Metabolism and Distribution of [2,3-\textsuperscript{14}C]Acrolein in Sprague-Dawley Rats

II. Identification of Urinary and Fecal Metabolites\textsuperscript{1}

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Received January 20, 1998; accepted March 30, 1998


The metabolites of [2,3-\textsuperscript{14}C]acrolein in the urine and feces of Sprague-Dawley rats were identified after either intravenous administration in saline at 2.5 mg/kg or oral administration by gavage as an aqueous solution as either single or multiple doses at 2.5 mg/kg or as a single dose of 15 mg/kg. Selected urine and feces samples were pooled by sex and collection interval and profiled by combinations of reverse-phase, anion-exchange, cation-exchange, and ion-exclusion high-performance liquid chromatography (HPLC). Feces were also profiled by size-exclusion chromatography. Metabolites were identified by comparison with well-characterized standards by HPLC and by mass spectrometry. The urinary metabolites were identified as oxalic acid, malonic acid, N-acetyl-S-2-carboxy-2-hydroxyethylcysteine, N-acetyl-S-3-hydroxypropylcysteine, N-acetyl-S-2-carboxyethylcysteine, and 3-hydroxypropionic acid. The fecal radioactivity from the oral dose samples was partitioned into methanol-soluble, water-soluble, and insoluble radioactivity, some of which could be liberated by dilute acid hydrolysis. HPLC analysis of these extracts revealed no discrete metabolites. Size-exclusion chromatography indicated a molecular weight range of 2,000 to 20,000 Da for the radioactivity, which was unaffected by hydrolysis at reflux with 6 M acid or base. This radioactivity was thought to be a homopolymer of acrolein, which was apparently formed in the gastrointestinal tract. The pathways of acrolein metabolism were epoxidation followed by conjugation with glutathione, Michael addition of water followed by oxidative degradation, and glutathione addition to the double bond either following or preceding oxidation or reduction of the aldehyde. The glutathione adducts were further metabolized to the mercapturic acids.

Apart from its use as a chemical intermediate, acrolein (propanal) has been utilized as an aquatic herbicide for more than 40 years (Bowmer and Sainty, 1977; Bowmer et al., 1974; Unrau et al., 1965; van Overbeek et al., 1959). Because it is cytotoxic to plant tissue and decomposes rapidly in water (Bowmer and Higgins, 1976; Bowmer et al., 1974; Kissel et al., 1978), it is an effective herbicide which leaves virtually no residue which would be harmful to the environment (U.S. EPA, 1980; Nordone et al., 1996a,b).

Acrolein is commonly present at low concentrations in cooking oil, wood smoke, tobacco smoke, alcoholic beverages including beer (Greenhoff and Wheeler, 1981) and wine (Avent, 1961), fish and shellfish (U.S. EPA, 1978), salt pork (Cantoni et al., 1969), and raw chicken muscle (Grey and Shrimpton, 1967).

We have undertaken a study of the toxicology of acrolein including studies of reproductive toxicity (Parent et al., 1992a), teratology (Parent et al., 1993), mutagenicity (Parent et al., 1991a, 1996a), chronic toxicity and oncogenicity in the rat (Parent et al., 1992c) and mouse (Parent et al., 1991b), and chronic toxicity in the dog (Parent et al., 1992b). Furthermore, we have previously reported on the metabolism, excretion, and distribution of \textsuperscript{14}C-labeled acrolein in the rat (Parent et al., 1996b), goat (Sharp et al., 1996), and hen (Berge et al., 1996). Acrolein is thought to be a cytotoxic agent resulting from treatment with cyclophosphamide, an antitumor agent used extensively in the United States (Blomgren and Hallstrom, 1991; Fraiser et al., 1991; Cox, 1979). Hemorrhagic cystitis of the bladder limits the human dose of cyclophosphamide and this toxic effect is thought to be due to the cytotoxicity of acrolein liberated from cyclophosphamide metabolites in the bladder (Brock et al., 1979; Sakata et al., 1989; Cox, 1979). Acrolein can also be generated in the liver by oxidation of cyclophosphamide by cytochrome P450 (Wildenauer and Oehlmann, 1982). Acrolein formed by oxidative metabolism is also thought to be responsible for liver toxicity in animals dosed with allyl alcohol (Jaeschke et al., 1987; Sarafini-Cessi, 1972; Silva and O'Brien, 1989).

\textsuperscript{1} Part of this work was presented at the 6th North American ISSX Meeting, Raleigh, NC, October 23-27, 1994.

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Little is known about the metabolism of acrolein. Acrolein was shown to be metabolized to glyceraldehyde, glyceraldehyde, and acrylic acid in rat liver microsomes, although the metabolites were identified only by cochromatography (Patel et al., 1980). No other Phase I metabolites of acrolein have been reported. In this paper we hypothesize the existence of unidentified Phase I metabolites of acrolein in an in vivo system, the rat.

While the acrolein–gluthathione adduct, 3-oxopropyl-S-glutathione, has also not been isolated in vivo (Ramu et al., 1995), mercapturic acids resulting from hepatic metabolism of glutathione adducts have been reported for acrolein and for several compounds thought to be in vivo precursors of acrolein. 3-Hydroxypropyl mercapturic acid [S-(3-hydroxypropyl)-N-acetyl-cysteine] has been reported to be present in the urine of rats treated with acrolein (Kaye, 1973; Sanduja et al., 1989; Linhart et al., 1996). It has also been reported in both human (Honjo et al., 1988; Al-Rawithi et al., 1993; Ramu et al., 1995; Kaye and Young, 1974) and rat urine (Giles, 1979; Ramu et al., 1995; Kaye and Young, 1974; Hashmi et al., 1992; Alarcon, 1976; Giles, 1979; Sanduja et al., 1989) after treatment with cyclophosphamide. Other acrolein precursors which have been reported to produce this urinary metabolite include allyl bromide, chloride, amine and cyanide (Sanduja et al., 1989), and allyl alcohol (Giles, 1979; Sanduja et al., 1989).

Another mercapturic adduct that has been identified in the urine of acrolein-exposed rats is the carboxyethyl mercapturic acid [S-(3-carboxyethyl)-N-acetyl-cysteine]. Draminski et al. (1983) identified this metabolite by comparison of the gas chromatographic retention time and mass spectrum of the methyl ester to that of a synthetic standard. He did not find the hydroxypropyl mercapturic acid after dosing rats orally with acrolein. This is because the alcohol moiety would not be methylated by diazomethane (March, 1985); thus the hydroxypropyl metabolite would not be volatile for GC/MS analysis. Only limited characterizations were conducted on standard materials used for HPLC and MS comparisons. Linhart et al. (1996) has recently described this metabolite in the urine of rats following both inhalation and intraperitoneal administration of acrolein.

The carboxyethyl mercapturic acid metabolite or esters thereof have also been identified in the urine of rats treated with methyl acrylate (Delbressine et al., 1981), acrylic acid (deBethizy et al., 1987; Winter et al., 1992), acrylonitrile (Fennel et al., 1991), acrylamide (Fennel et al., 1991), and ethyl acrylate (Ghanayem et al., 1987). This metabolite has not been found in the urine of rats treated with cyclophosphamide (Giles, 1979). Crotonaldehyde, a homolog of acrolein, has been reported to be metabolized to the corresponding homologous hydroxypropyl and carboxyethyl mercapturates (Gray and Barnsley, 1971).

This work tests the hypothesis that there are several metabolic pathways of acrolein that have not been previously elucidated. In this paper, we report the identification of the urinary and fecal metabolites of acrolein from the excreta samples generated in the rat metabolism study cited above (Parent et al., 1996b). We have synthesized and fully characterized potential metabolites by infrared and nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and elemental analysis. Four previously unknown urinary metabolites have been identified and the first study of fecal metabolites was undertaken. These results offer additional insights into the pathways of metabolism of acrolein.

**MATERIALS AND METHODS**

**Test Materials**

The nonradiolabeled acrolein (stabilized with 0.25% hydroquinone)* used was 96.1% acrolein by analysis (the remainder was mostly water). Radiolabeled acrolein (14C in the 2,3-positions) was supplied as a solution in dimethyl formamide with a specific activity of 8.9 mCi/mmol and a stated radiochemical purity of 93%. Upon receipt, radiolabeled acrolein was analyzed by HPLC and was found to have a radiochemical purity of 88%.

The dosing solutions were prepared by dissolving the acrolein in either normal saline (for the intravenous doses) or deionized water (for the oral doses). The analysis of dosing solutions was reported previously (Parent et al., 1996b).

**Metabolite Standards**

3-Hydroxypropionic acid,*6 malonic acid,*7 glyceraldehyde,*7 glycic acid,*7 allyl alcohol,*6 acrylic acid,*7 1,3-propanediol,*7 glycidol,* dimethyl malonate,*7 oxalic acid (dipotassium salt monohydrate),*7 lactic acid,*7 acrylic acid,*7 allyl alcohol, and glycerol*7 were obtained from commercial sources for comparison with the urinary and fecal metabolites. Their identity was confirmed by mass spectrometry. The following standards were synthesized.

S-3-Hydroxypropylcysteine was prepared by the procedure of Banks with minor modifications (Barnsley, 1966), S-3-Hydroxypropylcysteine: mp 207°C; \( ^{1} \)H NMR (D\(_2\)O): 3.15 (pentet, 2H, HOCH\(_2\)CH\(_2\)S), 2.68 (t, 2H, SCH\(_2\)CH\(_3\)), 3.10 (m, 2H, SCH\(_2\)CH\(_3\)), 3.69 (t, 2H, HOCH\(_2\)), 3.94 (m, 1H, SCH\(_2\)CH\(_3\)), IR (KBr): 3050 (m), 2935 (m), 1680 (m), 1595 (mv), 1586 (ms) 1480 (ms), 1342 (ms) cm\(^{-1}\). Found: C, 40.89; H, 5.69; N, 5.77.

N-acetyl-S-2-carboxyethylcysteine and N-acetyl-S-3-hydroxypropylcysteine were prepared by the method of Onkenhout et al. with minor modifications (Onkenhout et al., 1986). N-acetyl-S-2-carboxyethylcysteine: Thick syrup; The \(^{1} \)H NMR has been previously reported (Ramu et al., 1995). IR (neat): 3335 (m), 3085 (m), 2930 (m), 1719 (s), 1624 (ms), 1541 (m), 1419 (m), 1225 (m) cm\(^{-1}\); CI-MS (CH\(_4\))(M + 1) 369; Elemental analysis: Calculated for C\(_{8}\)H\(_{12}\)NO\(_{3}\): C, 40.21; H, 7.31; N, 7.81. Found: C, 39.98; H, 7.20; N, 7.75.

N-acetyl-S-3-hydroxypropylcysteine: Thick clear syrup; The \(^{1} \)H NMR has been previously reported (Ramu et al., 1995) IR (neat): 3330 (m), 2930 (m), 1730 (ms), 1563 (ms), 1554 (m) cm\(^{-1}\); CI-MS (CH\(_4\))(M + 1) 222; Elemental analysis: Calculated for C\(_{8}\)H\(_{12}\)NO\(_{3}\): C, 43.42; H, 6.83; N, 6.33. Found: C, 43.40; H, 6.76; N, 6.40.

S-2-Carboxyethylcysteine was prepared according to the following procedure: To L-cysteine (6.0 g, 50 mmol) in water (35 mL) was added acrylic acid (3.6 g, 50 mmol), followed by 5% NaOH (8.0 g, 100 mmol). The mixture was stirred under nitrogen for 24 h. Concentrated HCl was added dropwise until the pH was 4.4. White crystals formed. The mixture was filtered and the solid washed with water (5 mL) and ethanol to yield 5.5 g (56%) of S-2-carboxyethylcysteine: mp 217°C; \(^{1} \)H NMR (D\(_2\)O): 3.25-3.60 (m, 4H, \(-\text{COCH\(_{2}\text{CH}_{2}\text{S}\)}\)), 3.10 (m, 2H, \(-\text{SCH\(_{2}\text{CH}_{3}\)}\)), 3.93 (m, 1H, \(-\text{SCH\(_{2}\text{CH}_{3}\)}\)); IR (KBr): 3050 (m), 2935 (m), 1680 (m), 1595 (m), 1560 (ms) 1540 (ms) cm\(^{-1}\).

* Baker Performance Chemicals, Houston, TX.
* Sigma Chemical Co., St. Louis, MO.
* American Tokyo Kasei, Portland, OR.
* Aldrich Chemical Co., Milwaukee, WI.
Study Design

Four groups of 10 rats (5 male, 5 female) were dosed with radiolabeled acrolein either intravenously or orally by gavage at 2.5 mg/kg, either as a single dose or after 14 daily oral doses of unlabeled acrolein or as a single oral dose by gavage at 15 mg/kg. All doses were administered at a volume of 2 mL/kg. Oral doses were administered using a stainless-steel ball-tipped needle and a disposable syringe. Intravenous doses were injected into the lateral tail vein.

After being dosed with radiolabeled acrolein, animals were placed in glass Roth-type metabolism cages for the collection of expired air, urine, and feces. Three 4-h collections were made followed by a 12-h collection period and six 24-h collection periods for a total of 168 h. Urine and feces were collected over ice and stored frozen at -20°C. Expired air was passed through activated charcoal in order to trap any organics and then into a solution of ethanolamine and ethoxyethanol (1:1) to trap expired carbon dioxide.

Radioanalysis

Fecal samples were homogenized in two to three times the sample weight of water, and aliquots of the homogenate were combusted in Packard Model 306 or 307 oxidizers10 before analysis by liquid scintillation counting (LSC). Liquid samples were analyzed directly by LSC.

Samples were analyzed for 5 min or 100,000 counts on Packard Model 4640 liquid scintillation counters.10 Scintillation counting data (cpm) were automatically converted to dpm using the external standardization technique and an instrument-stored quench curve generated from a series of sealed quenched standards.

High-Performance Liquid Chromatography (HPLC)

HPLC was performed with the solvent systems indicated below. All solvents were HPLC grade. Detection was either by ultraviolet (UV) absorbance at 210 nm or by refractive index (RI) detection as many of the potential metabolites of acrolein had little or no UV absorbance at 210 nm. Radioactivity was detected either by fraction collection followed by static LSC or by on-line detection using Radiomatic radioHPLC detectors and FloScint II liquid scintillant.11 Better reproducibility of the HPLC profiles was obtained if the pH of the samples was adjusted to 3.0 before injection, so this was generally done.

1. Reverse phase.

Column: Spherex C1812, 250 × 4.6 mm, 5 μm particle size.

Temperature: ambient.

Mobile phase: Solvent A: 95% 5 mM KH₂PO₄ adjusted to pH 3 with phosphoric acid, 5% acetonitrile (ACN). Solvent B: Acetonitrile.

Gradient: isocratic at 100% A for 20 min, followed by 5 min at 100% B over 15 min, followed by 5 min at 100% B. The column was allowed to equilibrate with solvent A for at least 15 minutes before each injection.

Flow rate: 0.8 mL/min.

Scintillation cocktail flow rate: 3.2 or 4.0 mL/min.

This system was most useful for the relatively nonpolar metabolites such as the mercapturic acids.

II. Anion exchange.

Column: Supelcogel C610H-SP anion exchange,13 300 × 7.8 mm.

Temperature: 60°C.

Mobile phase: isocratic with 0.1% phosphoric acid.

Flow rate: 0.5 mL/min.

Scintillation cocktail flow rate: 2.0 mL/min.

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8 Charles River Laboratories, Portage, MI.
9 Certified Rodent Chow No. 5002, Purina Mills Inc., St. Louis, MO.
10 Packard Instrument Co., Downers Grove, IL.
11 Radiomatic Instrument Co., Tampa, FL.
12 Phenomenex, Sunnyvale, CA.
13 Supelco, State College, PA.
This system gave the best overall separation of the urinary metabolites.

### III. Ion exclusion.

- **Column:** ORH-801 organic acids column, 300 × 6.5 mm.
- **Temperature:** 35°C.
- **Mobile phase:** isocratic 1 mM H$_2$SO$_4$.
- **Flow rate:** 0.8 mL/min.
- **Scintillation cocktail flow rate:** 3.2 mL/min.

### IV. Cation exchange.

- **Column:** Bakerbond aliphatic sulfonic acid column, 250 × 4.6 mm, 5 μm particle size.
- **Temperature:** ambient.
- **Mobile phase:** isocratic 0.05 M H$_2$SO$_4$.
- **Flow rate:** 1.0 mL/min.
- **Scintillation cocktail flow rate:** 3.0 mL/min.

This column was the only one that retained the standard cysteine conjugates and was used to verify their absence.

**Size-Exclusion Chromatography**

Size exclusion chromatography was performed using either Sephadex G-10, G-25, or G-200 gels. The gels were washed with methanol and then soaked in deionized water to allow the gels to swell. The columns were slurry packed in 300 dpmbi and 2,2,3,3,-d$_4$-3-(trimethylsilyl)-propionic acid sodium salt as an internal standard.

**Mass Spectrometry**

Mass spectra were obtained on a Quattro triple quadrupole mass spectrometer. Positive or negative electrospray liquid chromatography mass spectrometry (LC/MS) used either direct flow injection or partial separation on a C18 HPLC column. Gas chromatography–mass spectrometry was performed in electron impact mode at 70 eV using a 25 m DB-1 column with He as the carrier gas, a head pressure of 10 psi, and an initial column temperature of 80°C. The mass spectra used for characterizing the synthetic metabolites was carried out by Oneida Research Services.

**NMR and Infrared Spectroscopy**

Proton NMR spectra were acquired with a Varian Gemini 200 using tetramethyl silane or 2,2,3,3,-d$_4$-3-(trimethylsilyl)-propionic acid sodium salt as an internal standard. Infrared spectra were acquired with a Shimadzu IR 460.

**Elemental Analyses**

Elemental analyses were performed by Oneida Research Services.

**Pooling of Urine and Feces and Preparation for Analysis**

The urines and feces containing the majority of the radioactivity were pooled by sex and time point for metabolite profiling and identification.
The pooled urine samples and the various extracts of pooled fecal samples were filtered through a 0.45-μm filter, analyzed by LSC, and injected directly into the appropriate HPLC system.

Isolation of Urinary Metabolites

A 500 mg, 12 mL Bonded Phase C18 Bond Elut cartridge was attached to a Vac-elut device and was rinsed with one column volume of methanol, followed by one column volume of deionized water adjusted to pH 3 with phosphoric acid. Pooled urine from the repeated dose male rats was adjusted to pH 3 with phosphoric acid and was applied to the column, followed by two 0.5-mL washes of the urine vial. The wash, which contained any unretained radioactivity, was collected, and the column was then eluted with one column volume of pH 3 water and one column volume of methanol. The aqueous fraction was reextracted with dichloromethane. The organic layer was concentrated and was submitted to LC/MS without further purification. The aqueous and methanol fractions from the two Bond Elut cartridges were combined. The aqueous fractions were lyophilized and reconstituted in deionized water and were isolated by HPLC using System II. The individual metabolites were isolated by HPLC using System II with fraction collection. The methanol fraction was evaporated to dryness and reconstituted in deionized water and was further purified. Metabolite 2 was methylated with methanol/sulfuric acid at reflux for 2 h. The reaction was neutralized with NaHCO3 and was applied to the column, followed by two 0.5-mL washes of the urine vial. The wash, which contained any unretained radioactivity, was collected, and the column was adjusted to pH 3 with phosphoric acid and was rinsed with one column volume of methanol, followed by one column volume of deionized water adjusted to pH 3 with phosphoric acid. The column was washed with two 0.5-mL washes of the urine vial. The wash, which contained any unretained radioactivity, was collected, and the column was washed with one column volume of deionized water adjusted to pH 3 with phosphoric acid. The column was then eluted with one column volume of pH 3 water and one column volume of methanol. The aqueous fraction was reextracted with dichloromethane. The organic layer was concentrated and was submitted to LC/MS without further purification. Metabolite 2 was methylated with methanol/sulfuric acid at reflux for 2 h. The reaction was neutralized with NaHCO3 and extracted with dichloromethane. The organic layer was concentrated and was submitted to LC/MS without further purification. Metabolite 2 was methylated with methanol/sulfuric acid at reflux for 2 h. The reaction was neutralized with NaHCO3 and extracted with dichloromethane. The organic layer was concentrated and was submitted to LC/MS without further purification. Metabolite 2 was methylated with methanol/sulfuric acid at reflux for 2 h. The reaction was neutralized with NaHCO3 and extracted with dichloromethane. The organic layer was concentrated and was submitted to LC/MS without further purification.

The pooled urine samples and the various extracts of pooled fecal samples were filtered through a 0.45-μm filter, analyzed by LSC, and injected directly into the appropriate HPLC system.

### TABLE 1

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<th>Hours</th>
<th>2.5 mg/kg intravenous</th>
<th>2.5 mg/kg single oral</th>
<th>2.5 mg/kg multiple oral</th>
<th>15 mg/kg oral</th>
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<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
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<td>NA</td>
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<td>Metabolite 5</td>
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<sup>a</sup> Oxalic acid.
<sup>b</sup> Malonic acid.
<sup>c</sup> N-Acetyl-S-2-carboxy-2-hydroxyethylcysteine.
<sup>d</sup> N-Acetyl-S-2-carboxyethylcysteine.
<sup>e</sup> N-Acetyl-S-3-hydroxypropylcysteine.

Note. NA, not analyzed; ND, not detected.
Metabolites of [2,3-14C]acrolein in urine and feces of rats

Results

Urine

Typical radiochromatograms of selected urine samples from orally and intravenously dosed male rats in the HPLC solvent systems used in the study are found in Figs. 1 and 2. The quantitative distribution of the metabolites in the urine samples profiled is found in Table 1.

There were only minor differences between the profiles from urine from male and female rats, as well as among the oral dose groups, whereas there were considerable differences between the profiles of the oral and intravenous dose groups, the most striking being the absence of Metabolites 1 and 2 from the intravenous dose groups.

Metabolite 1 was tentatively identified as oxalic acid by cochromatography with authentic material in the three HPLC systems used in the study. The presence of oxalic acid only in the orally dosed groups suggests that it is formed in the gastrointestinal tract, presumably by the gut flora. Oxalate has been identified as a metabolite of acrolein in fish and shellfish (Smith, 1993) and goat muscle (Sharp et al., 1996).
FIG. 6. Negative electrospray mass spectrum of Metabolite 4 isolated from urine.

Metabolite 2 was identified as malonic acid by cochromatography with authentic material in the three HPLC systems, although it was only resolved from coeluting metabolites in System II. The isolated metabolite was methylated and subjected to electron impact GC/MS (Fig. 3). While there were other volatile compounds seen in the spectrum, both the molecular ion \((m/z 132)\) and the base peak \((m/z 101)\) of dimethyl malonate were observed. Because this metabolite was present only in trace amounts, we were unable to obtain a purer sample.

Metabolite 3 eluted at the column void volume in System I and coeluted with malonate in System III. It had an \((m + 1)^+\) of 252 in positive electrospray mode indicating a metabolite of 16 mass units greater than Metabolite 4 (Fig. 4). The MS/MS showed fragments at \(m/z 130\) and 88 (Fig. 5). The presence of the fragment of \(m/z 88\) indicates that the oxygen is attached to the propionate moiety as opposed to the possibility of a sulfoxide. Based on the fragmentation pattern, Metabolite 3 was identified as N-acetyl-2-carboxy-2-hydroxyethylcysteine. The analogous amide to this metabolite had previously been reported as a metabolite of acrolein (Cheng, 1994).

Metabolite 4 was identified as N-acetyl-2-carboxyethylcysteine by coelution with a synthetic standard in the three HPLC systems used in the study as well as comparison of the negative ion electrospray mass spectra to that of the synthetic material (Fig. 6). Both the \((M-1)\) and daughter ion spectra of the isolated metabolite were identical with those of the synthetic material (Fig. 7).

Metabolite 5 was identified as N-acetyl-3-hydroxypropylcysteine by coelution with a synthetic standard in the three HPLC systems used in the study as well as comparison of the negative ion electrospray mass spectrum with that of the synthetic standard (Fig. 8).

Metabolite 6 was identified as 3-hydroxypropionic acid by coelution with authentic material in the three HPLC solvent systems used in the study, although it eluted at the void volume in System I and very near Metabolites 2 and 3 in System III. The isolated Metabolite 6 had the same negative ion electrospray LC mass spectrum as that of authentic material (Fig. 9). 3-Hydroxypropionic acid has not previously been identified as a metabolite of acrolein, but has been reported as a metabolite of acrylic acid (DeBethnizy et al., 1987).

Feces

Fecal homogenate was partitioned into methanol-soluble, water-soluble, and acid hydrolyzed fractions as outlined under Materials and Methods. All three fractions showed similar HPLC profiles: essentially complete elution at the void volume.
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with high column recoveries in System I and unacceptably low recoveries from Systems II and III. There was no indication of the presence of any of the urinary metabolites in the feces. Size-exclusion chromatography indicated a molecular weight range of 2,000 to 20,000 Da. The fecal radioactivity was refractory to several enzyme treatments. The radioactivity was then subjected to 6 M acid or base hydrolysis, which failed to change the molecular weight range.

DISCUSSION

The proposed pathways of metabolism of acrolein are illustrated in Fig. 10. The main pathway appears to be Michael addition of glutathione to the activated double bond, followed by processing to the mercapturic acid, which is excreted in the urine after either oxidation or reduction of the aldehyde, with reduction predominating. Both of these compounds have previously been reported as metabolites of acrolein (Kaye, 1973; Sanduja et al., 1989; Linhart et al., 1996; Draminski et al., 1983). Although we had synthetic standards of the corresponding cysteine and glutathione adducts, we were unable to detect them in either urine or feces. The mercapturic acid adducts have been found as metabolites in similar compounds, such as acrylamide, cinnamaldehyde, acryl acid, allyl alcohol, allyl amine, and allyl acetate.

Another pathway of acrolein metabolism is that of epoxidation of the double bond followed by attack of glutathione on the epoxide. Glycidaldehyde was previously reported to be a substrate for glutathione S-transferase, but the product was not identified (Patel, 1980). Although in theory, products of attack of glutathione at either end of the epoxide are possible, only one metabolite was found. In addition, the aldehyde could be either oxidized or reduced after epoxidation, yet only oxidation products are found. The structure reported is the product of opening of the epoxide by attack of glutathione on the least sterically hindered terminus; additively, attack from this end would produce a stabilized carbanion intermediate.

None of the unconjugated metabolites resulting from the epoxidation of acrolein, such as those reported by Patel et al. (1980), were found in the excreta. Presumably these compounds are incorporated into normal metabolic pathways resulting in either incorporation into tissues or transformation into CO\textsubscript{2}.

A third pathway involves Michael addition of water to acrolein to form 3-hydroxypropionaldehyde, which is further oxidized to malonic and ultimately oxalic acid. The addition of water to acrolein is a well-studied nonenzymatic process, which can occur in the GI tract. The 3-hydroxypropionaldehyde is further oxidized to 3-hydroxypropionic, malonic, and oxalic acids. The latter two transformations are most likely accomplished by the gut flora, as these metabolites are not present in the urine of intravenously dosed rats. On the other hand, 3-hydroxypropionic acid is found in the urine of both intravenously and orally dosed rats, indicating that either the...
nonenzymatic Michael addition is also occurring inside the body or that there is an enzymatic component to the addition reaction.

There are none of the usual acrolein metabolites present in significant quantities in feces. HPLC of both water and methanol extracts on several columns failed to detect cysteine or mercapturic acid adducts or other expected small molecule metabolites. The separations did show two fractions, one polar and one nonpolar. Gel permeation chromatography showed a molecular weight range of 2,000–20,000 Da. Our initial thought was that these fractions were polysaccharide or protein adducts resulting from the reaction of acrolein with food components in the gut. However, the high molecular weight radioactivity was refractory to both enzymatic and acid or base hydrolysis. It was then concluded that this radioactivity had no biological components and in fact represented homopolymers of acrolein. The initial 1 M acid hydrolysis appeared to liberate radioactivity from the feces residue; but this hydrolysate also shared the same molecular weight range as the methanol or water extracts. It is suspected that the dilute acid hydrolysis was destroying the matrix, causing better extractability. Another possibility is that the polyacrolein in this fraction was originally a copolymer with a natural polymer, either a protein or a polysaccharide, which was hydrolyzed, liberating the acrolein. The polymerization or acrolein is known to occur under acid, base, or radiation catalysis (Schulz, 1985). Commercial acrolein (as was the test material in this study) is stabilized against radiation-induced polymerization with hydroquinone, but the hydroquinone might not be adequate to prevent the polymerization of radiolabeled acrolein. Alternatively, cationic polymerization could be occurring in the acidic environment of the stomach. Some of the polymers of acrolein are thought to have carbon–carbon bonds between the monomer units, which would make the polymer resistant to acid or

base hydrolysis. The amounts of radioactivity excreted in the feces of rats in the 2 mg/kg single and multiple oral dose groups (11.9 to 14.8%) of the administered dose (Parent et al., 1996b) are comparable to the amount of possible polymerized radioactive acrolein found in the [14C]acrolein, assuming that the 12% impurities in the radiolabeled acrolein is entirely polymeric. However, the feces from the 15 mg/kg dose group contained 28.4 to 30.6% of the dose (Parent et al., 1996b), which implies that polymerization has at least partly taken place in the GI tract. The higher concentrations of acrolein in the 15 mg/kg dose group should favor increased polymerization. It is assumed that inert, high molecular weight polymeric compounds such as these are not bioavailable and have no toxicologic importance.

Metabolites not seen by us include the S-3-oxypropylglutathione and S-3-oxypropylglutathione-S-oxide; the former represents the initial intermediate in the metabolism of acrolein to mercapturic acids. We have not seen the latter in our work and we did identify all of the metabolites present in any significant amounts (see Figs. 1 and 2). The presence or absence of the S-oxide metabolite is particularly significant since it is thought to be the immediate precursor to acrolein released at specific organ sites after treatment with cyclophosphamide. These sites include the bladder (Cox, 1979; Ramu et al., 1995; Brock et al., 1979; Sakata et al., 1989) and the kidney (Horvath et al., 1992; Hashmi et al., 1992). While it is quite clear that Horvath et al. (1992) produced significant renal toxicity by injecting rats intravenously with S-3-oxypropylglutathione, dosing levels based on acrolein ranged from 5.7 to 56.9 mg/kg. These dosing levels would appear to be quite high considering a reported LD50 (ip) in rats of 4 mg/kg acrolein (Murphy et al., 1983) and an oral LD50 which we have determined in the rat (unpublished).

We have not observed any acrolein related bladder or kidney toxicity in any of the long term oral dosing studies which we have completed (Parent et al., 1991b, 1992a,b,c) nor are there any effects reported by the NTP in a recently completed 90-day oral gavage study in rats and mice (unpublished). While the cytotoxicity of S-3-oxypropylglutathione-S-oxide has been demonstrated using in vitro systems (Hashmi et al., 1992; Ramu et al., 1996) and liberation of acrolein from this compound has been demonstrated chemically (Ramu et al., 1995), we have no evidence that S-3-oxypropylglutathione-S-oxide is a metabolite of acrolein.

ACKNOWLEDGMENTS

The authors acknowledge the Radioanalysis and Metabolism In-Life groups of Covance Laboratories—Madison for technical assistance and Michael Siener for his valuable aid with structuring the manuscript.

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