# Inhibition of *N*-Nitrosodimethylamine Demethylase in Rat and Human Liver Microsomes by Isothiocyanates and Their Glutathione, L-Cysteine, and *N*-Acetyl-L-cysteine Conjugates[1]

Ding Jiao,[†] C. Clifford Conaway,[†] Mong-Heng Wang,[‡] Chung S. Yang,[‡] Werner Koehl,[†,§] and Fung-Lung Chung*[,†]

*Division of Carcinogenesis and Molecular Epidemiology, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595, and Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, New Jersey 08854*

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Natural and synthetic isothiocyanates and their conjugates were examined for their inhibitory effects toward rat and human liver microsomal *N*-dimethylnitrosoamine demethylase (NDMAd) activity using a radiometric NDMAd assay. Substrate concentrations of 30 and 60 $\mu$M were used to probe the activity of cytochrome P4502E1 isozyme through the α-hydroxylation of NDMA. It was found that alkyl isothiocyanates such as sulforaphane and allyl isothiocyanate displayed very weak inhibition, whereas the arylalkyl isothiocyanates such as benzyl and phenethyl isothiocyanate showed significant inhibition toward rat liver NDMAd activity with $IC_{50}$ values of 9.0 and 8.3 $\mu$M, respectively. More interestingly, glutathione conjugates of benzyl, phenethyl, and 6-phenylhexyl isothiocyanates all inhibited NDMAd at the comparable concentrations. In the phenethyl isothiocyanate conjugates series, there exist marked differences in their inhibitory activity; *i.e.*, its conjugates with L-cysteine ($IC_{50}$ = 4.3 $\mu$M) and with glutathione ($IC_{50}$ = 4.0 $\mu$M) are more potent than its conjugate of *N*-acetylcysteine ($IC_{50}$ = 24.0 $\mu$M). The same trend was also observed for the human liver microsomal NDMAd activity. The half-lives of these conjugates were determined in the presence of other free thiols from L-cysteine or glutathione using an HPLC system. It was shown that isothiocyanates are released from their conjugates and react with the free thiols present in the solution. The longer half-life of *N*-acetylcysteine conjugate of phenethyl isothiocyanate as compared to the other conjugates is consistent with its lower inhibitory activity. The inhibition of NDMAd, and therefore cytochrome P4502E1, by isothiocyanate conjugates is most likely due to the action of the free isothiocyanates released from the conjugates. Since cytochrome P4502E1 and other isozymes play important roles in the activation of the tobacco-specific nitrosoamines, these results provide a basis for investigating the potential of isothiocyanate conjugates as chemopreventive agents.

## Introduction

Organic isothiocyanates from natural sources have been shown to inhibit tumorigenesis induced by environmental carcinogens such as polycyclic aromatic hydrocarbons and nitrosamines in rodents (*1*−*6*). Mechanistic studies of the inhibitory effects of arylalkyl isothiocyanates against lung tumorigenesis induced by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK),[2] showed that arylalkyl isothiocyanates are potent competitive inhibitors of mouse and rat P450s responsible for metabolic activation of NNK to methylating and pyridyloxobutylating species (*7*−*10*). Inhibition of metabolic activation of NNK reduces the level of DNA methylation and pyridyloxobutylation and, consequently, decreases the number of neoplasms in the lungs of the NNK-treated A/J mice and F344 rat (*3*, *7*). Previous studies from this laboratory showed that benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) decreased metabolic activation of *N*-nitrosodimethylamine (NDMA) and the NDMA-induced methylation of hepatic DNA in rats (*11*). NDMA, a widely-occurring environmental carcinogen, induces liver and lung cancer in rat and A/J mice, respectively (*12*, *13*). The α-hydroxylation of NDMA at a low concentration to generate the methanediazonium ion is primarily catalyzed by microsomal P4502E1 (*14*). PEITC is a competitive inhibitor and also a suicidal inhibitor of this reaction (*15*).

In this study, we examined several natural isothiocyanates for their activities in the inhibition of NDMA metabolism (structures in Chart 1). Allyl isothiocyanate (AITC), sulforaphane (SFO), BITC, and PEITC are of particular interest because they are found in various cruciferous vegetables consumed by humans (*4*). Furthermore, glutathione (GSH) conjugation is a major

* To whom correspondence should be addressed.
† American Health Foundation.
‡ Rutgers University.
§ Present address: Werner Koehl, Department of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, 67663 Kaiserslautern, Germany.
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[2] Abbreviations: NDMA, *N*,*N*-dimethylnitrosamine; NDMAd, *N*,*N*-dimethylnitrosamine demethylase; P450, cytochrome P450; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; SFO, (−)-1-isothiocyanato-4(*R*)-(methylsulfinyl)butane (sulforaphane); AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; PHITC, 6-phenylhexyl isothiocyanate; NAC, *N*-acetyl-L-cysteine; GSH, glutathione, SAR, structure−activity relationship.

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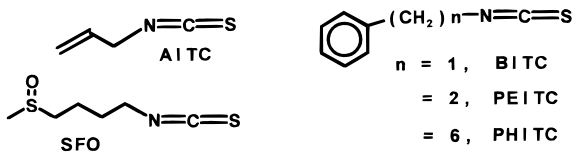
**Chart 1. Structures of the Isothiocyanates Studied**




metabolic pathway of isothiocyanates in rodents and humans since more than half of isothiocyanates administered were excreted as the *N*-acetylcysteine (NAC) conjugates in the urine (*16*−*19*). It is plausible that the GSH and L-Cys conjugates of isothiocyanates may be involved in the inhibition of P450s *in vivo*. To examine this possibility, the GSH, Cys, and NAC conjugates of several isothiocyanates were synthesized and structurally characterized. They were assayed for their inhibitory activities toward hepatic microsomal *N*-nitrosodimethylamine demethylase (NDMAd) *in vitro*. We also studied the exchange of isothiocyanate conjugate among thiol groups as a plausible mechanism for enzyme inhibition by the conjugates.

## Materials and Methods

**Instruments.** A Waters HPLC system (Waters Corp., Milford, MA) equipped with an automated gradient controller, two Model 501 pumps, and a Waters 990 photodiode array detector was used for the analysis. The 1D and 2D NMR spectra were recorded on a Bruker AM 360 MHz NMR spectrometer (Bruker Instruments Inc., Billerica, MA). A Finnigan MAT TSQ700 mass spectrometer (Finnigan Corp., San Jose, CA) was used to record electrospray mass spectra of all isothiocyanate conjugates.

**Chemicals**. AITC, BITC, and PEITC of the highest purity were purchased from Aldrich Chemical Co. (Milwaukee, WI). SFO was custom synthesized by LKT Laboratories, Inc. (St. Paul, MN). NDMA, $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, Cys, NAC, and GSH were from Sigma Chemical Co. (St. Louis, MO). [*methyl*-$^{14}$C]-*N*-Nitrosodimethylamine (49 mCi/mmol) was custom synthesized by SRI International Co. (Menlo Park, CA). The labeled NDMA was purified before use by passing through a chromatography column filled with anion exchange resin Dowex 1 (200−400 mesh, Sigma Chemical Co., St. Louis, MO). All other chemicals from commercial sources were reagent grade.

The GSH conjugates of AITC, BITC, PEITC, and PHITC and the Cys and NAC conjugates of PEITC were synthesized using published procedures (*16*, *20*). The GSH conjugate of SFO was not prepared due to a limited amount of SFO available. The purity of products was established by thin layer liquid chromatography (TLC) and reversed-phase HPLC. A single spot was observed under UV and after ninhydrin spray of TLC plates (Silica gel GHLF, 250 $\mu$m, 10 × 20 cm, purchased from Analtech, Inc., Newark, DE). Molecular weights and structures of the conjugates were confirmed by electrospray ionization MS and 1D and 2D NMR spectroscopy. For NMR measurements, the samples were dissolved in DMSO-$d_6$, and 2D COSY spectra were recorded with 1K data size and a total of 256 experiments each with 32 scans (*21*, *22*). Unambiguous NMR spectral assignments were made using 1D and 2D spectra. Molecular formula, weights, and capacity factors ($k'$) of all conjugates are summarized in Table 1. Their $^1$H-NMR spectral assignments are listed in Table 2.

**Hazardous Materials:** *NDMA is a volatile carcinogen. Precautions were taken when using radioactive compounds and carcinogens, e.g., wearing double disposable gloves, performing all experiments under a fume hood, and disposing waste according to appropriate radiation safety guidelines. In the handling of human tissues, proper precautions and safety procedures were exercised to avoid personnel contamination by infectious agents (23).*

**Table 1. Molecular Formula, Mass, and HPLC Capacity Factors ($k'$) of Isothiocyanate Conjugates**

| | formula | mass calcd | mass obsd[a] | HPLC $k'$ [b] |
|---|---|---|---|---|
| AITC-GSH | $C_{14}H_{22}N_4O_6S_2$ | 406.48 | 407.0 | 1.09 |
| BITC-GSH | $C_{18}H_{24}N_4O_6S_2$ | 456.54 | 457.2 | 3.33 |
| PEITC-GSH | $C_{19}H_{26}N_4O_6S_2$ | 470.57 | 471.3 | 4.15 |
| PHITC-GSH | $C_{23}H_{34}N_4O_6S_2$ | 526.68 | 527.3 | 7.76 |
| PEITC-Cys | $C_{12}H_{16}N_2O_2S_2$ | 284.40 | 285.1 | 4.32 |
| PEITC-NAC | $C_{14}H_{18}N_2O_3S_2$ | 326.40 | 327.0 | 6.24 |

[a] The molecular mass was determined by using an electrospray ionization MS spectrometry. [b] The capacity factor was calculated as $k' = (t_R - t_0)/t_0$, where $t_0$ is the retention time of the unretained peak (formaldehyde was used), and $t_R$ is the retention time of the conjugate. The compounds were eluted with a gradient of mobile phase (20% to 50% of acetonitrile over 30 min) at 1 mL/min.

**Microsomes.** Male F344 rats were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY). Rats were fed NIH-07 diet and maintained under the following standard conditions: 20 ± 2 °C, 50 ± 10% relative humidity, and a 12-h light, 12-h dark cycle. Microsomes were prepared as previously described from livers of 2 rats at 3 months of age and stored at −80 °C until use (*24*). The human liver microsomes were prepared using a published procedure (*25*) from a sample kindly provided by Dr. F. Peter Guengerich through the Tennessee Donor Service (Nashville, TN). The protein concentrations were determined by Bradford method (*26*) using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).

**Radiometric NDMA Demethylase Assay.** A previous published procedure was used for this assay (*27*). The final incubation mixture (100 $\mu$L) in a 2-mL Eppendorf tube contained 60 $\mu$M of radioactive NDMA (a 1:2 mixture of [$^{14}$C]NDMA and NDMA, 0.1 $\mu$Ci), rat liver microsomal proteins (129 $\mu$g), an NADPH generating system (0.4 mM $NADP^+$, 10 mM glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase), buffer at pH 7.4 (50 mM Tris, 10 mM $MgCl_2$, 150 mM KCl), and an inhibitor (1−100 $\mu$M). AITC, BITC, and conjugates were dissolved in water or buffer at the assay concentrations. PEITC has much lower solubility in water than the other two isothiocyanates. Most organic solvents cannot be used since they are inhibitors of NDMA demethylase (*28*). Acetonitrile was used as a vehicle to deliver PEITC into the Eppendorf tubes, and then was removed with a stream of $N_2$ before adding the other ingredients. Aqueous PEITC solution (up to 1 mM) could also be prepared by adding methyl-$\beta$-cyclodextrin (Sigma Chemical Co.) at a concentration that did not inhibit NDMA demethylase. These two methods of introducing PEITC into the aqueous incubation system gave comparable results. The result for PEITC was the average value. The former procedure was applied in the assay of all other isothiocyanates. However, neither of these methods was successfully applied to the more lipophilic PHITC. All incubations were carried out for 20 min and done in duplicate and the average values were used. In experiments using human liver microsomes, 97 $\mu$g of microsomal protein and 30 $\mu$M [$^{14}$C]NMDA with the same composition as the previously described were used. All operations were conducted in a fume hood, and safety procedures for handling radioactive chemicals and biohazards were followed as described in the "Hazardous Materials" section. The incubations were carried out in triplicate with each inhibitor concentration. The NDMAd activity measured by the amount of HCHO formed was plotted against concentration of the inhibitor. The $IC_{50}$ values, the concentrations that inhibit HCHO formation by 50%, were determined from these results. The standard deviation of $IC_{50}$ was calculated from three separate experiments.

**Exchange Reaction of Isothiocyanate Conjugates with L-Cysteine or GSH under Physiological Conditions.** The exchange reaction of isothiocyanate conjugates with thiol was conducted in a 2.0-mL Eppendorf tube at 37 °C in a water bath shaker. The isothiocyanate−GSH and L-cysteine were dissolved

**Table 2. $^1$H-NMR Assignments of Isothiocyanate Conjugates in DMSO-$d_6$ Solution[a]**

| | | AITC-GSH | BITC-GSH | PEITC-GSH | PHITC-GSH | PEITC-Cys | PEITC-NAC |
|---|---|---|---|---|---|---|---|
| $\gamma$-Glu | αH(1H) | 3.31 | 3.33 | 3.30 | 3.31 | | |
| | $\beta$H(2H) | 1.92, 1.83 | 1.90, 1.83 | 1.91, 1.83 | 1.92, 1.83 | | |
| | $\gamma$H(2H) | 2.30 | 2.30 | 2.30 | 2.30 | | |
| | $J_{\alpha b}$ | 6.8 | 6.8 | 6.8 | 6.8 | | |
| Cys | NH(1H) | 8.52 | 8.59 | 8.50 | 8.53 | | 7.55 |
| | αH(1H) | 4.50 | 4.52 | 4.48 | 4.49 | 3.53 | 4.10 |
| | $\beta$H(2H) | 3.30, 3.87 | 3.28, 3.79 | 3.23, 3.77 | 3.23, 3.75 | 3.61, 3.27 | 3.38, 3.19 |
| | $J_{N\alpha}$ | 8.6 | 8.6 | 8.5 | 8.8 | | 5.9 |
| | $J_{\alpha\beta}$ | 4.6 | 4.6 | 4.6 | 4.7 | 4.6 | 4.8 |
| | $J_{\alpha\beta'}$ | 10.0 | 10.0 | 9.9 | 10.0 | 5.8 | 3.8 |
| | $J_{bb'}$ | 13.4 | 13.8 | 13.3 | 13.5 | 14.4 | 14.4 |
| Gly | NH(1H) | 8.56 | 8.61 | 8.59 | 8.59 | | |
| | αH(2H) | 3.70 | 3.70 | 3.75 | 3.69 | | |
| | $J_{N\alpha}$ | 5.7 | | 5.7 | 5.7 | | |
| NHC(=S)S | NH(1H) | 10.21 | 10.58 | 10.18 | 10.03 | 8.50 (br pk) | 11.81 |
| alkyl | | 3 2 1<br>$CH_2$=$CHCH_2$− | 2 1<br>$PhCH_2$− | 2 1<br>$PhCH_2CH_2$− | 6 5 4 3 2 1<br>$PhCH_2CH_2CH_2CH_2CH_2CH_2$− | 2 1<br>$PhCH_2CH_2$− | 2 1<br>$PhCH_2CH_2$− |
| | | 1,(2H) 4.21 | 4.38 | 1,(2H) 3.58 | 1,(2H) 3.51 | 1,(2H) 3.75 | 1,(2H) 3.70 |
| | | 2,(1H)5.83 | $^2J_{HH}$ = 14.2 | 2,(2H) 2.87 | 2,4,(4H) 1.55 | 2,(2H) 2.91 | 2,(2H) 2.91 |
| | | 3,(2H) 5.17,5.10 | | $^3J_{1,2}$ = 7.6 | 3,5,(4H) 1.30 | $^3J_{1,2}$ = 7.7 | $^3J_{1,2}$ = 7.7 |
| | | $^3J_{2,3cis}$ = 17.2 | | | 6,(2H) 2.55 | | $CH_3CO$(3H) 1.60 |
| | | $^3J_{2,3trans}$ = 10.3 | | | $^3J_{5,6}$ = 7.2 | | |
| | | $^2J_{3,3}$ = 5.6 | | | | | |
| | ArH(5H) | | 7.30 | 7.25 | 7.22 | 7.25 | 7.25 |

[a] Chemical shifts ($\delta$) are in ppm relative to (TMS) and coupling constants ($J$) are in Hz. The NMR spectra were recorded on a 360 MHz spectrometer.

in a phosphate buffer (20 mM, pH 7.4, degassed) to make a 1.0 and 3.0 mM solution, respectively. The reaction was then initiated by mixing 0.75 mL of each solution. A 5 $\mu$L aliquot of the reaction mixture was taken every 20 min for HPLC analysis. The HPLC system consisted of a Vydac guard column (4.6 × 20 mm), a Vydac 218TP54 reverse-phase $C_{18}$ analytical column (4.6 × 250 mm) and a Partsil 5 ODS-3 reverse-phase $C_{18}$ analytical column (4.6 × 250 mm), (Whatman, Clifton, NJ) in sequence. Samples were eluted isocratically with a mobile phase of 40% of acetonitrile in water (0.1% trifluroacetic acid (TFA)), or a 1:1 ratio of acetonitrile and water (0.1% TFA) for PHITC-GSH and PEITC-NAC at a flow rate of 1 mL/min. The sample peaks were monitored by UV detection at 254 nm wavelength. These conditions offered the best separation of the GSH and L-Cys conjugates. In the case of reaction of PEITC-GSH with L-Cys, a synthetic standard of the cysteine conjugate of PEITC was used as a reference. The newly formed peak of this reaction was found to coelute with the standard conjugate, and they had identical UV spectra. The formation of PEITC-GSH when PEITC-Cys was incubated with GSH solution was also established using the synthetic standard of PEITC-GSH. Other isothiocyanate−Cys conjugates were identified by their characteristic UV spectra and HPLC retention times. For the reaction between BITC-GSH and PEITC-Cys, their solutions (1.0 mM in phosphate buffer) were mixed at 1:1 ratio. The reaction course was followed by the HPLC method as the above.

## Results

**Inhibition of Rodent Hepatic NDMA Demethylase Activity by Isothiocyanates and Their Conjugates.** The inhibition of NDMAd activity in rat liver microsomes was determined by a radiometric NDMAd assay (*27*). It has been reported that most organic solvents used for dissolving substrates or inhibitors in the enzyme assays are inhibitors of P4502E1 (*28*). Isothiocyanates such as SFO, AITC, and BITC were sufficiently soluble in aqueous solution at the concentrations examined. PEITC and more lipophilic isothiocyanates such as PHITC, however, were poorly soluble in water. PEITC was assayed using methods described in the Materials and Methods to circumvent the problem of poor solubility. The solubility also significantly increased upon conjugation with GSH. The $IC_{50}$ values of each isothiocyanate and their conjugates were determined and are summarized in Table 3. SFO, a major isothiocyanate in broccoli and a potent phase II enzyme inducer in hepatoma cell culture (*29*), was not inhibitory toward P4502E1 activity. Similarly, AITC, abundant in brown mustard and also a phase II enzyme inducer (*30*, *31*), showed very weak inhibitory activity. In contrast, both BITC and PEITC significantly inhibited P4502E1 activity in rat liver microsomes. GSH conjugates of AITC, BITC, and PEITC were more potent than their parent isothiocyanates. Although our previous studies showed that PHITC was a much stronger inhibitor of NNK-induced lung tumorigenesis in A/J mouse and of NNK metabolism in A/J mouse lung microsome than PEITC (*7*, *32*), its GSH conjugate was not as potent as PEITC-GSH. PEITC-Cys and PEITC-GSH were both potent inhibitors of NDMAd activity, whereas PEITC-NAC was much less inhibitory. Similar trends were also observed in the mouse liver microsomes.[3] In summary, GSH conjugates of BITC, PEITC, and PHITC and PEITC-

**Table 3. Inhibition ($IC_{50}$) of NDMA Demethylase Activity in Rat Liver Microsomes and the Half-Lives ($t_{1/2}$) of Isothiocyanate Conjugates in the Aqueous Solution Containing Thiol at Physiological Condition**

| inhibitors | $IC_{50}$ ± SD ($\mu$M)[a] | $t_{1/2}$ (min)[b] |
|---|---|---|
| SFO | >100 | |
| AITC | 77.0 ± 4.4 | |
| BITC | 9.0 ± 0.3 | |
| PEITC | 8.3 ± 1.9 | |
| AITC-GSH | 24.7 ± 2.2 | 27 |
| BITC-GSH | 2.2 ± 0.2 | 25 |
| PEITC-GSH | 4.0 ± 1.3 | 18 |
| PHITC-GSH | 8.8 ± 0.5 | 48 |
| PEITC-CYS | 4.3 ± 1.2 | 14 |
| PEITC-NAC | 24.0 ± 1.7 | >50 |

[a] Determined using the radiometric NDMA demethylase assay described in Materials and Methods. Each value was obtained from three determinations. [b] Determined by the reaction of conjugates (0.5 mM) and free thiol (1.5 mM) in a phosphate buffer (20 mM, pH 7.4) and at 37 °C.

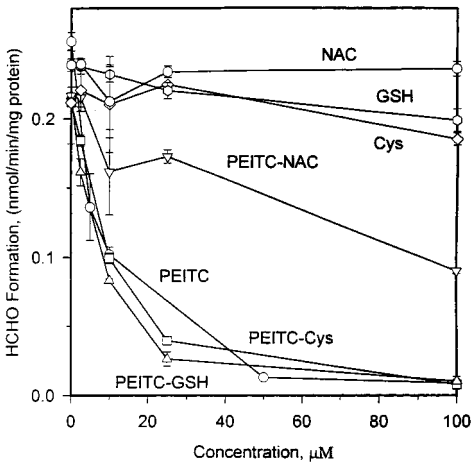



**Figure 1.** Dose-dependent inhibition of NDMA demethylation activity in human liver microsomes by PEITC and its conjugates. Each data point represents the average of three incubations. Incubation mixture (0.1 mL) contains 97 $\mu$g of human liver microsomal protein suspended in a pH 7.4 Tris buffer, a NADPH generating system, 30 $\mu$M radioactive NDMA, and an inhibitor (1−100 $\mu$M). Blanks were done without adding NADPH generating system, and controls were done without adding inhibitors. Incubation was carried out at 37 °C for 20 min. While Cys, NAC, and GSH had little effect on the NDMA demethylase activity, PEITC and its conjugates inhibited the enzyme activity with $IC_{50}$ values: 6 $\mu$M (PEITC), 9 $\mu$M (PEITC-Cys), 8 $\mu$M (PEITC-GSH), and 85 $\mu$M (PEITC-NAC).

Cys all significantly inhibited NDMAd activity, but AITC-GSH and PEITC-NAC were considerably less active.

**Inhibition of NDMA Demethylase Activity in Human Liver Microsomes by PEITC and Its Conjugates.** It has been shown that activation of NDMA in human liver microsomes was primarily catalyzed by P4502E1 (*33*, *34*). To examine the ability of isothiocyanates and their conjugates to inhibit human NDMAd activity, *i.e.*, P4502E1 activity, we assayed PEITC and its conjugates in human liver microsomes. The inhibition curves for the selected compounds were plotted in Figure 1, together with those of Cys, GSH, and NAC as controls. While Cys, GSH, and NAC had little effect on human P4502E1 activity within the concentration range tested, PEITC and its conjugates significantly inhibited the P4502E1 activity in a dose-dependent manner. Consistent with results obtained from rat liver microsomes, PEITC, PEITC-Cys, and PEITC-GSH had similar $IC_{50}$ values (6−9 $\mu$M), whereas PEITC-NAC was much less potent ($IC_{50}$ = 85 $\mu$M).

**GSH Conjugates of Isothiocyanate Exchange with Free Thiol Groups in Solution.** The observation that GSH conjugates of isothiocyanate inhibited NDMAd activity prompted us to examine the reaction of isothiocyanate−GSH conjugates with free thiols of proteins, one of the reactions believed to be involved in the inactivation of enzymes (*35*). Upon incubation of PEITC-GSH conjugate with L-cysteine in pH 7.4 buffer at 37 °C, we observed a time-dependent formation of the L-Cys conjugates of isothiocyanates by using a RP-HPLC system (Figure 2). The newly formed peak coeluted with the synthetic standard of PEITC-Cys and showed a UV spectrum identical to that of PEITC-Cys. Conversely, PEITC-GSH was formed when PEITC-Cys was incubated with GSH under the same conditions. Similarly, when a mixture of BITC-GSH and PEITC-Cys was incubated, both BITC-Cys and PEITC-GSH peaks were observed.

[3] D. Jiao, C. Conaway, and F.-L. Chung, unpublished data.

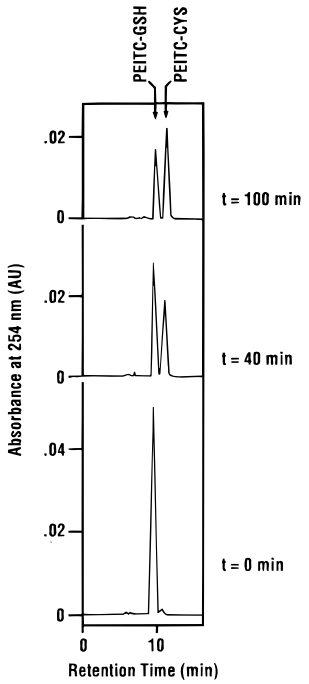



**Figure 2.** HPLC chromatograms showing time-dependent formation of PEITC-Cys upon reaction of PEITC-GSH with L-Cys. The reaction was initiated by mixing 0.5 mM of PEITC-GSH and 1.5 mM of L-Cys in a phosphate buffer (1.5 mL, pH 7.4) at 37 °C. Aliquots of sample (5 $\mu$L) were analyzed by HPLC at an interval of 20 min.

Since the latter two compounds have to be formed through the reaction between free isothiocyanates and free sulfhydryl groups of Cys or GSH, this result firmly established that the exchange of isothiocyanate conjugates is via the release of free isothiocyanates as intermediates.

The kinetics of the exchange reaction of different conjugates varies. For example, the three conjugates of PEITC exhibited marked differences for the exchange process, *i.e.*, the rate to reach equilibrium in descending order: PEITC-Cys, PEITC-GSH, and PEITC-NAC (Figure 3). To compare the exchange processes among different conjugates, the half-life for each conjugate to reach equilibrium in the presence of free thiol groups (of Cys in most cases) was determined (Table 3). Their half-lives vary depending upon the particular isothiocyanate conjugate. In the GSH conjugate series, the PHITC conjugate showed a much longer half-life than the conjugates of other isothiocyanates.

## Discussion

Our data showed that NDMAd in rat liver microsomes was significantly inhibited by BITC and PEITC at micromolar concentrations, but weakly inhibited by SFO and AITC. Thus, arylalkyl isothiocyanates appeared to have stronger inhibitory effects than the alkyl isothiocyanates. In agreement with these observations, the GSH conjugate of AITC showed considerably weaker inhibition of NDMAd than the GSH conjugates of BITC and PEITC. In the inhibition of NNK metabolism in mouse lung microsomes, PHITC is a much more potent inhibitor than PEITC (*10*). However, such a trend was not observed in the conjugated series for NDMA demethylation, probably due to the retarded dissociation of PHITC-GSH. Another possibility is that a different

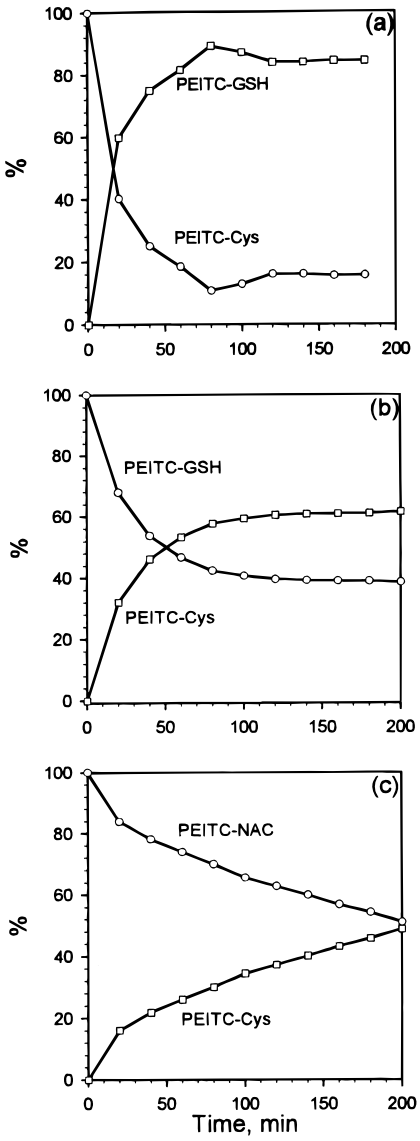



**Figure 3.** Time-dependent changes of the composition of PEITC conjugates in reaction mixture. (a) PEITC-Cys (0.5 mM, pH 7.4) + GSH (1.5 mM, pH 7.4); (b) PEITC-GSH (0.5 mM, pH 7.4) + L-Cys (1.5 mM, pH 7.4); (c) PEITC-NAC (0.5 mM, pH 7.4) + L-Cys (1.5 mM, pH 7.4).

structure−activity relationship may be involved in inhibiting the activation of different carcinogens. Current evidence indicates that the metabolism of NDMA is primarily catalyzed by P4502E1 in liver microsomes, whereas the metabolism of NNK in mouse lung is primarily catalyzed by P4502A1 and 2B1 (*8*, *36*). More investigations regarding the selectivity of isothiocyanates toward the inhibition of specific P450 enzyme are needed for a better assessment of their chemopreventive potentials.

In this study we have demonstrated that isothiocyanate conjugates are inhibitory toward NDMAd in rat liver microsomes. Similar trends were observed for PEITC and its conjugates in human liver microsomes. The inhibitory activities of both isothiocyanates and their conjugates in the microsomal systems appear to operate through a common mechanism. It was postulated that isothiocyanates interact with the substrate binding site of P450 and react with thiol, amino, and hydroxyl groups of the protein (*37*). Since there are more than a half dozen Cys and many amino and hydroxyl group containing residues in rat and human P4502E1 proteins (*38*), it is likely that some of these functional groups are modified by isothiocyanates. Moreover, the NADPH−P450 reductase is also susceptible to the modification of sulfhydryl groups and may also be a target for isothiocyanates (*39*). The GSH conjugates of isothiocyanates have been shown to release isothiocyanates with or without the catalysis of glutathione *S*-transferases (*20*, *40*, *41*). The inhibitory effect of isothiocyanate conjugates in the NDMAd assay could be attributed to the ability of conjugates to generate free isothiocyanate. This notion is supported by the present observation that the isothiocyanates released by their GSH or other thiol conjugates are subsequently conjugated with free thiol groups via this pathway. The lower $IC_{50}$s of the conjugates as compared to those of the corresponding isothiocyanates suggest, however, the possibility that conjugates can directly inhibit NMDAd. The differences in $IC_{50}$ between the conjugates and the parent isothiocyanates could be due partially to the poor solubility of the isothiocyanates (see Materials and Methods). Our observations that the conjugates were considerably less active than the isothiocyanates in pentoxyresorufin dealkylase (2B1) and ethoxyresorufin dealkylase (1A) assays in which dimethyl sulfoxide was used to solubilize the substrates seem to support this possibility.[3] Additional evidence against the conjugates being inhibitors comes from studies using an *S*-hexyl-GSH analog with a thioether linkage which precludes dissociation reaction. This compound showed no inhibitory activity against 2E1 in mouse liver microsome incubations.[3] Although the direct inhibition by the conjugates cannot be completely ruled out, the results so far obtained are consistent with the mechanism of dissociation in the inhibition by the conjugates.

Since the exchange of conjugation among different thiol groups requires release of the free isothiocyanate from one conjugate and subsequent formation of a new conjugate, the difference in their half-lives to reach equilibrium could have resulted from either the different dissociation rate of isothiocyanate conjugates or the different reactivity of isothiocyanates toward free thiols or a combination of both. Although this system does not allow us to examine the detailed kinetic features of either equilibrium involved, it may reflect a certain complexity of the interactions between isothiocyanate conjugates and protein thiols in microsomes. When examined carefully, however, the data may reveal some qualitative information on the dissociation of the conjugates. For instance, in the series of PEITC conjugates, it may be assumed that the reactivity of PEITC toward the sulfhydryl group of Cys and GSH is similar or identical; the different half-lives could be attributed solely to different dissociation rates of PEITC-Cys, PEITC-GSH, and PEITC-NAC in descending order. It is noted that PEITC-NAC was much less potent than PEITC-Cys and PEITC-GSH in the inhibition of NDMAd in both rodent and human liver microsomes. Since most of incubations in the assay were completed within an hour, PEITC-Cys and PEITC-GSH dissociated readily and had sufficient time to interact with microsomal enzymes. Thus, they were more potent inhibitors than PEITC-NAC, which had a much longer half-life (>50 min) and probably would be much less facile to interact with enzymes during the incubation period. However, such an interpretation based on the different dissociation rates of conjugates needs to be confirmed with an accurate measurement of dissociation constants for those conjugates.

It has become increasingly evident that many chemically reactive metabolites may be transported as GSH conjugates *in vivo* (*42*). It is plausible that the previously observed inhibitory activity of isothiocyanates against NNK-induced tumorigenesis in mouse lung may be, in part, mediated by transportation and subsequent dissociation of their respective GSH conjugates. Therefore, the discovery of the inhibitory activity of NDMAd by isothiocyanate conjugates provides an important insight into the molecular mechanism by which isothiocyanates inhibit nitrosamine-induced carcinogenesis. The PEITC-Cys conjugate is less toxic than PEITC itself and has been shown to be absorbed upon oral administration to mice resulting in the stimulation of glutathione *S*-transferase production (*43*). It is of considerable interest to study whether isothiocyanate conjugates act as inhibitors in animal tumor bioassays. Future studies will focus on the *in vivo* metabolism of isothiocyanates and the potential of isothiocyanate conjugates as chemopreventive agents.

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