# APPLICATION OF CHEMICAL IONIZATION MASS SPECTROMETRY AND THE TWIN-ION TECHNIQUE TO BETTER DEFINE A MECHANISM IN ACETYLHYDRAZINE TOXICITY

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SUMMARY - Chemical ionization mass spectrometry was used to show that N-acetylcysteine is formed in microsomal incubations containing NADPH, oxygen, cysteine and acetylhydrazine. The twin-ion technique was used to compare the ratio of hydrogen and deuterium in the N-acetylcysteine product with the initial ratio in a mixture of acetylhydrazine-trideuteroacetylhydrazine used as substrate. The results showed that the entire acetyl group was trapped by cysteine, thereby eliminating ketene as the reactive acylating agent formed during the oxidation of acetylhydrazine by liver microsomes.

Hydrazines are an important class of chemical compounds, some of which are used as therapeutic agents, as rocket fuels and as herbicides. Many of these compounds produce toxicities including hemolysis, fatty liver, mutagenesis, and carcinogenesis (1,2,3). In recent studies (4,5,6) we have found evidence that acetylhydrazine is a toxic metabolite of isoniazid and may be responsible for the serious hepatitis that has led to curtailment of the use of the drug in the prophylactic treatment of tuberculosis (7).

In experimental animals acylation of hepatic macromolecules and acute hepatic necrosis occur after the administration of [$^{14}$C]-acetylhydrazine. Both necrosis and acylation were potentiated by pretreatment of animals with phenobarbital and prevented by inhibitors of microsomal metabolism such as cobalt chloride. Studies in vitro with rat and human liver microsomes indicated that acetylhydrazine was oxidized by a microsomal cytochrome P-450 enzyme to produce the reactive acylating agent (8). The electrophilic nature of the reactive intermediate was shown by trapping experiments with the nucleophilic

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sulfhydryl-containing amino acid cysteine, which decreased the in vitro covalent binding of acetylhydrazine to microsomes.

To better define the nature of the reactive intermediate, we have studied the structure of possible adducts formed between cysteine and the reactive intermediate. Stable isotope labeling, combined with a chemical ionization mass spectrometry twin-ion technique, was used to establish the structure of the cysteine adduct. In addition, this technique ruled out ketene as the reactive acylating agent formed by microsomal oxidation of acetylhydrazine.

## MATERIALS AND METHODS

Synthesis. [$^{14}$C]-Acetylhydrazine was prepared as the fumarate salt by a transacetylation reaction. [$^{14}$C]-Carboxyl-labeled ethylacetate (Amersham-Searle) was diluted with ethylacetate to give 2 mmoles of ethylacetate with an activity of 1 mCi/mmole. This was refluxed for 6 hr in 2 ml of ethanol with 2.2 mmoles of hydrazine hydrate (85% solution, Fischer). Fumaric acid (1.1 mmoles) was added to the solution and the fumarate salt precipitated upon cooling. The product was recrystallized to constant specific activity from ethanol and then from methanol:ether, m.p. 122-123°C. Radiochemical purity > 99% was confirmed by thin-layer chromatography on Avicel developed in 4:1:1 n-butanol: ethanol:0.4 N ammonium hydroxide, $R_f$ = 0.49, and by gas liquid chromatography of the free hydrazine on 10% OV-17 (Gas Chrom. Q, 100-120 mesh, 6 ft. x 1/4 in. O.D. glass column, retention time 2.8 min) in a Perkin-Elmer 900 series gas chromatograph ($N_2$ carrier flow, 30 ml/min; hydrogen and air adjusted to give maximal detector response).

Trideuteroacetylhydrazine was prepared as the hydrochloride salt by the following reaction sequence. A solution of 2.0 mmoles of tertiary-butyl-carbazate (Aldrich) in a slurry of 5 ml of methylene chloride containing 2.5 mmoles of sodium carbonate, was acetylated with 2.1 mmoles of $d_3$-acetic anhydride (Stohler Isotopes). After 4 hr at room temperature the reaction mixture was washed with 2 ml of water and the methylene chloride was dried over anhydrous magnesium sulfate. After rotary evaporation of the solvent under reduced pressure, the product, $N^2$-$d_3$-acetyl-tertiary-butylcarbazate, was recrystallized from hexane and subsequently hydrolyzed in 2 ml of 2 N HCl at room temperature. After 20 min the acidic solution was lyophilized to yield a hygroscopic crystalline mass that was recrystallized from methanol:ether to give white rhombic crystals, m.p. 131-133°C. Electron impact mass spectrometry on a V.G. Micro Mass 16F instrument (accelerating voltage, 4 kV; ionization current, 100 μA; electron energy, 70 eV; ion source temperature, 200°C) showed 84% $d_3$, 15% $d_2$, and 1% $d_1$-acetylhydrazine.

Trapping experiments. Liver microsomes were obtained from male Fischer rats (180-200 g) as previously described (9). The incubation mixture, in a total volume of 3.0 ml, contained 2 mg/ml of microsomal protein and the following concentrations of buffer and substrate: 20 mM phosphate buffer (pH 7.4), 75 mM KCl, and 1 mM acetylhydrazine (approximately equimolar mixture of deuterated acetylhydrazine and radiolabeled acetylhydrazine containing 1 μCi of $^{14}$C-activity). Reactions were carried out at 37°C in air in a Dubnoff shaking incubator for 15 min, during which time the reaction rate remained constant.

Four sets of incubations were carried out. One set contained substrate and an NADPH-generating system consisting of 4 units of glucose-6-phosphate dehydrogenase and concentrations of 0.83 mM NADP, 10 mM $MgCl_2$, and 20 mM glucose-6-phosphate per 3 ml incubation. Another set contained substrate, NADPH-generating system and cysteine (1 mM). A control set contained cysteine and substrate but lacked the NADPH-generating system in order to demonstrate that no direct acetylation of cysteine occurred. The final set of incubations contained substrate and microsomes but no NADPH-generating system in order to determine the amount of nonspecific binding of radiolabel to microsomes. Reactions were started by transferring incubation vessels from an ice bath to the incubator and were stopped by the addition of 4 ml of ice-cold methanol. Covalent binding of radiolabel was determined as previously described (9).

Cysteine conjugates were isolated by the following procedure. The supernatants from 10 replicates of each set of incubations were combined and evaporated under a stream of nitrogen to remove methanol and then lyophilized to remove water and most of the excess acetylhydrazine. The residues were dissolved in 1 ml of water and applied to a Sephadex G-10 column (1.5 x 90 cm) equilibrated previously with deionized water. Elution was performed with deionized water in a cold room using a Buchler polystaltic pump adjusted to give a flow rate of 18 ml/hr. Two ml fractions were collected. Approximately 50% of the radioactivity applied to the column was eluted in fractions 54-58 from those incubations containing microsomes, NADPH-generating system, substrate and cysteine, whereas almost no radioactivity was found in fractions from the other three sets of incubations that lacked the NADPH-generating system or cysteine.

Fractions 54-58 were combined and lyophilized to yield a small amount of residue. This was applied to 5 ml column of BioRad AG 1-X4 (200-400 mesh. $Cl^-$ Form) and eluted with 10 ml water, followed by 5 ml of 0.5 N acetic acid, 5 ml 1 N acetic acid and 5 ml of 2 N acetic acid; all the elution solvents contained 1% dithiodiglycol to prevent oxidation of cysteine derivatives. Approximately 80% of the radioactivity was recovered in the first ml of the 2 N acetic acid eluate. This fraction was lyophilized and purified further by thin-layer chromatography on 500 μ Avicel F plates developed with 100% n-propanol. Approximately 90% of the activity was recovered by scraping a band ($R_f$ 0.85-0.95) from the region corresponding to that of authentic N-acetylcysteine ($R_f$ 0.89) and eluting the radiolabeled material with 10 ml of anhydrous methanol. The eluate was carefully evaporated under a stream of nitrogen, and the residue was dissolved in 10 μl of glass-distilled ethylacetate and subjected to chemical ionization mass spectrometry.

Mass spectrometry. Chemical ionization mass spectrometry was performed by direct insertion probe on a VG Micro Mass 16F instrument at an accelerating voltage of 4 kV, an electron energy of 100 eV, and an ionizing current of 200 μA. Ion source pressure was ~0.3 Torr (isobutane reactant gas) and the temperature was 200°C. Selected ion monitoring was carried out by focusing the spectrometer on the quasimolecular ions at m/e 75 and 78 of the acetylhydrazine substrate mixture and m/e 164 and 167 of the isolated N-acetylcysteine.

## RESULTS

Previous work has shown that acetylhydrazine and other monosubstituted hydrazine derivatives are oxidized by a microsomal P-450 enzyme to reactive intermediates that covalently bind to tissue macromolecules (8). The present experiments with a mixture of acetylhydrazine and trideuteroacetylhydrazine

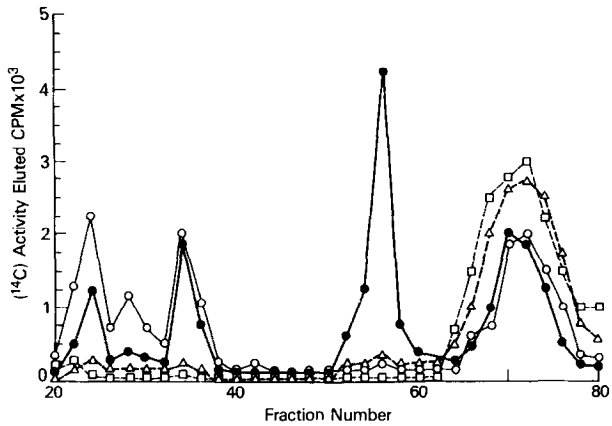


Fig. 1. The elution pattern of radioactivity from Sephadex G-10 of lyophilates of the supernatant from microsomal incubations of $^{14}$C-acetylhydrazine containing 1) NADPH cofactor and cysteine ( ● ), 2) NADPH cofactor ( O ), 3) cysteine ( Δ ), and 4) only microsomes and substrate ( □ ). Each fraction represents two ml of eluate. For ease of reading only every other fraction is plotted.

provided quantitatively similar results on the covalent binding reaction. Incubation mixtures containing microsomes, substrate, and NADPH gave covalent binding values of 0.495 nmoles/mg/15 min. Those reaction mixtures containing microsomes, substrate, NADPH and cysteine showed greatly reduced binding levels of 0.246 nmoles/mg/15 min. Control incubations lacking NADPH showed insignificant amounts of covalent binding either alone (0.034 nmoles/mg/15 min) or when cysteine was included (0.020 nmoles/mg/15 min).

Elution profiles from Sephadex G-10 of the lyophilized supernatants from the four incubation groups are shown in Fig. 1. A major new peak of radioactivity (fractions 52-60) was found only in those incubations containing enzyme, NADPH, substrate and cysteine. Further purification by ion exchange chromatography and thin-layer chromatography yielded a compound with chromatographic characteristics identical to those of N-acetylcysteine.

A chemical ionization mass spectrum of a sample of the purified material (Fig. 2B) was identical to N-acetylcysteine except for the isotope doublets. The spectrum shows the base peak to be the quasimolecular ion (QM+) at m/e 164

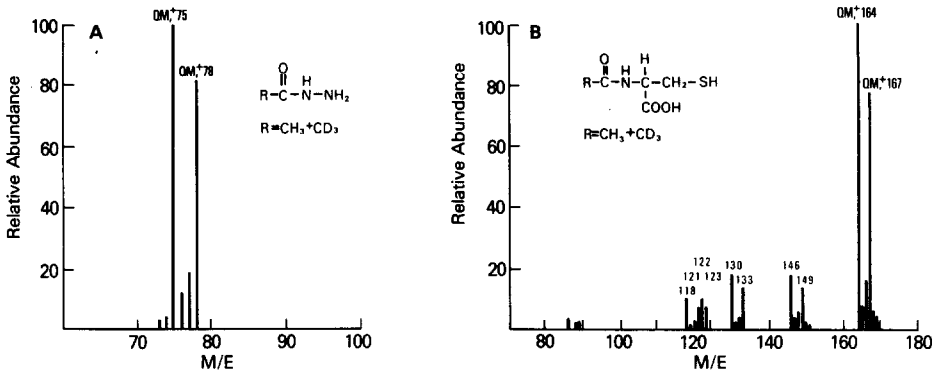


Fig. 2. Chemical ionization mass spectra (isobutane reagent gas) of a sample of the substrate mixture of acetylhydrazine and trideuteroacetylhydrazine (A) and of the cysteine adduct isolated from a microsomal incubation containing the substrate mixture, NADPH cofactor and cysteine (B). Spectrum B is identical to a spectrum of N-acetylcysteine run under the same conditions, except for the deuterium doublets.

and 167 for N-acetylcysteine and its trideuterated analog. Other ions are found at m/e 146 and 149 (QM+-$H_2O$), m/e 130 and 133 (QM+-$H_2S$), m/e 122 (QM+ of $d_3$-N-acetylcysteine less ketene), m/e 123 (QM+ of N-acetylcysteine less $d_2$-ketene), and m/e 118 and 121 (QM+ - $COOH_2$). A metastable peak (not shown) was found centered approximately at m/e 90.7 in the X3 spectrum representing the loss of ketene from the quasimolecular ions. A chemical ionization mass spectrum of a sample of the acetylhydrazine substrate (Fig. 2A) shows quasimolecular ions at m/e 75 and 78. Comparison of spectra 2A and 2B indicates that deuterium was fully retained in the product N-acetylcysteine. This was confirmed by selected ion monitoring of the quasimolecular ions in substrate and product, because H/D ratios were 1.25 for acetylcysteine and 1.31 for acetylhydrazine (based on peak heights).

DISCUSSION

From these studies we postulate the reaction sequence outlined in Fig. 3 for the oxidation of acetylhydrazine. The hydrazine is oxidized by a microsomal cytochrome P-450 enzyme to an N-hydroxyhydrazine that dehydrates to

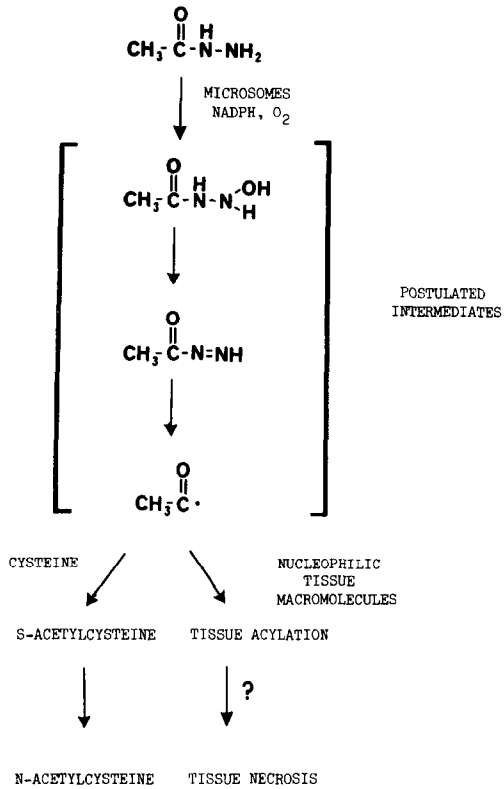


Fig. 3. Postulated reaction scheme for the metabolic activation of acetylhydrazine and trapping of a reactive intermediate with cysteine.

produce acetyldiazene. Although acetyldiazene could be the reactive species, monosubstituted diazenes are known to fragment rapidly in the presence of oxygen, most likely to radicals (10). The reactive acetyl radical could then acylate tissue macromolecules. An alternate nucleophile, such as cysteine, can trap the electrophilic intermediate, thus producing an acetylated cysteine derivative. The ions of m/e 130 and 133 in the mass spectrum of the product mixture (2B) indicate the loss of $H_2S$ from the quasimolecular ions, thereby excluding S-acetylcysteine as the isolated derivative. The work of Smith and Gorin (11), which showed that S-acetylcysteine rearranges rapidly at neutral pH values to N-acetylcysteine, suggests that the initial product might have been S-acetylcysteine which subsequently rearranged to the observed product, N-acetylcysteine.

Another mechanism that can be postulated is a second oxidation of the diazene to form a diazohydroxide. This intermediate would be similar to that envisioned by Magee and Barnes (12) for carcinogenic nitrosamines and by Preussman (13) and Druckrey (2) for activating 1,2-dialkylhydrazines. Although this mechanism seems reasonable for the activation of nitrosamines and 1,2-disubstituted hydrazines, monosubstituted hydrazines form much less stable diazenes (10). The results obtained in the present work combined with our studies showing correlations between propane formation and binding of an isopropylhydrazine metabolite to tissue molecules (8) suggests to us that radicals are the proximate reactive species for these monoalkylhydrazines.

The twin-ion technique has proven to be a useful tool when applied to biotransformation studies. Hammar and Holmstedt (14) used the naturally occurring isotopic doublet of chlorine to study the metabolism of chlorpromazine. Morfin et al. (15) were the first to use stable isotope standards in studies of the pathways of testosterone metabolism; several other stable isotope studies have followed (16,17,18).

Recently, Pohl et al. (19) coupled the twin-ion technique with chemical ionization mass spectrometry to determine a new pathway of warfarin metabolism. In the present studies this technique again proved useful because no derivatization was required to obtain good spectra by chemical ionization mass spectrometry of N-acetylcysteine, the product formed by trapping the reactive acylating intermediate with cysteine. As pointed out by Milne et al. (20), the chemical ionization mass spectra of amino acids are highly diagnostic, with most fragmentations arising from quasimolecular ions. The isotopic doublet formed by these ions in the chemical ionization mass spectra of a mixture of acetylhydrazine and trideuteroacetylhydrazine and in the spectra of the N-acetylcysteine product has made it possible to determine that: 1) N-acetylcysteine is formed in incubation mixtures containing acetylhydrazine, liver microsomes, NADPH and cysteine. 2) The acetyl group in the product arises from the substrate acetylhydrazine without apparent loss of deuterium, which eliminates ketene as the reactive acylating intermediate.

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