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# Effect of Cytosolic Components on the Metabolism of the Hydrazide Iproniazid

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SUMMARY

The effects of thiols, such as glutathione (GSH), and the cytosolic glutathione *S*-transferases on the microsomal metabolism of the hydrazide iproniazid to hydrocarbon products were investigated. Thiol compounds stimulated propane production and depressed propylene production. Addition of preparations of cytosolic proteins to the microsomal reaction mixtures in the presence of GSH depressed production of propane by more than 80% and propylene by 50% compared to the GSH-mediated reaction. The purified glutathione *S*-transferases A and B were most potent in eliciting this effect; isozymes AA, C, and E had little or no effect on hydrocarbon production. Further, a mixture of these purified isozymes in the concentrations known to exist in cytosol affected hydrocarbon production in a manner similar to cytosol. Experiments performed with isolated hepatocytes and an inhibitor of these cytosolic enzymes further supported the involvement of these enzymes in altered hydrocarbon production. These isozymes were subsequently shown to catalyze the formation of a GSH conjugate, *S*-(2-propyl)glutathione. The decreases in hydrocarbon production by microsomes in the presence of the glutathione *S*-transferases and GSH were accompanied by production of slightly larger amounts of conjugate. These data indicate that the cytosolic glutathione *S*-transferases interact with an oxidative microsomal metabolite of iproniazid to enzymatically produce an *S*-(2-propyl)glutathione conjugate and thus prevent formation of a reactive species which would otherwise chemically decompose to yield hydrocarbons or to covalently bind to cellular macromolecules.

## INTRODUCTION

Iproniazid (1-isopropyl-2-isonicotinylhydrazine) is a hydrazide that has been extensively used as an antidepressant and antitubercular drug (1). Iproniazid has been removed from clinical usage in the United States because its use led to a high incidence of hepatocellular damage in humans (2). However, it is still used in Europe and serves as a model compound for the metabolism of other hydrazides.

To date, all of the hydrazine and hydrazide compounds tested in animal bioassay have been shown to be carcinogenic, including iproniazid (3, 4). Iproniazid has also be noted to be hepatotoxic in rats, although only when large dosages are administered (5). Other carcinogenic hydrazines, such as procarbazine and 1,2-dimethylhydrazine, have similarly been observed to be only marginally toxic to hepatocytes in primary culture and *in vivo* at moderate dosages (6, 7). These results suggest that although metabolic activation may be important in the carcinogenicity and toxicity of these compounds, efficient biological systems exist for the detoxication of the reactive metabolites formed.

The *N*-oxidation of hydrazines to their azo or diazene intermediates has been documented to be an important initial event in the activation of many hydrazine compounds to chemically reactive metabolites (8-10). Prough *et al.* (11) proposed that alkyldiazene intermediates formed from biological *N*-oxidation can decompose to yield the parent hydrocarbon and Nelson *et al.* (5) noted that propane is a product of the oxidative microsomal metabolism of iproniazid. Experiments with iproniazid, labeled on the methine carbon or hydrogen of the isopropyl group, have previously indicated that this portion of the molecule is responsible for both covalent binding to protein and hydrocarbon production (5). Iproniazid oxidation by horseradish peroxidase and prostaglandin synthetase has recently been shown to yield first a nitrogen-centered iproniazid radical and subsequently a carbon-centered isopropyl radical (12). The isopropyl radical can abstract a hydrogen atom to form propane. This radical product was proposed to result from decomposition of

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the azo ester intermediate formed during the oxidation of iproniazid. Recently, we observed the iproniazid indeed undergoes direct oxidation to hydrocarbon products by liver microsomal fractions.[2] Activation of hydrazine derivatives to unstable azo, diazene, or azo ester intermediates with subsequent formation of other reactive products may thus be an important initial event in hydrazine toxicity.

Efficient biological components exist for the detoxication of the oxidative metabolites of many foreign compounds, including hydrazines. Nelson *et al.* (14) have implicated GSH and cysteine as components of the biological system involved in the detoxication of hydrazines. GSH and cysteine were able to effectively decrease covalent binding of the alkyl or acyl moieties of isopropylhydrazine or acetylhydrazine to rat liver microsomal protein. Moloney *et al.*[3] have indicated that GSH is also able to decrease binding of the methyl portion of azoprocarbazine to microsomal protein. The cytosolic glutathione *S*-transferases are known to interact in a GSH-dependent reaction with many electrophilic compounds (16). The present study characterizes the effects of thiols on hydrocarbon (propane and propylene) production from iproniazid, explores the interaction of the glutathione *S*-transferases with the putative azo ester intermediate of iproniazid metabolism, and presents a mechanism for the enzymic detoxification of the reactive metabolites of this hydrazide.

## MATERIALS AND METHODS

*Chemicals.* GSH,[4] GSSG, penicillamine, cysteamine, DL-dithiothreitol, hyaluronidase, NADP, bovine serum albumin, DL-sodium isocitrate, isocitrate dehydrogenase (type IV), and iproniazid phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). [$^3$H]GSH labeled in the glycine moiety was purchased from New England (Boston, MA) at a specific activity of 5.0 Ci/mmol. Sodium PB was obtained from Merck and Co., Inc. (Rahway, NJ). Isopropyl magnesium chloride in ether was purchased from Alpha Products (Danvers, MA). Modified L-15 medium without glutamine was purchased from Flow Laboratories (McLean, VA); collagenase was from Worthington Diagnostics (Freehold, NJ), fetal calf serum was from Biocel Laboratories (Carson, CA), and tryptose phosphate broth was from GIBCO (Grand Island, NY). 1-Butanol and 2-propanol were purchased from Mallinckrodt, Inc. (Paris, KY) and methanol was obtained from Burdick and Jackson, Inc. (Muskegon, MI). Silica gel G TLC plates (20 × 20 cm, 0.25-mm thickness) were purchased from Analtech (Newark, DE). All other chemicals were of the highest purity commercially available.

*Animal and tissue preparations.* Male Sprague-Dawley rats [Crl:CD (SD)BR, 100–150 g] and male Fischer 344 rats (COBS CDF F344/Crl, 80–120 g) were purchased from Charles River Breeding Labs (Wilmington, MA), and were maintained on tap water and lab chow *ad libitum*. Animals pretreated with PB were given daily intraperitoneal injections of PB in 0.9% NaCl solution (80 mg/kg) for 4 days and were fasted 18 hr prior to death. Animals used for the preparation of purified glutathione *S*-transferases were not fasted prior to death. The livers were perfused *in situ* with 0.9% NaCl solution to remove hemoglobin; microsomes were prepared by the method of Remmer *et al.* (17). Hepatocytes were isolated according to the method of Fry *et al.* (18). The initial cell viability was always greater than 92% as measured by trypan blue dye exclusion; viability following a 1-hr incubation with or without iproniazid was routinely about 82–85%. Glutathione *S*-transferase isozymes AA, A, B, C, and E were purified to homogeneity from Fischer 344 rats and isozymes A and B from Sprague-Dawley rats according to Spearman and Leibman (19). Specific activities with several commonly used substrates were similar to those reported earlier (data not shown; refs. 16 and 19).

*Synthesis of S-(2-propyl)glutathione.* The GSH-isopropyl conjugate was synthesized in a three-step procedure in which the four free carboxyl groups of GSSG (1 g free acid) were methylated according to Watanabe *et al.* (20). This derivative was subsequently converted to the *N*-trifluoroacetyl derivative in methylene chloride as described by Corey *et al.* (21). After washing this methylene chloride solution with saturated NaCl solution (3 × 500 ml), the organic phase was dried over anhydrous $Na_2SO_4$ overnight. The $Na_2SO_4$ was removed by filtration and the derivative was recovered by rotary evaporation (yield, 126 mg). This material was dissolved in dry tetrahydrofuran and placed in a sealed flask under an atmosphere of argon. One equivalent of isopropyl magnesium chloride in ether was added to yield carboxymethylated and *N*-trifluoroacetylated *S*-(2-propyl)glutathione and GSH. The methyl and *N*-trifluoroacetyl groups were removed by vigorously stirring with 10% $Na_2CO_3$ for 2 hr. *S*-(2-Propyl)GSH and GSH were separated by HPLC with a Waters Associates (Milford, MA) liquid chromatograph and a Radial Pak A column in an RCM 100 module. An isocratic system of methanol/glacial acetic acid/water, 2:1:97 (v/v) was utilized at a flow rate of 2 ml/min for 13 min followed by a linear gradient from 2–100% methanol over 2 min. The retention times were: GSH, 3 min; *S*-(2-propyl)GSH, 14.5 min; GSSG, 17 min. The synthetic *S*-(2-propyl)GSH was analyzed by NMR and electron impact mass spectrometry (by direct probe). The proton NMR spectrum of the synthetic standard is shown in Fig. 1. Resonance assignments for individual protons were made according to published literature values (22–24). Decoupling experiments were not performed with this conjugate, but have been

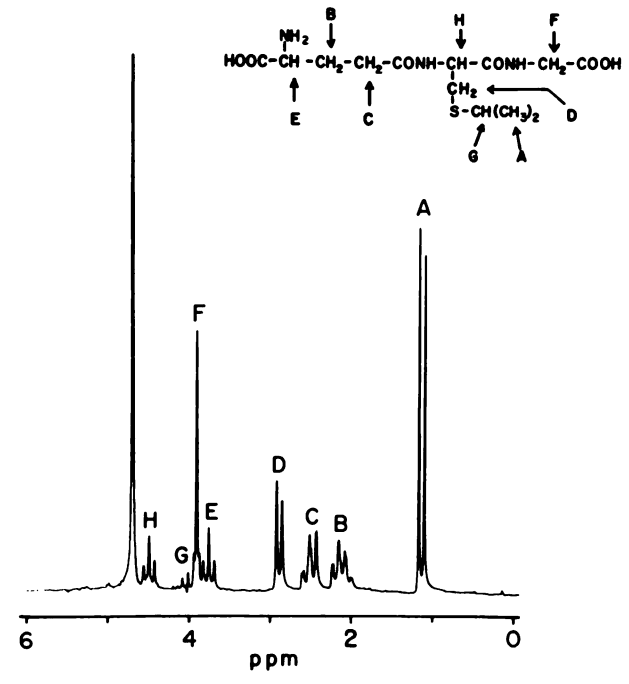


FIG. 1. *NMR spectrum of S-(2-propyl)glutathione*

The proton NMR spectrum of the GSH-isopropyl conjugate is shown with 0 ppm chemical shift corresponding to trimethylsilylpropionic acid. *A–H* correspond to the protons indicated. Spectra were recorded on a JEOL FX 90Q Fourier transform NMR spectrometer with 16 accumulations. The samples were dissolved in $D_2O$. The downfield peak at 4.68 is due to trace amounts of $H_2O$ and HDO.

[2] S. J. Moloney, F. P. Guengerich, and R. A. Prough, submitted.

[3] S. J. Moloney, J. P. Wiebkin, S. W. Cummings, and R. A. Prough, submitted.

[4] The abbreviations used are: GSH and GSSG, glutathione, reduced and oxidized forms; HPLC, high performance liquid chromatography; PB, phenobarbital; BSP, bromosulfophthalein.

previously performed to aid in resonance assignments (22–24). The downfield shift of the methine proton of the isopropyl is similarly seen with the proton adjacent to the sulfur with other GSH conjugates. The electron impact mass spectra of the synthetic standard (carboxymethylated with ethereal diazomethane for 15 min in methanol followed by treatment with 10% HCl in methanol for 2 hr) is shown in Results.

*Analysis of metabolites.* Propane and propylene were analyzed by gas chromatography according to Moloney *et al.*[2] with a 10-foot metal column packed with Porapak-Q in a Hewlett-Packard model 5750B gas chromatograph. Head space analysis was performed by withdrawing a 1-ml volume of gas into an air-tight syringe. The metabolites were quantitated by comparison with known amounts of propane and propylene. The *S*-(2-propyl)GSH was analyzed by HPLC and TLC. With HPLC, the standard and the metabolite co-migrated and were reactive with fluorescamine (indicative of the presence of a primary amine). With TLC, the standard and the metabolite also co-migrated, were reactive with ninhydrin (indicative of the presence of peptide bonds), but were unreactive with 5,5′-dithiobis(2-nitrobenzoic acid), (indicative of the lack of a free sulfhydryl group). Production of *S*-(2-propyl)[$^3$H] GSH was quantitated by TLC on silica gel G TLC plates with a solvent system of 1-butanol/2-propanol/glacial acetic acid/water, 4:3:1:2 (v/v). The $R_F$ values were: *S*-(2-propyl)GSH, 0.50; GSH, 0.34, GSSG, 0.16. The respective areas on the TLC plates were scraped into glass scintillation vials, Budget-Solve was added, and radioactivity was quantitated in a Beckman model LS-230 scintillation counter. Conjugate formation was also quantitated with the HPLC assay described above. This conjugate was found to be stable to freezing, drying, or storage in aqueous media for 18 hr and did not readily decompose to yield hydrocarbons.

*Enzyme assays.* The microsomal reaction mixtures (5 ml) consisted of 0.5 mM NADP, 5 mM DL-sodium isocitrate, 0.8 IU of isocitrate dehydrogenase per ml, 1.5 mM $MgCl_2$, and 0.1 M potassium phosphate (pH 7.5). Experiments designed to measure conjugate formation contained 1 mM [$^3$H]GSH (specific radioactivity, 0.5 $\mu$Ci/$\mu$mol). With incubation mixtures containing the purified glutathione *S*-transferases, the concentrated enzymes were added in a buffer consisting of 100 mM sodium phosphate (pH 7.4), 5 mM GSH, 1 mM EDTA, and 30% glycerol. In experiments with hepatocytes, the isolated cells were routinely preincubated for 60 min in L-15 medium (containing 10% fetal calf serum and 5% tryptose phosphate broth) at a concentration of $10^6$ cells/ml prior to the addition of iproniazid. Gas production was measured by incubation of the reaction mixtures of stoppered 25-ml flasks (hepatocyte experiments) or 10-ml flasks (all other assays). All of the reactions conducted were linear with respect to time and protein concentration.

Potential loss of glutathione *S*-transferase activity, due to binding of a reactive metabolite to the enzyme, was ascertained by assay of cytosol or the purified isozymes with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates in a Cary model 14 spectrophotometer according to Spearman and Leibman (25). Formation of GSSG and GSH was monitored by the HPLC system described above.

*Measurement of protein concentration.* Protein was measured according to the method of Lowry *et al.* (26) with bovine serum albumin as the standard.

## RESULTS

*Effects of thiols on propane and propylene production.* Four thiol compounds, GSH, penicillamine, cysteamine, and dithiothreitol, were studied for their effects on propane and propylene production from microsomal reaction mixtures that contained iproniazid (Fig. 2). With all of the thiols tested, a stimulation of propane production and a depression of propylene production was observed. With GSH and cysteamine, this effect appeared to become maximal at a concentration of about 5 mM. With penicillamine, no apparent maximal amount of stimulation of propane production was noted, although maximum depression of propylene production apparently did occur. With dithiothreitol, higher concentrations of this compound depressed propane production, perhaps due to the interaction of this thiol with microsomal disulfides which may alter metabolism. These results demonstrate that added thiols potentiate metabolism of iproniazid to the saturated hydrocarbon product.

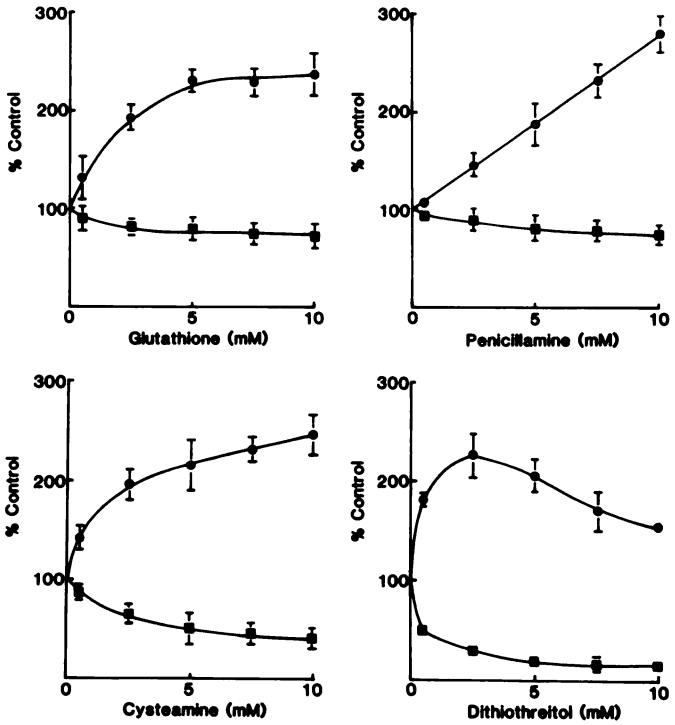


FIG. 2. *Effect of thiols on propane and propylene production from iproniazid*

Propane (●) and propylene (■) production with microsomal protein (1.5 mg/ml) was measured with increasing concentrations of glutathione, penicillamine, cysteamine, and dithiothreitol and are expressed as the per cent control activity (± standard deviation) obtained with iproniazid phosphate (12 mM) and $n = 4$. Reactions were incubated for 10 min at 37°. Control production of propane was 355 ± 25 pmol/min/mg microsomal protein and of propylene was 99 ± 12 pmol/min/mg.

*Effects of cytosol.* Varying concentrations of dialyzed cytosol were added to microsomal reaction mixtures and production of propane and propylene was measured (Fig. 3). Increasing concentrations of cytosol, in the presence of 5 mM GSH, were found to depress both propylene and propane formation by about 45 and 80% compared to the GSH-stimulated rate, respectively. In the absence of added GSH, dialyzed cytosol only slightly stimulated the rate of propylene formation, but did not affect the rate of propane formation. Boiled cytosol, in the presence of added 5 mM GSH, did not affect propane or propylene production beyond the effects noted by the addition of 5 mM GSH alone (data not shown), thus indicating the enzymic nature of the inhibition of hydrocarbon formation.

*Effect of GSH concentration on the cytosolic effects.* Varying concentrations of GSH were added to microsomal incubation mixtures containing dialyzed cytosol (1 mg/ml cytosolic protein) and propane and propylene

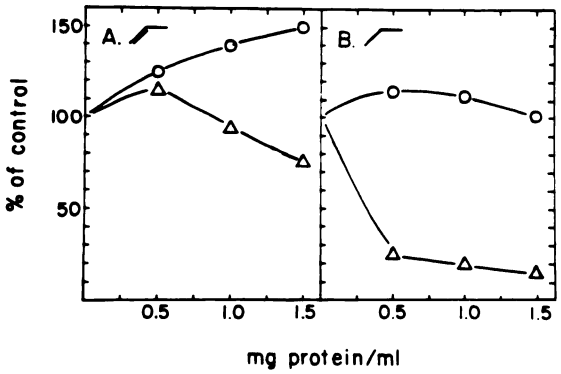


FIG. 3. *Effect of cytosolic protein on hydrocarbon formation*

Increasing concentrations of dialyzed cytosolic protein were added to microsomal reaction mixtures in the absence (O) or presence (Δ) of 5 mM GSH. Propylene (panel A) and propane (panel B) production was measured. The incubation conditions were otherwise identical to those of Fig. 2.

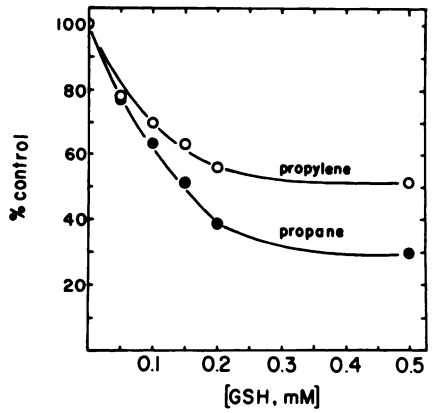


FIG. 4. *Effect of increasing GSH concentrations on hydrocarbon formation*

Increasing concentrations of GSH were added to microsomal incubation systems similar to those in Fig. 3 which contained dialyzed cytosolic protein (1 mg/ml). Propylene (O) and propane (●) production was measured.

were subsequently quantitated. Increasing concentrations of GSH in the presence of cytosol resulted in decreases in both propane (by about 75% at 1 mM) and propylene (by about 45% at 1 mM) production when compared with values seen in the presence of cytosol, but absence of GSH (Fig. 4). With both of these hydrocarbons, this effect was half-maximal at a GSH concentration of approximately 0.1 mM. Habig *et al.* (16, 27) reported that the apparent $K_m$ values for the cytosolic glutathione *S*-transferase isozymes AA, A, B, and C (measured with 1-chloro-2,4-dinitrobenzene as the second substrate) were approximately 0.1–0.2 mM. However, they demonstrated that isozyme E had a $K_m$ value of 2 mM (with 1,2-epoxy-3-(*p*-nitrophenoxy)propane as the second substrate). Thus, these data regarding GSH concentration effects on hydrocarbon production suggested that some of the cytosolic glutathione *S*-transferases might be interacting with a metabolite(s) of iproniazid to alter hydrocarbon production at low concentrations of GSH.

*Effect of purified glutathione S-transferases.* Glutathione *S*-transferase isozymes AA, A, B, C, and E were purified to apparent homogeneity from Fischer 344 rat livers and isozymes A and B were purified from Sprague-Dawley rat livers. Isozymes A and B from either strain were catalytically equivalent when their specific activity was determined with 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene as substrates (data not shown). In addition, the effects of added cytosol or added purified transferases on hydrocarbon production from iproniazid were virtually identical when cytosolic proteins or purified isozymes from either strain (Fischer 344 or Sprague-Dawley) were used (data not shown). Thus, for the subsequent studies, purified isozymes from the Fischer 344 rats were used. Glutathine *S*-transferase isozymes AA and E did not alter propane production from the microsomal reactions in the presence of 5 mM GSH (Fig. 5). Isozyme C had a small effect at an enzyme concentration of 25 μg/ml, but not at 10 μg/ml. However, isozymes A and B had pronounced effects in decreasing propane production (similar to that found with cytosol). A concentration-dependent loss in the rates of propane production occurred with increasing amounts of isozyme A.

*Comparison of cytosol and purified glutathione S-transferase effects.* A mixture of 16.4 μg of isozyme AA, 14.5 μg of A, 113 μg of B, 44.5 μg of C, and 14.8 μg of E per ml is approximately equal to the concentration of glutathione *S*-transferases estimated to exist in 1 mg/ml of PB-induced rat liver cytosol (19, 28–31). These concentrations of purified isozymes were added to the microsomal reaction mixture to determine whether they alone could account for the effect of added cytosol in decreasing the production of propane. In the presence of 5 mM GSH, this mixture of purified isozymes decreased propane production to the same extent as a 1 mg/ml cytosol fraction from PB-induced rats (Fig. 6). Thus, these enzymes appear to account for the decreased yield of propane and propylene found in the presence of GSH and cytosol.

*Metabolism of iproniazid in freshly isolated hepatocytes.* Hepatocytes were isolated and pre-incubated as described in Materials and Methods. Bromosulfophthalein (50 μM), an inhibitor of isozymes A, B, C ($I_{50}$ values of

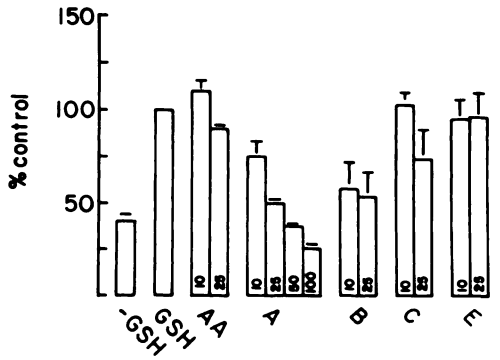


FIG. 5. *Effects of purified glutathione S-transferase isozymes on hydrocarbon formation*

Increasing concentrations of glutathione *S*-transferases AA, B, C, and E (10 or 25 μg/ml) or isozyme A (10, 25, 50, and 100 μg/ml) were added to microsomal reaction mixtures containing 5 mM GSH similar to those described in Fig. 2. Propane production is expressed as per cent activity seen with microsomal reaction mixtures containing 5 mM GSH in the absence of added transferases ± standard deviation. All experiments were performed in duplicate and $n = 3$.

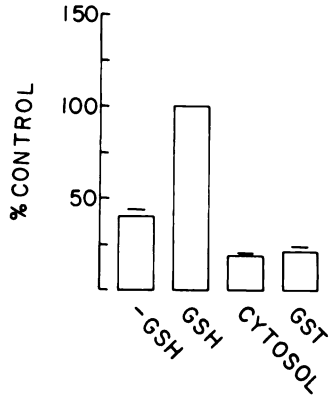


FIG. 6. *Comparison of the effects of cytosol and mixtures of glutathione S-transferase isozymes on propane formation*

The incubation mixtures used were as described in Fig. 3 and contained 5 mM GSH (*GSH*) and either 1 mg/ml cytosol (*CYTOSOL*) or a mixture of glutathione *S*-transferase isozymes (*GST*) were added. All of the values for propane production were expressed relative to the microsomal reaction mixtures containing 5 mM GSH alone. Data are expressed as the per cent control ± standard deviation, $n = 3$.

TABLE 1

*Effect of an inhibitor of glutathione S-transferases on propane and propylene production from iproniazid in isolated rat hepatocytes*

| Condition | Metabolic rate[a] | |
|---|---|---|
| | Propane | Propylene |
| Control | 2.3 ± 0.8 | 1.2 ± 0.2 |
| BSP (50 μM) | 4.5 ± 1.4 (196)[b] | 2.1 ± 0.4 (175) |

[a] The rates are expressed as nanomoles of hydrocarbon produced per $10^6$ cells per 60 min ± standard deviation. $n = 6$ with values determined in duplicate.

[b] The values in parentheses are the per cent increases in hydrocarbon production due to the presence of bromosulfophthalein.

10, 10, and 6 μM, respectively; ref. 32), was preincubated with the cells for 5 min. Iproniazid was added, the cell suspension was incubated for 60 min, and hydrocarbon production was measured. Inhibition of isozymes A, B, and C would be expected to result in increased propane and propylene formation. Indeed, the data in Table 1 indicate that in experiments with isolated hepatocytes, inhibition of these isozymes with BSP resulted in an approximate doubling of propane and propylene formation. This concentration of BSP had no effect on hydrocarbon production from microsomal incubation mixtures containing NADPH and iproniazid in the absence of cytosol (data not shown). Thus, the reactions characterized in this more intact system, the hepatocyte, were similar to those noted in the *in vitro* systems involving microsomes, GSH, and cytosol or purified glutathione *S*-transferases.

*Formation of S-(2-propyl)glutathione conjugates in vitro.* Iproniazid was incubated with [$^3$H]GSH in the presence of microsomes and cytosolic protein as described earlier. Hydrocarbon production was quantitated and the reaction mixture was placed on ice at the end of the incubation period. A portion of this incubation mixture was then applied to silica gel G TLC plates and the chromatogram was developed. Alternatively, the reaction mixture was mixed with an equal volume of acetone to precipitate protein and was centrifuged, and the aqueous phase was reduced to the original volume under a stream of nitrogen prior to injection onto the Radial Pak A ($C_{18}$) HPLC column (Fig. 7). With both the TLC and HPLC systems, a metabolite containing tritium radioactivity was found to co-migrate with the synthetic *S*-(2-propyl)GSH standard. This metabolite required the presence of iproniazid, microsomal protein, cytosolic protein, and NADPH for its formation. In contrast with results of Nelson *et al.* (14) there appeared to be little or no conjugate formed from iproniazid in the absence of cytosolic protein. Further, isopropylhydrazine did not form significant amounts of conjugate in the presence of cytosolic protein and GSH (data not shown). The GSH conjugate formed from this reaction was collected from the HPLC effluent, dried by rotary evaporation, carboxymethylated as described for the synthetic standard in Materials and Methods, and analyzed by mass spectrometry. The results of analysis of both the synthetic and biologically generated materials yielded similar spectra which are shown in Fig. 8. A molecular ion of 377 was found corresponding to carboxymethylated *S*-(2-propyl)GSH. Also found were ions at 362 (loss of 15, $CH_3$), 318 (loss of 59, $COOCH_3$), 302 [loss of 75, S-$CH(CH_3)_2$], and 287 [loss of 90, $CH_3$-S-$CH(CH_3)_2$].

The rates of hydrocarbon production and rate of conjugate formation in the presence and the absence of added cytosol are shown in Table 2. The results obtained from TLC and HPLC experiments are in close agreement. Loss of propane and propylene in the presence of added cytosol and GSH accounted for approximately 55% of the amount of conjugate produced in the presence of GSH. These results may reflect the ability of the glutathione *S*-transferases to shift the metabolic pathways away from several other pathways, including those that can yield hydrocarbon, those resulting in protein covalent binding (which was not quantitated), and those leading to other unmeasured metabolites.

*Glutathione S-transferase binding and formation of oxidized glutathione.* Experiments performed in the presence of microsomes, GSH, NADPH, and cytosol or pur-

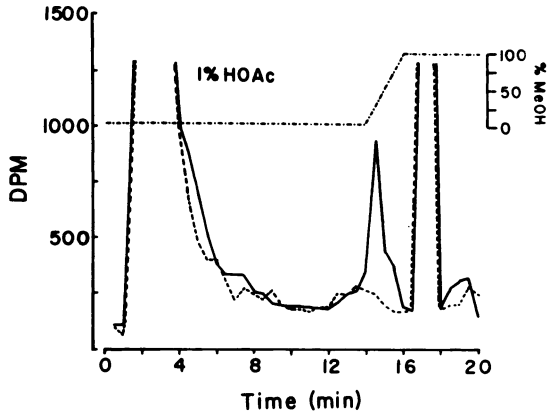


FIG. 7. *Elution profile of radioactivity from microsomal reaction mixtures in the absence and presence of cytosol*

The reactions contained [$^3$H]GSH/GSH (1 mM) and the microsomal reaction conditions were as described in Fig. 3. ---, no cytosol added; ——, cytosol added.

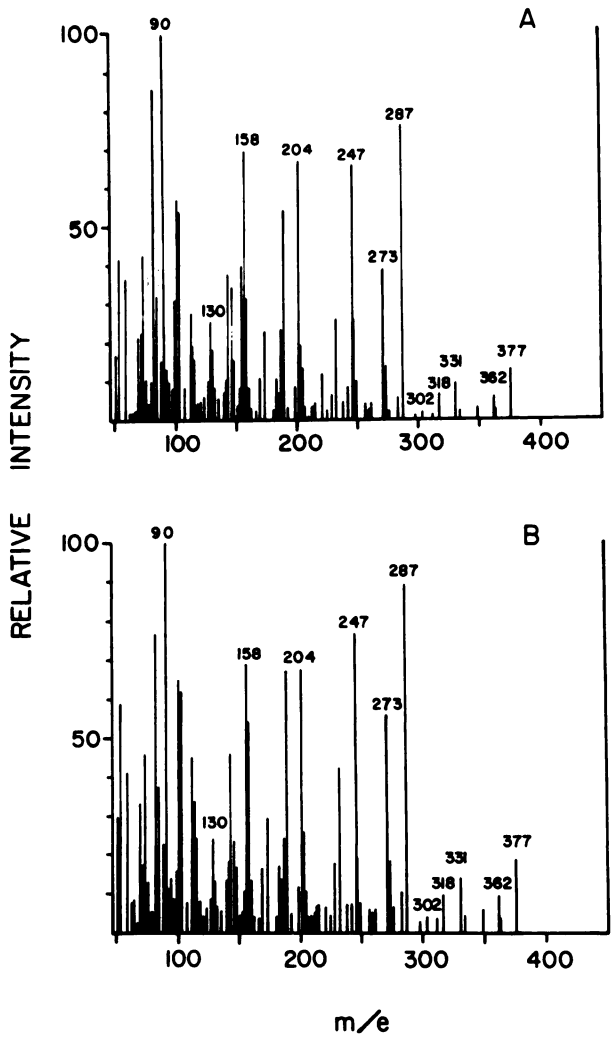


FIG. 8. *Mass spectra of synthetic (A) and biologically produced (B) S-(2-propyl)glutathione*

The mass spectra were recorded with the solid samples on the probe in a Finnigan automated electron impact/chemical ionization GC/MS system coupled to an INCOS data system. Analyses were performed at 69 eV with an ionizer temperature of 250° while the probe was heated from ambient to 320°.

TABLE 2

*Comparison of hydrocarbon loss and conjugate formation in the presence of cytosol*

| Experiment | Analytical technique | Metabolic rate[a] | |
|---|---|---|---|
| | | Hydrocarbon loss[b] | Conjugate formation |
| 1 | TLC | 0.39 | 0.69 |
| 2 | TLC | 0.45 | 0.80 |
| | HPLC | 0.45 | 0.76 |
| 3 | HPLC | 0.36 | 0.78 |

[a] The specific activities shown are expressed as nanomoles of product formed per min per mg of microsomal protein determined in duplicate. GSH concentration used was 1 mM.

[b] Measured by head space analysis with gas chromatography.

ified isozymes A or B (25 μg/ml) failed to show any alteration in glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene caused by incubation of these enzymes during the microsomal metabolism of iproniazid (data not shown). Although previous work (33–35) has indicated that binding to the transferases may occur at sites other than the active conjugating site, binding to these allosteric sites has been shown to alter catalytic activity. If the mechanism of these enzymes in decreasing propane and propylene production was due to binding of reactive metabolites, altered enzyme activity should have been noted.

Production of GSSG from an azo ester metabolite, as suggested by Kosower and Kosower (36), was also examined. There appeared to be no substantial differences in the content of GSSG in microsomal reaction mixtures in the presence or absence of cytosol (data not shown). Due to the presence or glutathione reductase in the cytosol, the experiment was also repeated using the mixture of purified glutathione *S*-transferase isozymes. There was no difference between the GSSG contents of reaction mixtures with or without iproniazid (data not shown). However, the variability in GSSG formation seen with microsomal reaction mixtures in the absence of added cytosol or transferase isozymes was nearly as large as the small amount of GSSG that would have resulted from the chemical oxidation in the presence of added purified isozymes or cytosol. Thus, the effects of cytosol or of the purified isozymes on hydrocarbon production appeared not to be due to the reduction of azo ester compound back to the hydrazide with subsequent formation of GSSG.

## DISCUSSION

Hydrazine oxidation to azo and diazene intermediates has previously been shown to be an important reaction in the initial metabolism of these toxic compounds (9, 10, 37, 38). Ethylhydrazine oxidation by rat liver microsomes (39) and iproniazid oxidation by horseradish peroxidase and prostaglandin synthetase (12) have each been shown to yield carbon-centered alkyl free radicals. Thus, hydrazine and hydrazide metabolism results initially in the formation of azo or diazene intermediates which can decompose to form carbon-centered free radicals.

The isopropyl free-radical that could result from iproniazid metabolism may chemically abstract a hydrogen atom from GSH to form propane. GSH is known to be one source of hydrogen atoms for abstraction by free radicals (40), although other fates such as interaction with microsomal proteins or lipids may also occur (41). Indeed, earlier work by Nelson *et al.* (14) has indicated that exogenously added thiols decreased protein covalent binding of the isopropyl moiety of iproniazid. Results presented here have shown that thiols, when added to microsomal reaction mixtures in the absence of added cytosol, potentiate propane formation. More potent thiols, such as dithiothreitol or cysteamine, increased propane and decreased propylene similarly, indicating that a common intermediate, such as an azo ester, may exist in the formation of the two products (Fig. 9). The

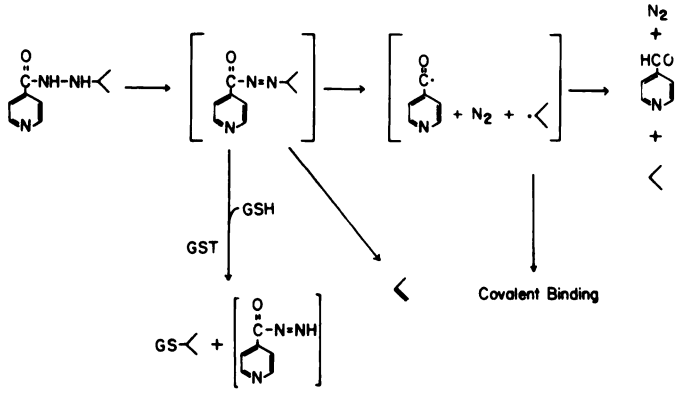


FIG. 9. *Proposed reaction scheme for the metabolism of iproniazid GST*, mixture of GSH *S*-transferase isozymes; *GS*, glutathione.

propane and propylene may thus result from chemical decomposition of the unstable diazene intermediate. GSH apparently serves specifically as a chemical source of hydrogen atoms for abstraction by the isopropyl free radical to yield propane. Propylene production was affected less by GSH than was propane production, suggesting that it may arise directly from decomposition of the azo ester (Fig. 9). The origin of propylene is as yet not clearly defined.

In addition, cytosolic enzymes may interact with this putative azo ester derivative of iproniazid. Glutathione *S*-transferases A and B apparently accounted for most, if not all, of the effects of cytosolic protein. The consequences of the interaction of the enzymes with a microsomal metabolite, in the presence of GSH, were the enzymatic formation of a GSH conjugate (*S*-(2-propyl)glutathione), a decrease in hydrocarbon formation, or presumably a decrease in protein covalent binding as indicated by Nelson *et al.* (14). This reaction occurred in both the intact isolated hepatocyte as well as the reconstituted systems of GSH, NADPH, and microsomes plus cytosol or purified glutathione *S*-transferases. Our results appear to be the first report which demonstrates that a hydrazine metabolite can serve as a substrate for the glutathione *S*-transferases. In conclusion, if GSH levels are decreased or if enzymic function of the cytosolic glutathione *S*-transferase is inhibited, increased toxicity from iproniazid might be expected to occur due to the formation of chemically reactive species during metabolism of this therapeutic hydrazide.

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