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SEQUENTIAL ENZYME-CATALYZED METABOLISM OF 4-NITROTOLUENE TO S-(4-NITROBENZYL)GLUTATHIONE

J. Don deBethizy and Douglas E. Rickert

Department of General and Biochemical Toxicology, Chemical Industry Institute of Toxicology, P. O. Box 12137, Research Triangle Park, NC 27709

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Summary: [^{14}C]-4-Nitrotoluene was metabolized by rat liver postmitochondrial supernatant containing NADPH, reduced glutathione and a sulfate activating system to 4-nitrobenzyl alcohol, 4-nitrobenzyl sulfate, and S-(4-nitrobenzyl) glutathione. Formation of both sulfur-containing metabolites was dependent on the presence of a sulfate activating system. These results suggest that the glutathione conjugate was derived from 4-nitrobenzyl sulfate. Reaction of 4-nitrobenzyl sulfate with glutathione was not detected in pH 7.4 buffer, but rat liver cytosol catalyzed the formation of the glutathione conjugate from 4-nitrobenzyl sulfate. These results show that 4-nitrotoluene is metabolized in rat liver by sequential side chain oxidation, sulfation, and glutathione conjugation. Furthermore, they indicate that, unlike certain other arylmethyl sulfates, 4-nitrobenzyl sulfate is not highly reactive.

Freshly isolated rat hepatocytes convert 4-nitrotoluene (4NT) to 4-nitrobenzyl sulfate and S-(4-nitrobenzyl)glutathione (1). Recent studies have suggested that certain arylmethyl compounds can be activated to strong electrophiles by side chain oxidation and sulfation of the resulting benzyl alcohol (2,3), and it is possible that the S-(4-nitrobenzyl)glutathione, which was produced from 4NT by hepatocytes, resulted from a similar mechanism. Alternatively, benzyl sulfate has been shown to be a substrate for glutathione S-transferase (EC 2.8.11.18) to yield S-(benzyl)glutathione (4), suggesting that S-(4-nitrobenzyl)glutathione could be produced by a similar enzymic mechanism. Therefore, studies were conducted with rat liver subcellular fractions to determine (a) if sulfate conjugation is obligatory for glutathione conjugate formation and (b) if the reaction with glutathione proceeds by a nonenzymic, electrophilic reaction.

Abbreviations used: 4NT, 4-nitrotoluene; PMS, post-mitochondrial supernatant; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

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MATERIALS AND METHODS

Chemicals

Ring-labeled [U-^{14}C]4-nitrotoluene (52.5 mCi/mmole) was purchased from Midwest Research Institute (Kansas City, Missouri) and judged > 98% radiochemically pure by HPLC analysis. 4NT, 4-nitrobenzyl bromide, chlorosulfonic acid (99%) and 4-nitrobenzyl alcohol were purchased from Aldrich Chemical Co. (Milwaukee, WN). Reduced glutathione, ATP, arylsulfatase (Type H-2) and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). Pyridine, magnesium chloride, EDTA, and sodium sulfate were obtained from Fisher Scientific Co. (Fair Lawn, NJ). S-(4-nitrobenzyl)glutathione was synthesized as previously described (5) and 4-nitrobenzyl sulfate was synthesized by reacting 4-nitrobenzyl alcohol with chlorosulfonic acid in anhydrous pyridine (2).

Preparation of enzyme sources

Liver post-mitochondrial supernatant (PMS) and cytosol were prepared from untreated male Fischer-344 rats (CDF®(F-344)/CRLBr) 200-250 g by differential centrifugation (6,7). The subcellular fractions were treated with diethylmaleate (1 mM) for 30 min to deplete glutathione and then dialyzed at 4°C overnight against 2500 volumes of 2% NaCl containing 2 mM EDTA (pH 7.0). Protein concentrations were determined by the Biuret method using bovine serum albumin as the standard protein.

Metabolism of 4NT by PMS.

Incubation mixtures contained radiolabelled 4NT (100 µM, 0.25 µCi), ATP (5 mM), Na_2SO_4 (5 mM), $MgCl_2$ (3 mM), EDTA (0.1 mM), NADPH (2.5 mM), reduced glutathione (1 mM) and PMS protein (3.3 mg) in a total volume of 1.25 ml of Tris-HCl buffer (66 mM, pH 7.4). The mixture was preincubated in 7-ml screw-cap vials for 3 min at 37°C, and the reaction was started by the addition of 10 µl of 4NT in ethanol. After 45 min the reaction was stopped by the addition of an equal volume of methanol. The precipitated proteins were removed by centrifugation, and the supernate was analyzed by HPLC in the reversed phase mode. Elution was accomplished with a 20 min linear gradient from 10 to 90% methanol in 0.005 M phosphate buffer, pH 7.4, at a flow rate of 2.0 ml/min. Fractions (1.0 ml) of the HPLC effluent were collected directly into scintillation vials and scintillation fluid (ACS, Amersham/Searle, Arlington Heights, IL) was added to the vials. Radioactivity in the fractions was determined with a Packard Model 460 liquid scintillation counter. Metabolites were tentatively identified by coelution of authentic standards on HPLC. The identity of 4-nitrobenzyl sulfate was confirmed by hydrolysis with aryl sulfatase and GC-MS analysis of the resulting 4-nitrobenzyl alcohol. The identity of S-(4-nitrobenzyl)glutathione was confirmed by Raney nickel hydrolysis as previously described (8).

Metabolism of 4-nitrobenzyl sulfate by dialyzed cytosol

Incubations consisted of 4-nitrobenzyl sulfate (1 mM), EDTA (0.1 mM) reduced glutathione (5 mM), and dialyzed hepatic cytosol (3.0 mg of protein) in a total incubation volume of 2.0 ml Tris-HCl buffer (66 mM, pH 7.4). The reaction was started by the addition of 4-nitrobenzyl sulfate and incubated for 45 min at 37°C. The reaction was terminated and samples were analyzed as above except that the metabolites were detected at 254 nm using a fixed wavelength UV detector (Waters Associates, Milford, MA).

Table 1. The effect of cofactor or reactant omission on the metabolism of 4NT by rat hepatic postmitochondrial supernatant.

Cofactor or reactant omitted	Metabolite Formed[a] (nmole/mg protein/45 min)			Total Metabolism (nmole/mg protein/45 min)
	4-Nitrobenzyl alcohol	4-Nitrobenzyl sulfate	S-(4-nitrobenzyl) glutathione	
None[b]	4.28 ± 0.09	1.13 ± 0.09	2.40 ± 0.03	7.81 ± 0.21
Glutathione	4.77 ± 0.11*	2.76 ± 0.16*	1.08 ± 0.05*	8.61 ± 0.26
ATP	12.89 ± 0.17*	ND[c]	ND	12.89 ± 0.17*
NADPH	ND	ND	ND	ND

[a]Values are mean ± S.E.M. for 3 rats; asterisks represent significant difference from complete incubations using Student's "t"-test. ($p = 0.05$).

[b]The complete incubation contained [^{14}C]4NT (100 μM), ATP (5 mM), Na_2SO_4 (5 mM), $MgCl_2$ (3 mM), EDTA (0.1 mM), NADPH (2.5 mM), glutathione (1 mM), and PMS (3.3 mg protein).

[c]ND = not detected.

RESULTS AND DISCUSSION

Incubation of 4NT with rat liver PMS containing NADPH, an active sulfate (PAPS)-generating system, and reduced glutathione produced 4-nitrobenzyl alcohol, S-(4-nitrobenzyl)glutathione, and 4-nitrobenzyl sulfate (Table 1). The sulfur-containing metabolites comprised 45% of the total metabolites generated from 4NT. Omission of glutathione from the incubation mixture resulted in a decrease in glutathione conjugation and a concommitant increase in 4-nitrobenzyl sulfate. The absence of glutathione did not change the total amount of sulfur-containing metabolites and only a slight increase in total metabolism of 4NT was observed. When ATP was omitted, the formation of both the sulfate conjugate and the glutathione conjugate was abolished; only 4-nitrobenzyl alcohol was produced. Furthermore, boiled PMS failed to metabolize 4NT (data not shown). These results indicate that the formation of both 4-nitrobenzyl sulfate and S-(4-nitrobenzyl)glutathione was dependent upon the availability of the PAPS-generating system.

Watabe et al. (2) reported that 7-hydroxymethyl-12-methylbenz[a]anthracene is metabolized by rat liver cytosol in the presence of a PAPS-generating system to the 7-hydroxymethyl sulfate ester. This aralkyl sulfate ester as

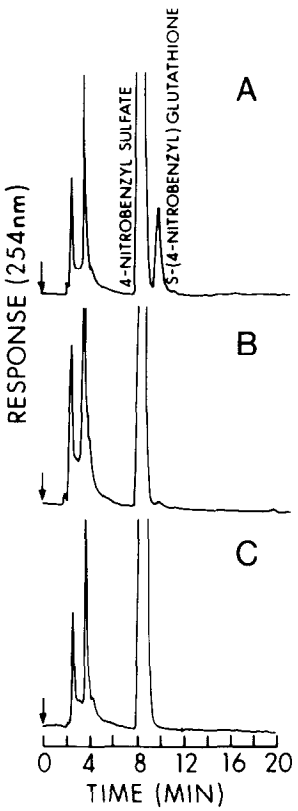

Figure 1. High pressure liquid chromatogram of incubations of dialyzed rat liver cytosol with 4-nitrobenzyl sulfate and glutathione.

Chromatograms are typical of those obtained in separate experiments. Panel A is from an incubation containing cytosol and both reactants. Panels B and C are from incubations containing no glutathione or boiled cytosol, respectively.

well as benzyl sulfate and 2-menaphthylsulfate were direct acting mutagens in Salmonella typhimurium strain TA98. These compounds also reacted nonenzymically with glutathione. These results led Watabe et al. (2) to suggest that aralkyl esters could directly form highly reactive electrophilic species.

Studies were conducted to examine the interaction of synthesized 4-nitrobenzyl sulfate and glutathione in the presence and absence of the cytosolic glutathione S-transferase, to determine whether 4-nitrobenzyl sulfate was also capable of reacting with glutathione nonenzymically. Incubation of only glutathione and 4-nitrobenzyl sulfate in Tris-HCl buffer, (pH 7.4), resulted in no detectable (minimum detectable conversion = 0.2%) formation of S-(4-nitrobenzyl) glutathione. These results indicate that 4-nitrobenzyl sulfate does not directly form an electrophilic species. The data presented in Figure

1A shows that S-(4-nitrobenzyl)glutathione was formed when 4-nitrobenzyl sulfate was incubated with glutathione and rat liver cytosol. When glutathione was omitted from the incubation, or the cytosol heat denatured, no glutathione conjugate was detected (Figure 1B and 1C, respectively).

Several aralkyl sulfate esters have been shown to be substrates for glutathione S-transferase partially purified from rat liver cytosol (4). The data presented here in conjunction with other studies (4) suggests that aralkyl sulfate esters with a single aromatic nucleus (i.e., benzyl sulfate) are good substrates for glutathione-S-transferase(s). In contrast to polycyclic aralkyl sulfate esters, single ringed aralkyl sulfate esters have been shown to be only weakly mutagenic (3) or inactive (9) in the Ames assay. Our results with 4-nitrobenzyl sulfate indicate that it is a stable compound at pH 7.4. Thus, sulfation of side chain alcohols on single-ring aromatic compounds may be more often associated with detoxification rather than with formation of a highly reactive metabolite.

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