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THE METABOLISM AND EXCRETION OF MONONITROTOLUENES BY FISCHER 344 RATS

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ABSTRACT:

The metabolism and excretion of 2-, 3-, and 4-nitrotoluene (2NT, 3NT, and 4NT, respectively) were studied in male Fischer 344 rats. Excreta were collected for 72 hr after an oral dose (200 mg/kg) of radiolabeled 2NT, 3NT, or 4NT. Radiolabel from each NT isomer was rapidly excreted (86, 73 and 74% of the dose for 2NT, 3NT, and 4NT, respectively in 24 hr). The urine was the major route of excretion with 70–85% of the dose being excreted by that route in 72 hr. Five to 13% and 0.0 to 0.1% of the dose was excreted in the feces and expired air, respectively, in 72 hr. The major metabolites excreted in urine in 72 hr after administration of 2NT were 2-nitrobenzoic acid (29% of the dose), an unidentified metabolite (16% of the dose), 2-nitrobenzyl glucuronide (14% of the dose), and S-(2-nitrobenzyl)-N-acetylcysteine (12% of the dose). The major metabolites excreted in urine in 72 hr after administration of 3NT were 3-nitrohippuric acid (24% of the dose), 3-nitrobenzoic acid (21% of the dose), and 3-acetamidobenzoic acid (12% of the dose). The major metabolites excreted in urine in 72 hr after administration of 4NT were 4-nitrobenzoic acid (28% of the dose), 4-acetamidobenzoic acid (27% of the dose), and 4-nitrohippuric acid (13% of the dose). Thus, the major urinary metabolites of 3NT and 4NT differed only quantitatively while those of 2NT were also qualitatively different.

The mononitrotoluenes are intermediates in the production of sulfur and azo dyes, as well as rubber and agricultural chemicals (1). The estimated amount of NT[1] produced in the United States is 44 million pounds per annum, of which 28 million pounds are 2NT, 15 million pounds are 4NT, and 1 million pounds are 3NT.

Several investigators have shown that each of the three isomers of NT produces different types of toxicity. When male and female Wistar rats were given 2NT (200 mg/kg/day), 3NT (300 mg/kg/day), or 4NT (400 mg/kg/day) 5 days/week for 6 months, a number of organ-, isomer-, and sex-specific toxicities were observed (2). All three isomers produced splenic lesions in male rats, but only 3NT produced lesions of the spleen in female rats. Testicular atrophy was seen after either 3NT or 4NT, but not 2NT. Lesions in kidney tubules were seen only after treatment with 2NT, and the frequency of lesions was greater in females. 2NT gave a positive response in the *in vivo-in vitro* hepatocyte unscheduled DNA synthesis assay in Fischer 344 rats at a dose of 200 mg/kg. No positive response was given by 3NT or 4NT at doses up to 500 mg/kg (3). 2NT-derived material binds extensively to Fischer 344 rat hepatic DNA, but binding of 3NT or 4NT is less than 10% that of 2NT.[2] Data such as these have led to the prediction that 2NT will be shown to be a hepatocarcinogen in Fischer 344 rats (3).

The differences in toxicity observed among the NT isomers suggest that there are substantial differences in metabolism, distribution, or elimination. Therefore, we have studied the *in vivo* metabolism and elimination of the NT isomers at a dose that induced hepatic unscheduled DNA synthesis after 2NT, but not after 3NT or 4NT. The data indicate that there are substantial differences among the NT isomers with respect to the metabolites excreted in the urine of male Fischer 344 rats.

[1] The abbreviation used is: NT, nitrotoluene.

[2] Kedderis, Dyroff, and Rickert, unpublished data.

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Materials and Methods

Animals. Male Fischer 344 rats (CDF(F-344)/CrlBR) (80–90 days old; 200 g) were purchased from the Kingston, NY colony of Charles River Breeding Laboratories. All animals were allowed free access to food (pelleted NIH-07 diet, Ziegler Brothers, Gardners, PA) and water. The rats were allowed to acclimate to the animal holding quarters in suspended wire-bottom cages for 2 weeks prior to initiation of the experiments. A constant 12-h light:dark cycle was maintained. Temperature and humidity were 70 ± 5°F and 50 ± 10%, respectively. Sentinel animals were routinely screened for viral and mycoplasm infection by serological techniques and determined to be free of infection (Microbiological Associates, Bethesda, MD).

Chemicals. The following compounds were obtained from Aldrich Chemical Company (Milwaukee, WI): 2- and 3-aminobenzyl alcohol, 2-, 3-, and 4-nitrobenzyl alcohol, 2-, 3-, and 4-nitrobenzoic acid, 3-hydroxy-4-nitrotoluene, and 2-, 3-, and 4-nitrotoluene. Compounds purchased from Pfaltz and Bauer Inc. (Stamford, CT) were 2-, 3-, and 4-acetamidobenzoic acid, and 2-aminobenzoic acid. 3- and 4-aminobenzoic acid were purchased from Fluka Chemical Corp. (Hauppauge, NY) and Fisher Scientific Company (Fair Lawn, NJ), respectively. β-Glucuronidase (bovine liver type B-10), arylsulfatase (*Helix pomatia* type H-2), and D-saccharic acid-1,4-lactone (saccharolactone), were purchased from Sigma Chemical Company (St. Louis, MO).

The following standards were synthesized in our laboratory according to published procedures: S-(2-nitrobenzyl)glutathione, S-(2-nitrobenzyl)-N-acetylcysteine, S-(3-nitrobenzyl)glutathione, S-(3-nitrobenzyl)-N-acetylcysteine, S-(4-nitrobenzyl)glutathione, and S-(4-nitrobenzyl)-N-acetylcysteine (4), and 2-, 3-, and 4-nitrohippuric acid (5).

Radiolabeled [*ring*-U-^{14}C]mononitrotoluenes were purchased from Midwest Research Institute (Kansas City, MO). The specific activities of the mononitrotoluenes were 52.5 mCi/mmol for 2NT and 4NT, and 55.8 mCi/mmol for 3NT. All three isomers were determined to be >99% radiochemically pure by HPLC.

Dose Administration and Sample Collection. Radiolabeled NT isomers

were diluted with the unlabeled isomer to specific activities of 1.50×10^5 dpm/μmol (2NT), 1.62×10^5 dpm/μmol (3NT), and 1.38×10^5 dpm/μmol (4NT), and dissolved in corn oil. Rats (3/treatment group) were given a single oral dose of NT by gavage (200 mg/kg) and immediately placed in glass metabolism cages (Jencon Scientific Limited, Hemel Hempstead, Hertfordshire, England). Air flow through the cages was 500 ml/min. Urine and feces were collected over dry ice and expired NT was trapped on activated charcoal. Urine was collected 2, 4, 8, 12, 24, 36, 48, 60, and 72 hr after the dose. Feces were collected 12, 24, 36, 48, 60, and 72 hr after the dose. Charcoal traps were changed 12, 24, 48, and 72 hr after the dose. Urine volumes were measured and all samples were immediately frozen and stored at −40°C until analyzed.

Quantitation and Identification of Metabolites. Aliquots of urine (10 μl) and chloroform extracts of the charcoal traps (6) were mixed with 10 ml of aqueous counting scintillant (Amersham, Arlington Heights, IL) and total radioactivity was determined by liquid scintillation spectrometry in a Packard 460CD scintillation counter. The external standard method was used to correct for quenching. The total radioactivity in the feces was determined as previously described (6).

The NT metabolites appearing in the urine were identified by specific enzyme hydrolysis of conjugates, co-elution with authentic standards on HPLC, and mass spectral analysis. The urine samples were treated with either β-glucuronidase, arylsulfatase, or arylsulfatase plus saccharolactone, in 0.2 M sodium acetate, pH 4.5, for 16 hr at 37°C (6). After the incubation, the samples were centrifuged in a Beckman Microfuge 11 (Palo Alto, CA). The supernatant fraction was removed and the pH was adjusted to 6–6.5 with 10% KOH.

Separation of the metabolites of NT in the supernatant fraction was achieved by an HPLC system consisting of two model 6000 A pumps, a model 660 solvent programmer, a model 440 UV absorbance detector from Waters Associates (Milford, MA), and a model 7125 Rheodyne injector (Berkeley, CA). The metabolites of 2NT were eluted from two 4.6 mm × 25 cm LiChrosorb columns connected in series (E. Merck, Darmstadt, West Germany, 10-μm particle size) by a 30-min linear gradient from 5 to 90% acetonitrile in 5 mM sodium phosphate buffer, pH 4.5, at a flow rate of 2 ml/min. The metabolites of 3NT were eluted from the same columns, but the solvent system consisted of a 20-min gradient (curve 8 on the Waters 660 solvent programmer) from 0 to 60% acetonitrile in 5 mM sodium phosphate buffer, pH 7.5, at a flow rate of 2 ml/min. The metabolites of 4NT were separated on the same columns as above with a solvent system consisting of a 20-min linear gradient from 0 to 80% methanol in 5 mM sodium phosphate buffer, pH 7.5, at a flow rate of 2 ml/min.

The identities of the metabolites were confirmed by mass spectrometry on a Finnigan 3200 mass spectrometer (Sunnyvale, CA). Electron impact spectra were obtained at 70 eV and 500 μA. Chemical ionization spectra were obtained using methane as the reagent gas.

Results

Elimination of NT. Radiolabel from the NT isomers was rapidly eliminated by the male Fischer 344 rat. By 72 hr after treatment, 80–90% of the dose was excreted in the urine, feces, and expired air. The major route of excretion for all isomers was the urine, and more radioactivity derived from 2NT than from 3NT or 4NT was excreted in the urine (table 1). Most of the excretion of NT occurred during the first 24 hr (73–86% of the dose). The peak excretion rate of radiolabel in the urine occurred between 3 and 6 hr after the dose for 2NT, and between 3 and 10 hr after the dose for 3NT and 4NT. The peak excretion rate of NT-related material in the feces occurred in the first 24 hr after the dose for each isomer, while the peak excretion rate of NT in expired air occurred in the first 12 hr after the dose (data not shown).

Identification of the Urinary Metabolites of 2NT. Seven metabolites were found in the urine of animals given 2NT (fig. 1). Peaks 1 and 6 co-eluted on HPLC with authentic 2-nitrobenzoic acid and 2-aminobenzoic acid, respectively. Their identities were confirmed by GC/MS (electron impact ionization) analysis of the methyl ester derivative of the metabolites. Peak 7 increased after β-glucuronidase hydrolysis of the urine and co-eluted with 2-nitrobenzyl alcohol; its identify was confirmed by GC/MS (electron impact ionization). Peak 5 gave a pseudo-molecular ion at m/z 299 by positive ion chemical ionization mass spectrometry, and a molecular ion at m/z 298 on negative ion chemical ionization mass spectrometry. A molecular weight of 298 is consistent with an *N*-acetylcysteine conjugate of 2-nitrotoluene. Therefore, *S*-(2-nitrobenzyl)-*N*-acetylcysteine was synthesized and subjected to HPLC and mass spectral analysis. Authentic *S*-(2-nitrobenzyl)-*N*-acetylcysteine co-eluted with peak 5 on HPLC and gave negative and positive chemical ionization spectra identical to that of peak 5.

Since a mercapturic acid conjugate of 2-nitrotoluene was identified in the urine, it seemed possible that one of the remaining unidentified peaks was a glutathione conjugate. When *S*-(2-nitrobenzyl)glutathione was synthetized and subjected to HPLC, it co-eluted with peak 4. However, electron impact and chemical ionization mass spectrometry failed to yield definitive spectra of the authentic glutathione standard, or of peak 4 isolated from urine by HPLC. Therefore, the isolated metabolite and the synthesized glutathione conjugate were treated with Raney nickel

TABLE 1

Excretion of radioactivity by male Fischer 344 rats 72 hr after a single oral dose of radiolabeled 2-, 3-, or 4NT

	Per Cent of Dose Excreted[a]		
	2NT	3NT	4NT
Urine	85.8 ± 1.7	67.8 ± 2.3	76.7 ± 2.6
Feces	4.6 ± 1.3	12.5 ± 2.3	6.1 ± 0.7
Expired air	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Total	90.5 ± 0.4	80.4 ± 0.4	82.8 ± 2.3

[a] Values represent the mean ± SEM for three rats. The dose of each isomer was 200 mg/kg.

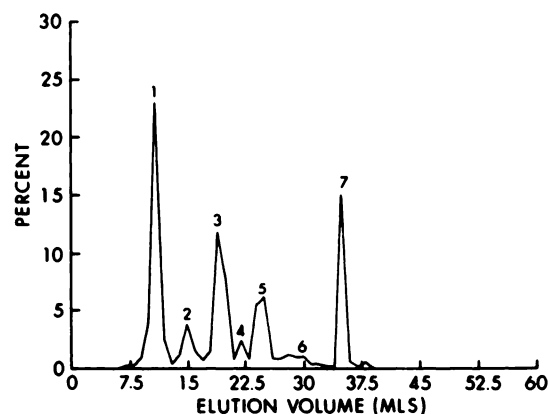

FIG. 1. *Radiochromatogram of β-glucuronidase-hydrolyzed urine from rats treated with 2NT.*

The metabolites of 2NT were separated by HPLC as described in *Materials and Methods*, and identified as described in *Results*. *Peak 1* is 2-nitrobenzoic acid. *Peaks 2* and *3* are unidentified. *Peak 4* is *S*-(2-nitrobenzyl)glutathione. *Peak 5* is *S*-(2-nitrobenzyl)-*N*-acetylcysteine. *Peak 6* is 2-aminobenzoic acid. *Peak 7* is 2-nitrobenzyl alcohol.

as previously described (7). Specific cleavage of the benzyl carbon-sulfur bond and reduction of the nitro group to amine would be expected in the reaction with Raney nickel. Peak 4 and the synthesized *S*-(2-nitrobenzyl)glutathione yielded 2-aminotoluene on reaction with Raney nickel (identified by co-elution on HPLC and by GC/MS). These results suggest that peak 4 is *S*-(2-nitrobenzyl)glutathione.

The compounds yielding peaks 2 and 3 could not be extracted from aqueous solution into either diethyl ether or ethyl acetate, were not affected by either β-glucuronidase or arylsulfatase treatment, and did not give readily interpretable mass spectra by electron impact or chemical ionization mass spectrometry. When these metabolites were isolated by HPLC and treated with Raney nickel, peak 2 yielded 2-acetamidotoluene and peak 3 yielded 2-aminotoluene (by co-elution on HPLC and GC/MS analysis). These results are consistent with identities of sulfur-containing conjugates of 2-acetamidotoluene and 2-aminotoluene for peaks 2 and 3, respectively. However, attempts to synthesize the *N*-acetylcysteine and glutathione conjugates of 2-acetamidotoluene and 2-aminotoluene *via* reduction of the corresponding conjugates of nitrotoluene or *via* reaction of *N*-acetylcysteine and glutathione with 2-aminobenzyl bromide were unsuccessful.

Quantitative analysis showed that 2NT was excreted in rat urine as the following metabolites (mean % of the dose ± SEM for 3 rats in 72 hr): 2-nitrobenzoic acid (28.6 ± 0.3); peak 2 (15.9 ± 1.5); 2-nitrobenzyl glucuronide (14.1 ± 0.7); *S*-(2-nitrobenzyl)-*N*-acetylcysteine (11.6 ± 0.2); peak 3 (6.0 ± 0.6); *S*-(2-nitrobenzyl)glutathione (3.9 ± 0.4); 2-aminobenzoic acid (1.8 ± 0.2); 2-nitrobenzyl sulfate (0.5 ± 0.2); and 2-nitrobenzyl alcohol (0.4 ± 0.3). The peak excretion rates of the metabolites of 2NT which had not undergone nitro group reduction occurred within the first 4 hr following the dose (fig. 2). 2-Aminobenzoic acid and peaks 2 and 3 had maximal excretion rates between 4 and 12 hr after administration of the dose.

Identification of the Urinary Metabolites of 3NT. Rats given 3NT excreted eight metabolites in the urine (fig. 3). Peaks 1, 3, and 4 co-eluted on HPLC with authentic standards of 3-aminobenzoic acid, 3-acetamidobenzoic acid, and 3-nitrobenzoic acid, respectively. Their identities were confirmed by GC/MS analysis of the methyl ester derivatives of the metabolites. Peak 8 appeared after hydrolysis of the urine with β-glucuronidase and co-eluted with 3-nitrobenzyl alcohol. GC/MS analysis confirmed peak 8 as 3-nitrobenzyl alcohol. Since glutathione and *N*-acetylcysteine conjugates were found in the urine of animals treated with 2NT, *S*-(3-nitrobenzyl)glutathione and *S*-(3-nitrobenzyl)-*N*-acetylcysteine were synthesized and subjected to HPLC analysis. *S*-(3-Nitrobenzyl)glutathione was found to co-elute with peak 7. Treatment of the synthetic *S*-(3-nitrobenzyl)glutathione as well as peak 7 with Raney nickel yielded 3-aminotoluene, as confirmed by HPLC and GC/MS. This suggests that peak 7 is *S*-(3-nitrobenzyl)glutathione. Peak 6 co-eluted with and gave a mass spectrum (chemical ionization) identical to that of 3-nitrohippuric acid. Peaks 2 and 5 did not co-elute with any of the authentic or synthesized standards.

Rats excreted 3NT in the urine as the following metabolites (mean % of the dose ± SEM for 3 rats 72 hr after treatment): 3-nitrohippuric acid (23.6 ± 2.0); 3-nitrobenzoic acid (21.1 ± 1.1); 3-acetamidobenzoic acid (11.6 ± 0.4); peak 5 (2.6 ± 0.3); peak

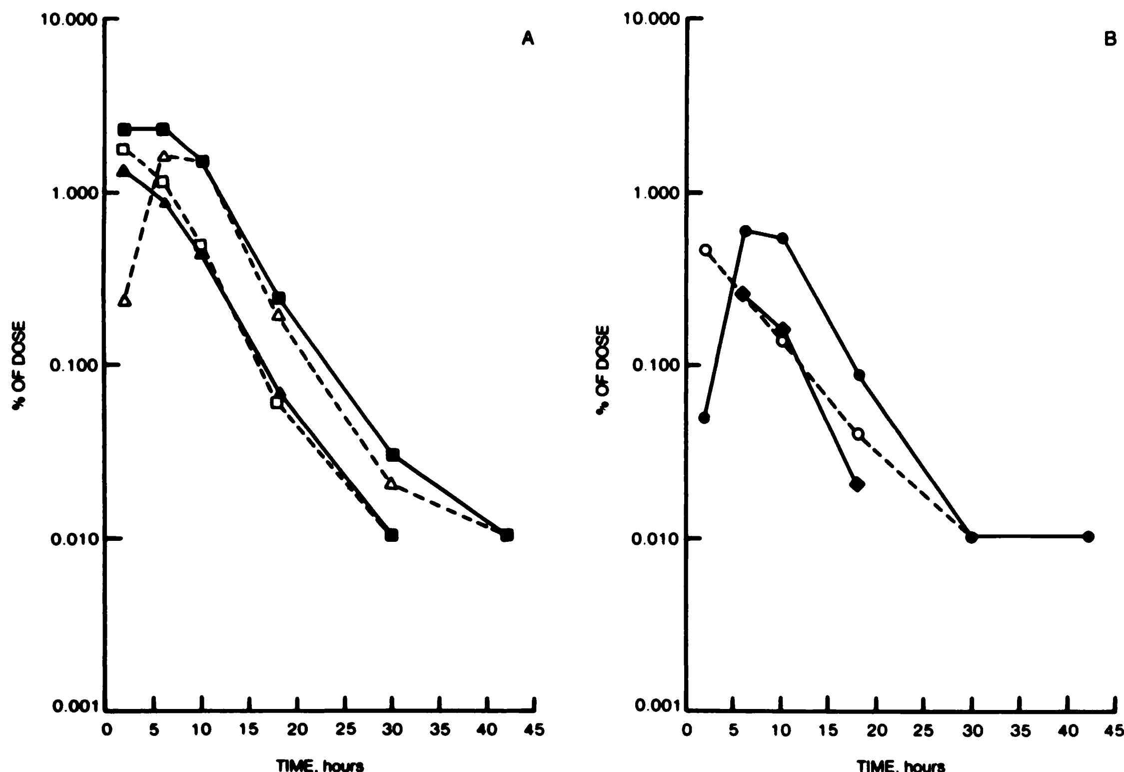

FIG. 2. *Excretion rates of metabolites of 2NT in the urine.*

A shows the excretion rates of: 2-nitrobenzoic acid, ■; *Peak 3*, △; *S*-(2-nitrobenzyl)-*N*-acetylcysteine, ▲; and 2-nitrobenzyl alcohol glucuronide, □. *B* shows the excretion rates of: *Peak 2*, ●; *S*-(2-nitrobenzyl)glutathione, ○; and 2-aminobenzoic acid, ◆. Values are the percentage of the dose/hr excreted in the urine. SEM are ≤30%.

2 (2.2 ± 0.3); 3-nitrobenzyl glucuronide (2.0 ± 0.1), S-(3-nitrobenzyl)glutathione (1.3 ± 0.1); and 3-aminobenzoic acid (1.2 ± 0.3). As with 2NT, the reduced metabolites of 3NT showed a delayed peak excretion rate (fig. 4). The peak excretion for 3-aminobenzoic acid, 3-acetamidobenzoic acid, peak 2, and peak 5 occurred between 8 and 24 hr after the dose. 3-Nitrobenzyl glucuronide and S-(3-nitrobenzyl)glutathione showed peak excretion rates which occurred within the first 4 hr after the dose. The excretion of 3-nitrobenzoic acid was paralleled by that of its glycine conjugate, 3-nitrohippuric acid, and appeared to be slower than that of the corresponding metabolite of 2NT.

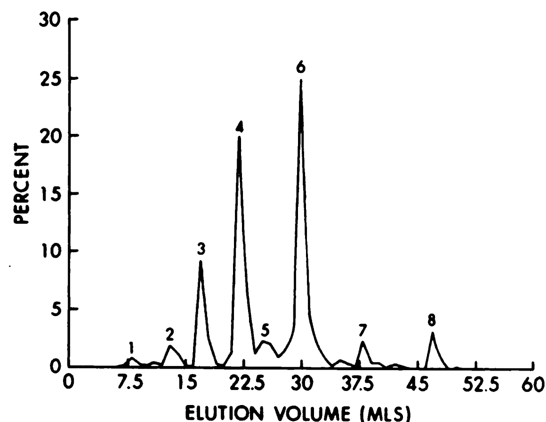

FIG. 3. *Radiochromatogram of β-glucuronidase-hydrolyzed urine from rats treated with 3NT.*

The metabolites of 3NT were separated by HPLC as described in *Materials and Methods*, and identified as described in *Results*. *Peak 1* is 3-aminobenzoic acid. *Peak 2* is unidentified. *Peak 3* is 3-acetamidobenzoic acid. *Peak 4* is 3-nitrobenozic acid. *Peak 5* is unidentified. *Peak 6* is 3-nitrohippuric acid. *Peak 7* is S-(3-nitrobenzyl)glutathione. *Peak 8* is 3-nitrobenzyl alcohol.

Identification of the Urinary Metabolites of 4NT. Seven metabolites were found in urine from rats which had been treated with 4NT (fig. 5). Authentic standards of 4-aminobenzoic acid, 4-acetamidobenzoic acid, and 4-nitrobenzoic acid co-eluted on HPLC with radioactive peaks 1, 2, and 3, respectively. GC/MS (electron impact) analysis of the methyl ester derivatives of the radioactive peaks showed that each peak gave spectra identical to the methyl ester derivative of the standard with which it co-eluted. Peak 6 appeared after hydrolysis of the urine with β-glucuronidase and co-eluted with 4-nitrobenzyl alcohol. GC/MS (electron impact) confirmed this identification. Peak 7 appeared after hydrolysis of the urine with either β-glucuronidase or arylsulfatase plus saccharolactone, and co-eluted with authentic 5-methyl-2-nitrophenol. Analysis of peak 7 from hydrolyzed urine by electron impact mass spectrometry confirmed its identify as 5-methyl-2-nitrophenol. Peak 5 co-eluted on HPLC with synthetic S-(4-nitrobenzyl)-N-acetylcysteine and, when isolated by HPLC, gave chemical ionization mass spectra (positive and negative ion) identical to those for authentic S-(4-nitrobenzyl)-N-acetylcysteine. Authentic 4-nitrohippuric acid co-eluted on

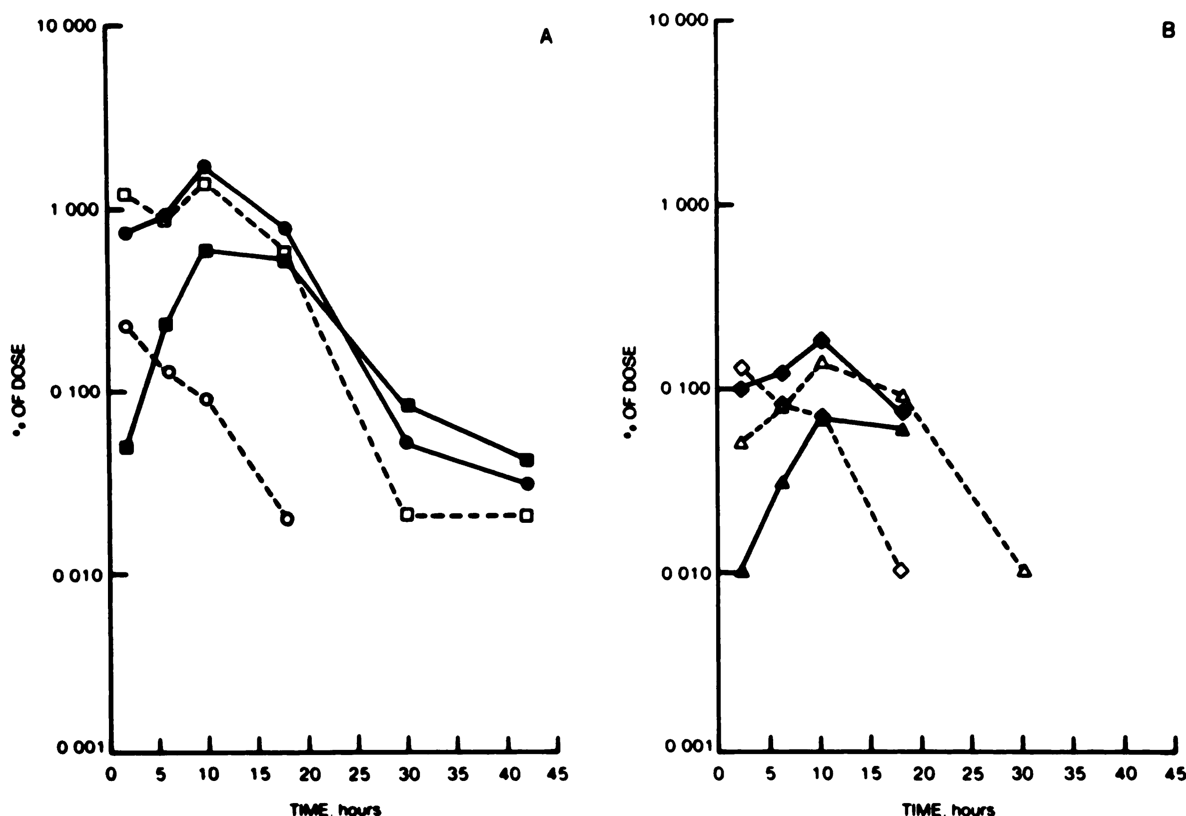

FIG. 4. *Excretion rates of metabolites of 3NT in the urine.*

A shows the excretion rates of: 3-acetamidobenzoic acid, ■; 3-nitrobenzoic acid, □; 3-nitrohippuric acid, ●; and 3-nitrobenzyl alcohol glucuronide, ○. *B* shows the excretion rates of: 3-aminobenzoic acid, ▲; *peak 2*, △; *peak 5*, ◆; and S-(3-nitrobenzyl)glutathione, ◇. Values are the mean percentage of the dose/hr excreted in the urine. SEM are ≤30%.

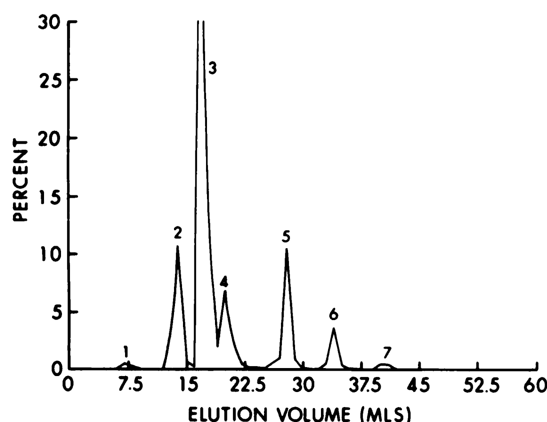

FIG. 5. *Radiochromatogram of β-glucuronidase-hydrolyzed urine from rats treated with 4NT.*

The metabolites of 4NT were separated by HPLC as described in *Materials and Methods*, and identified as described in *Results*. *Peak 1* is 4-aminobenzoic acid. *Peak 2* is 4-acetamidobenzoic acid. *Peak 3* is 4-nitrobenzoic acid. *Peak 4* is 4-nitrohippuric acid. *Peak 5* is *S*-(4-nitrobenzyl)-*N*-acetylcysteine. *Peak 6* is 4-nitrobenzyl alcohol. *Peak 7* is 5-methyl-2-nitrophenol.

HPLC with peak 4, and the identity of peak 4 was confirmed by positive and negative chemical ionization mass spectrometry.

The following metabolites appeared in the urine after a dose of 4NT (mean % of dose ± SEM for 3 rats 72 hr after treatment): 4-nitrobenzoic acid (28.0 ± 2.6); 4-acetamidobenzoic acid (27.1 ± 3.0); 4-nitrohippuric acid (13.0 ± 0.7); *S*-(4-nitrobenzyl)-*N*-acetylcysteine (3.7 ± 0.1); 4-nitrobenzyl glucuronide (1.4 ± 0.1); 4-aminobenzoic acid (0.8 ± 0.1); 5-methyl-2-nitrophenyl glucuronide (0.3 ± 0.0); and 5-methyl-2-nitrophenyl sulfate (0.2 ± 0.0). 4-Nitrobenzoic acid was excreted at a rate similar to the 3-isomer, but more slowly than 2-nitrobenzoic acid (fig. 6). 4-Nitrohippuric acid was excreted at a rate similar to that of 4-nitrobenzoic acid. The other nonreduced metabolites of 4NT, 4-nitrobenzyl alcohol glucuronide, *S*-(4-nitrobenzyl)-*N*-acetylcysteine, and the sulfate and glucuronide conjugates of 5-methyl-2-nitrophenol showed peak excretion rates within the first 4 hr after the dose. The reduced metabolites of 4NT, 4-acetamidobenzoic acid, and 4-aminobenzoic acid had peak excretion rates which occurred between 4 and 12 hr after the dose.

Discussion

The data presented here on the urinary metabolites of NT are summarized in fig. 7, which presents plausible routes of metabolism of NT in male Fischer 344 rats. With the exception of small amounts of phenolic metabolites of 4NT, all metabolism of the mononitrotoluenes appears to be the result of further biotransformation of nitrobenzyl alcohol. This suggestion is supported by the nature of the metabolites found here and by data showing that all three isomers of NT are hydroxylated at the methyl group by rat hepatic microsomes at similar rates (4). Isolated rat hepatocytes metabolize the resulting nitrobenzyl alcohol by three different routes depending on the isomer (4). There also appear to be three main pathways of metabolism of the nitrobenzyl alcohol *in vivo*.

Nitrobenzyl alcohol is oxidized to nitrobenzoic acid in the first pathway. In isolated hepatocytes, further oxidation of nitrobenzyl alcohol to nitrobenzoic acid is the predominant route only for 3NT (4) but appears to be important to all three isomers *in vivo*. There were equivalent amounts of nitrobenzoic acid excreted in the urine among the isomers, but 3- and 4-nitrobenzoic acid were further metabolized and excreted as their glycine conjugates, while 2-nitrobenzoic acid was not. The nitrobenzoic acids or their glycine conjugates appearing in the urine account for 28 to 44% of the dose, or 33 to 65% of the urinary metabolites of the nitrotoluenes.

A second pathway of nitrobenzyl alcohol metabolism is conjugation with glucuronic acid. This reaction occurs with the dinitrobenzyl alcohols formed from 2,4- and 2,6-dinitrotoluene (6, 8, 9), and is the primary pathway for 2-nitrobenzyl alcohol metabolism in isolated rat hepatocytes (4). Glucuronidation of nitrobenzyl alcohol apears to be the primary pathway of metabolism *in vivo* for 2NT. Glucuronidation of nitrobenzyl alcohol in the liver may result in the formation of amino- and acetamidobenzoic acids by the following reactions. Biliary excretion of the nitrobenzyl alcohol glucuronide might result in deconjugation and nitro group reduction by intestinal microflora. The resulting aminobenzyl alcohol could be reabsorbed, further oxidized, and acetylated to yield aminobenzoic acids or acetamidobenzoic acids. An alternative pathway for the formation of amino- and acetamidobenzoic acids is active transport of nitrobenzoic acid from the systemic circulation into the intestinal lumen where reduction by microflora could take place. Gardner and Renwick (10) have shown that rats given either an ip or an oral dose of 2-, 3-, or 4-nitrobenzoic acid excreted the corresponding aminobenzoic and acetamidobenzoic acids, but excretion of the reduced metabolites was much greater after administration of 3- or 4-nitrobenzoic acid than after 2-nitrobenzoic acid. They demonstrated that reduction of the nitro groups was dependant upon gut microflora and suggested that the nitrobenzoic acids gained access to the microflora by active transport across the intestinal wall. Thus, it is possible that the relatively greater amounts of 3- and 4-acetamidobenzoic acids formed *in vivo* are a result of gut flora reduction of 3- and 4-nitrobenzoic acid, followed by acetylation of the amino groups. While the latter explanation for the formation of amino- and acetamidobenzoic acids seems to fit existing data well, the data we have presented here do not allow an unequivocal assignment of pathway to be made.

The third pathway of nitrobenzyl alcohol metabolism is conjugation with glutathione. The relatively high amounts of conjugation with glutathione following a dose of 2NT compared to 3NT or 4NT may reflect an affinity of 2-nitrobenzyl alcohol or 2-aminobenzyl alcohol for cytosolic sulfotransferase. The resulting sulfate conjugate may then be a substrate for glutathionetransferase in the liver. Similar sulfate conjugates are substrates for glutathionetransferase in rat liver (11–13), and the sequence of sulfation and glutathione conjugation may be responsible for the appearance of the *S*-(nitrobenzyl)glutathiones and the *S*-(nitrobenzyl)-*N*-acetylcysteines found in rat urine after treatment with NT. Peaks 2 and 3 which appear in rat urine after treatment with 2NT are depicted in fig. 7 as sulfur-containing metabolites which arise through a benzyl sulfate intermediate. As noted in *Results*, this assignment is tentative, since only indirect evidence as to their identities is available. More work is needed to identify these compounds and the pathway(s) by which they arise.

The requirement for microflora in the reduction of related single ring nitroaromatics has been previously demonstrated by Rickert *et al.* (14) and Levin and Dent (15). The delay in the peak excretion rates of the reduced metabolites of NT may reflect the time required for the unreduced compounds to gain access

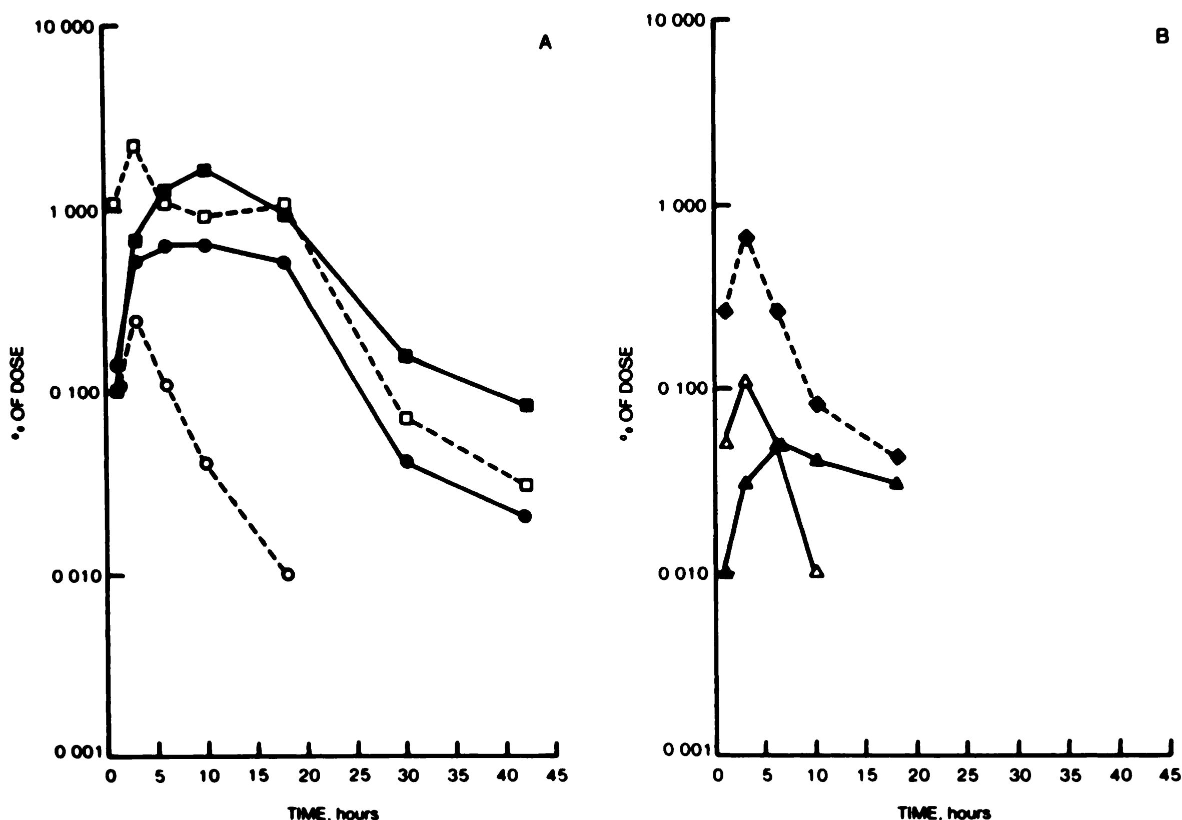

FIG. 6. *Excretion rates of metabolites of 4NT in the urine.*

A shows the excretion rates of: 4-acetamidobenzoic acid, ■; 4-nitrobenzoic acid, □; 4-nitrohippuric acid, ●; and 4-nitrobenzyl alcohol glucuronide, O. *B* shows the excretion rates of: 4-aminobenzoic acid, ▲; 5-methyl-2-nitrophenol (glucuronide and sulfate conjugates added together), Δ; and *S*-(4-nitrobenzyl)-*N*-acetylcysteine, ◆. Values are the mean percentage of the dose/hr excreted in the urine. SEM are ≤30%.

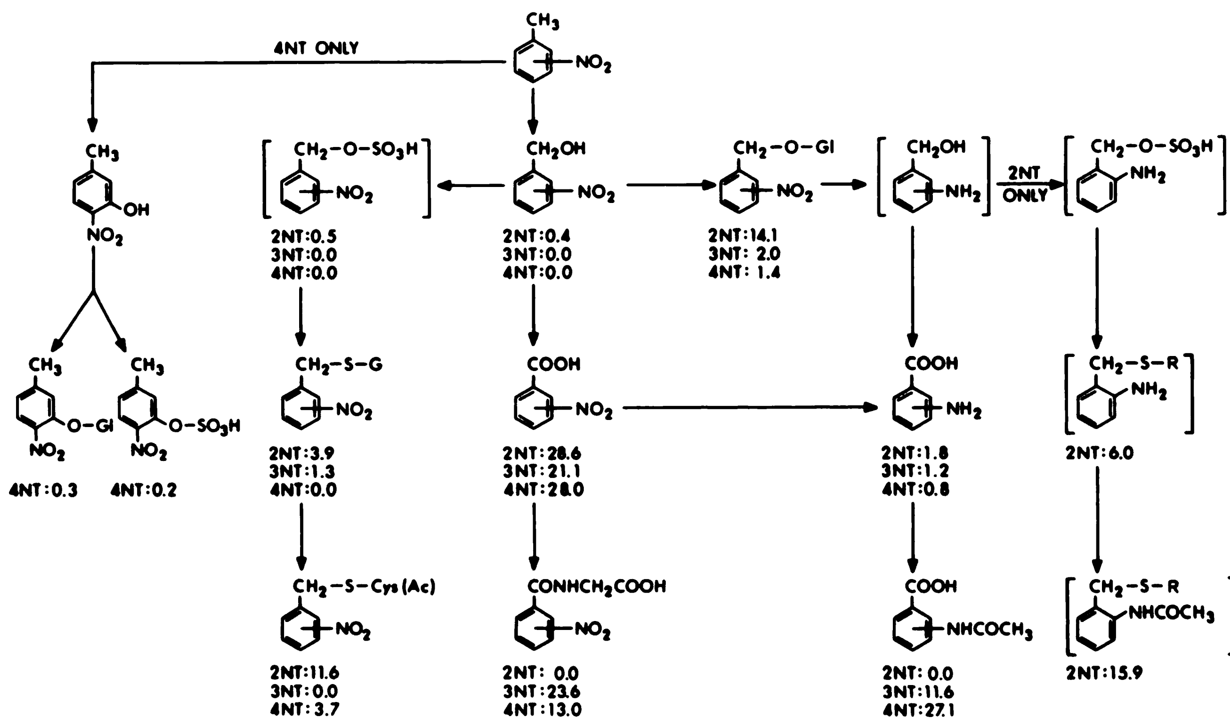

FIG. 7. *Proposed pathway of metabolism of NT in male Fischer 344 rats.*

The *values* below each *structure* represent the percentage of the dose of each metabolite excreted in the urine 72 hr after a 200 mg/kg oral dose of NT. *Structures* in *brackets* are postulated intermediates or inconclusively identified metabolites (see text). *S-G*, glutathione; *S-Cys(Ac)*, *N*-acetylcysteine; *Gl*, glucuronide; *S-R*, sulfur-containing conjugate.

to intestinal microflora. The delay in the peak excretion rates of the unknown metabolites of 2NT and 3NT, peaks 2 and 3 (fig. 1) and peaks 2 and 5 (fig. 3), respectively, suggests that these metabolites have also been reduced. The observation that 3- and 4-nitrobenzoic acids are excreted more slowly than 2-nitrobenzoic acid suggests that 3NT and 4NT are either metabolized more slowly than 2NT, or that 3- and 4-nitrobenzoic acids are more slowly eliminated by the kidneys.

Hepatic covalent binding and hepatic unscheduled DNA synthesis is greater in rats after administration of 2NT than after 3NT or 4NT (3).[2] Our data do not clearly delineate a pathway of metabolism which would result in such isomer-specific toxicity. 2NT requires intestinal microflora for genotoxic activity (3) and its metabolism may occur by pathways similar to those described for the dinitrotoluenes (8, 9). It is thus possible that isomeric differences in toxicity, as measured by the *in vivo-in vitro* unscheduled DNA synthesis assay and by covalent binding, may arise from the relatively greater percentage of the dose of 2NT which is excreted in the bile as 2-nitrobenzyl glucuronide, and converted to 2-aminobenzyl alcohol by the microflora. Further metabolism of 2-aminobenzyl alcohol to 2-aminobenzyl sulfate may yield a highly reactive compound as has been demonstrated for similar conjugates of 7,12-dimethylbenzanthracene (16). Alternatively, 2-aminobenzyl alcohol may be converted to a hydroxylamine and then to a reactive *N,O*-sulfate similar to that described for *N*-acetylaminofluorene (17). Kedderis *et al.*[2] have shown that pretreatment of rats with 2,6-dichloro-4-nitrophenol or pentachlorophenol, potent inhibitors of sulfotransferase (18, 19), significantly decreased, but did not abolish, the hepatic covalent binding of 2NT, and had no effect on the covalent binding of 3NT or 4NT. Thus, it appears that sulfation is an important step in the activation of 2NT, but whether the active metabolite is a benzyl sulfate or *N*-hydroxysulfate is unclear.

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