved when treated with this enzyme.

The urinary metabolites of butadiene are primarily derivatives of glutathione conjugates of intermediary butadiene metabolites. The detoxification of the initial butadiene metabolite, butadiene monoepoxide, is hypothesized to occur both by action of glutathione-S-transferase to produce glutathione conjugates and by hydrolysis via epoxide hydrolase to 1,2-dihydroxy-3-buten, which subsequently undergoes glutathione conjugation and is excreted as 1,2-dihydroxy-4-(N-acetylcycteiny1)-S-butane (BD-M-1). The proportions of metabolites produced by these two detoxification pathways in different species have been correlated with relative activities of epoxide hydrolase (Sabourin et al., 1992; Bechtold et al., 1994). Man and monkeys metabolize butadiene monoepoxide primarily to BD-M-1. Mice have much lower epoxide hydrolase activity and metabolize butadiene monoepoxide primarily by direct conjugation of butadiene epoxide with glutathione. Rats have intermediate epoxide hydrolase activity and produce similar amounts of metabolites by each of these detoxification routes (Bechtold et al., 1994).

Styrene and α-methylstyrene (AMS) are analogs of butadiene and isoprene, respectively, in which an ethenyl group has been replaced with a phenyl moiety. Like butadiene and isoprene, the major metabolic pathway of styrene and AMS in rodents has as its initial step the epoxidation of a conjugated vinyl group. These epoxides are further metabolized via pathways involving either epoxide hydrolase or glutathione-S-transferase. Approximately 85% of the metabolites of AMS formed following an 11 mg/kg i.v. dose to rats are products of the epoxide hydrolase-mediated pathway whereas the remaining 15% are formed by glutathione conjugation (Mathews and de Costa, 1998). Reports of the metabolism of styrene by rats after a variety of exposure routes and doses (James and White, 1967; Truchon et al., 1990; Summer and Fennell, 1994) indicate that glutathione addition to the initially formed styrene epoxide is involved in the formation of 10 to 40% of the styrene metabolites whereas 60 to 80% are formed via hydrolytic opening of the epoxide. The conditions responsible for the wide variation in the percentage of metabolites formed via glutathione conjugation are not yet understood.

Based on isoprene’s structural similarity to butadiene and the equally diverse rates of hydrolysis of isoprene monoepoxides by mouse, rat, and human microsomes (Bogaards et al., 1996), glutathione conjugation was anticipated to be important in the metabolism of isoprene in rats. However, this does not appear to be true under the conditions of our study. The identified urinary metabolites of isoprene, accounting for about 90% of the total isoprene metabolized, are all products of the hydrolytic opening of the C-1, C-2 epoxide. In this regard, the metabolism of isoprene most closely resembles that of AMS rather than butadiene or styrene. In both isoprene and AMS, the initial epoxide has a quaternary carbon, possibly sterically hindering the glutathione-S-transferase-mediated conjugation with glutathione. The C-1, C-2 epoxide of isoprene has been shown (Gervasi and Longo, 1990) to be much more reactive toward hydrolysis (half-life of 1.25 h at 37°C) than is butadiene epoxide (half-life of 13.7 h at 37°C). Thus hydrolysis of isoprene C-1, C-2 epoxide, proceeding through an S$_2$1 process, may play a significant role in the disposition of isoprene relative to butadiene (Bleasdale et al., 1996). In another difference with butadiene, no metabolite of isoprene was identified that was analogous to the major butadiene metabolite, BD-M-1. BD-M-1 is postulated to be formed via a pathway that includes 1-hydroxy-3-buten-2-one as an intermediate (Sabourin et al., 1992; Richardson et al., 1998). The analogous intermediate in the metabolism of isoprene is not possible for the pathway involving the C-1, C-2 epoxide (the methyl group at C-2 precludes formation of the ketone) but would be possible via the C-3, C-4 epoxide.

The presence of additional, unidentified metabolites in the urine of mice signifies a more complex series of metabolic pathways in this species. Likely processes that could give rise to the added complexity are epoxidation of isoprene at C-3, C-4 and/or a larger role of glutathione conjugation. The latter process normally results in detoxification; the possible involvement of the C-3, C-4 epoxide may be related to the increased sensitivity of mice to isoprene toxicities. The relationship between pathways of metabolism and species sensitivity observed with butadiene toxicity suggests the need for further characterization of the mouse versus rat urinary metabolites of isoprene.
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References


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