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Kinetics and Mechanistic Studies of the Hydrolysis of Diisocyanate-Derived Bis-thiocarbamates of Cysteine Methyl Ester

Itai Chipinda,[†,‡] Sarah J. Stetson,[†] Gary J. Depree,[†] Reuben H. Simoyi,[‡] and Paul D. Siegel*[,†]

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, West Virginia 26505-2888, and Department of Chemistry, Portland State University, Portland, Oregon 97201-0751

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Diisocyanates (dNCOs) are the most commonly reported cause of chemically induced occupational asthma, but the ultimate antigenic form is unknown. Reactions of the three most common monomeric dNCOs, hexamethylene dNCO (HDI), methylene diphenylisocyanate (MDI), and toluene dNCO (TDI), with cysteine methyl ester (CME) gave the corresponding bis-dithiocarbamates (HDI−CME, TDI−CME, and MDI−CME). The dissociation kinetics of these bis-thiocarbamates, in aqueous conditions, was followed spectrophotometrically under varying pH and temperature conditions. Reaction of the adducts with methylamine or human serum albumin (HSA) produced diurea, monourea, and diamine products, and this was consistent with the base-catalyzed elimination reaction (E1cB) pathway being the dominant, but not exclusive, dissociation mechanism. The hydrolysis of the adducts was first-order with respect to OH^- concentration and overall second-order (HDI−CME, $k = 3.36 \times 10^2$ M^{-1} min^{-1}; TDI−CME, $k = 2.49 \times 10^4$ M^{-1} min^{-1}; and MDI−CME, $k = 5.78 \times 10^4$ M^{-1} min^{-1} at pH 7.4) with deviation from second-order when the dNCO had an aromatic functional group. Arrhenius plots gave activation energies (HDI−CME, $E_a = 70.6$ kJ/mol; TDI−CME, $E_a = 46.1$ kJ/mol; and MDI−CME, $E_a = 44.5$ kJ/mol) that were consistent with the following order of stability: HDI−CME > TDI−CME > MDI−CME. Therefore, the stability of different dNCO-derived thiocarbamates in aqueous environments can vary greatly. Thiocarbamate dissociation rates and type of products formed may potentially influence antigenicity and subsequent hypersensitivity/toxic reactions following dNCO exposures.

Introduction

Diisocyanates (dNCO) are used extensively in industry in the manufacture of various polyurethane products including foams, wood binders, elastomers, coatings, and paints. These compounds are very reactive due to their electrophilic nature, making them susceptible toward nucleophilic attack. Isocyanates can react under aqueous physiological conditions with primary amines and thiols to form ureas and thiocarbamates (*1*), respectively. Reaction with hydroxyls, to form carbamates, is also physiologically feasible in hydrophobic regions of membranes and proteins (*1*).

Several health effects have been documented in workers exposed to dNCO (*2*). Mucous membrane irritation of the eyes,

* To whom correspondence should be addressed. Tel: 1-304-285-5855. Fax: 1-304-285-6126. E-mail: psiegel@cdc.gov.
[†] National Institute for Occupational Safety and Health.
[‡] Portland State University.

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respiratory tract, and gastrointestinal tract has been reported (*2*, *3*). Skin irritation/inflammation has also been reported following direct skin contact with the isocyanate (*4*). Two immunologically mediated diseases, hypersensitivity pneumonitis (HP) and asthma, have been reported in workers exposed to dNCOs. Reports on dNCO-mediated HP have been sporadic (*5*−*7*). In contrast to HP, dNCOs have been implicated as one of the most frequently reported causes of occupational asthma (*7*−*11*).

The pathological mechanism of dNCO-induced occupational asthma is not known. The lack of detectable specific IgE in dNCO asthmatics has led to theories concerning both disease mechanisms and antibody detection. Because specific IgE can be detected in only 5−20% of dNCO asthmatics, it has been proposed that only a portion of the asthma is IgE-mediated, with the remainder mediated by other mechanisms (*12*−*15*). Questions concerning the detection antigen(s) used have also been raised. dNCO-haptenized human albumins are commonly employed as the antigens for dNCOs specific antibody detection. Researchers have explored the effects of hapten density (*16*) and isocyanate polymer haptens for specific antibody detection (*17*). dNCOs have also been shown to bind to airway epithelial cell proteins (*18*), and toluene dNCO (TDI) has been colocalized with tubulin on the cilia of exposed differentiated human airway epithelial cells (*19*). The protein to which the dNCO is conjugated may be important with respect to the detection of dNCO specific antibodies from sensitized individuals.

Formation, in vivo, of mono- and bis-thiocarbamates following exposure to dNCO would be expected under physiological conditions because of the high density of intra- and extracellular protein and nonprotein thiols within the body and the avidity of isocyanates for thiols. Day et al. (*20*) demonstrated in a cell-free system that bis-thiocarbamates are the favored reaction product of dNCOs and thiol amino acids. Lange et al. (*19*) reported indirect in vivo and direct in vitro evidence for the formation of monothiocarbamates following exposure to TDI. Lung tissue isolated from TDI-exposed mice had a reduced capacity to quench phenoxyl radicals. Thiol levels were reduced in cultured human bronchial lung cells exposed to TDI vapor and identification of a mono-TDI S-glutathionyl adduct was reported. Loss of thiol reactivity and radical scavanging cannot be completely attributed to thiocarbamate formation. Mechanisms including oxidant stress, inhibition of disulfide reductases, thiol transport, and synthetic functions may also contribute to these observations. Loss of free reduced thiols due to thiocarbamate formation from dNCO exposure should be transient, since these are regenerated upon solvolysis.

It is important to study how bis-thiocarbamates may react under aqueous conditions to help understand potential reactions and reaction products that may participate in dNCO-mediated hypersensitivity diseases. Day et al. (*20*) and Reisser et al. (*21*) reported the synthesis, characterization, and solvolysis of bis-thiocarbamates made from the reaction of glutathione with TDI and 4,4-methylenediphenyl dNCO (MDI), respectively. The reported pseudo-first-order half-lives were 59 min for MDI−, 245 min for 2,4-TDI−, and 2460 min for 2,6-TDI−bis-thiocarbamates. The mono-MDI adduct was found to be significantly more stable than the bis-adduct. It was suggested in both studies (*21*, *22*) that the data were consistent with a base-catalyzed elimination reaction (E1cB) mechanism of hydrolysis; however, extensive modeling was not performed to explore or confirm the mechanism.

In the present study, model water/alcohol soluble bis-thiocarbamate compounds were synthesized using cysteine methyl ester (CME) and commercially important dNCOs, MDI, TDI, and HDI (1,6-hexamethylene dNCOs). Detailed kinetic modeling of these compounds was performed under varying aqueous conditions to study mechanisms of hydrolysis and to compare hydrolysis rates of the bis-thiocarbamates derived from the three dNCOs. Potential differences in dNCOs vs bis-thiocarbamate reactivity with primary amines were examined by comparison of urea type reaction products.

Experimental Procedures

Materials. Silver nitrate, 2,4-TDI, 4,4′-MDI 1,6-HDI, and L-CME hydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Methylamine hydrochloride, human serum abumin (HSA), picrylsulfonic acid, and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol and sodium phosphate dibasic, (anhydrous), came from Fisher Scientific (Pittsburgh, PA).

Instrumentation. Absorbance measurements to determine free thiol concentrations were carried out on a Beckman DU 650 Spectrophotometer (Fullerton, CA) at 412 nm. Temperature control was achieved by using a VWR Scientific circulating water bath (Niles, IL).

HPLC analysis of urea products was performed on a Shimadzu system (Kyoto, Japan) consisting of two LC-600 pumps, a SIL-10AD autoinjector, a LPI-6B interface, and an SPD-M10A diode array detector. An injection volume of 10 μL was used, and separations were performed on a 5 μm particle size, 250 mm × 4.6 mm Supelco Discovery C18 column (Bellefonte, PA) at a flow rate of 1 mL/min. An isocratic mobile phase consisting of 50% MeOH was employed. Absorbance at 247 nm was monitored.

IR spectra were determined as Nujol mulls between KBr disks on a Perkin-Elmer 1600 Series FTIR (Shelton, CT). Mass spectra were recorded on a Finnigan Mat LCQ mass spectrometer (Franklin, MA). All electrospray ionization mass spectrometry (ESI-MS) samples were run using methanol as the solvent. Solution state NMR spectra were also recorded on a Bruker DMX-300 spectrometer (Billerica, MA). ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were recorded as CD_3OD solutions. Chemical shifts were referenced internally with TMS using the following standard references for deuterated methanol: ^{1}H, 3.31 ppm; ^{13}C, 49.15 ppm. Full NMR assignments were made using COSY, HETCORR, HMBC, and NOE experiments.

Synthesis of Bis-thiocarbamates. 1. Preparation of MDI−CME. A solution of MDI (126 mg, 0.50 mmol) in acetone (2 mL) was added dropwise to a solution of L-CME hydrochloride (172 mg, 1.00 mmol) in CH_3CN/H_2O (7:3, 10 mL). The solution was stirred at room temperature for 30 min. Precipitation was induced by the addition of diethyl ether and acetone at −20 °C overnight. The white solid (219 mg, 74% Bis-CME−MDI) was collected by filtration, washed with diethyl ether, and dried under vacuum. MDI NMR: ^{1}H NMR: δ 7.41 (*d*, J = 8.5 Hz, 4H, Ar−H), 7.13 (*d*, J = 8.5 Hz, 4H, Ar−H), 4.40 (*dd*, J = 6.2 and 4.5 Hz, 2H, SCH_2CH), 3.89 (*s*, 2H, CH_2), 3.86 (*s*, 6H, CO_2CH_3), 3.61 (*dd*, J = 15.3 and 4.5 Hz, 2H), and 3.41 (*dd*, J = 15.3 and 6.2 Hz, 2H, SCH_2CH). ^{13}C NMR: δ 169.3 (*s*, CO_2CH_3), 165.3 (*s*, SCO), 139.0 (*s*, C4), 138.1 (*s*, Ar−C), 130.5 (*d*, Ar−C), 121.2 (*d*, Ar−C), 54.7 (*d*, SCH_2CH), 54.1 (*q*, CO_2CH_3), 41.7 (*t*, CH_2), 30.6 (*t*, SCH_2CH). ESI-MS: *m*/*z* 521.1 $[M + H]^+$, 261.1 $[M + 2H]^{2+}$.

2. Preparation of HDI−CME. HDI (84 mg, 0.50 mmol) was added dropwise to a solution of l-CME hydrochloride (172 mg, 1.00 mmol) in CH_3CN/H_2O (7:3, 10 mL). The solution was stirred at room temperature for 30 min. Acetone and diethyl ether were then added, and the reaction mixture was placed at −20 °C overnight. This yielded a colorless oil, which was washed with acetone and then dried under vacuum (196 mg, 77% Bis-CME−MDI). HDI NMR: ^{1}H NMR: δ 4.40 (*dd*, J = 6.2 and 4.6 Hz, 2H, SCH_2CH), 3.84 (*s*, 6H, CO_2CH_3), 3.55 (*dd*, J = 15.1 and 4.6 Hz, 2H) and 3.39 (*dd*, J = 15.1 and 6.2 Hz, 2H, SCH_2CH), 3.23 (m, 4H, $NHCH_2CH_2CH_2$), 1.50 (m, 4H, $NHCH_2CH_2CH_2$), 1.29 (m, 4H,

$NHCH_2CH_2CH_2$). ^{13}C NMR: δ 169.4 (*s*, CO_2CH_3), 167.0 (*s*, SCO), 54.8 (d, SCH_2CH), 54.2 (q, CO_2CH_3), 42.6 (t, $NHCH_2CH_2CH_2$), 30.5 (t, $NHCH_2CH_2CH_2$ and SCH_2CH), 27.6 (t, $NHCH_2CH_2CH_2$). ESI-MS: *m*/*z* 439.1 $[M + H]^+$, 220.1 $[M + 2H]^{2+}$.

3. Preparation of TDI−CME. TDI (87 mg, 0.50 mmol) was added dropwise to a solution of l-CME hydrochloride (172 mg, 1.00 mmol) in CH_3CN/H_2O (7:3, 10 mL). The solution was stirred at room temperature for 30 min. Acetone and diethyl ether were then added, and the reaction mixture was placed at −20 °C overnight resulting in the isolation of a colorless oil (196 mg, 76% Bis-CME−TDI). TDI NMR: ^1H NMR: δ 7.70 (s, 1H, Ar−H), 7.34 (m, 1H, Ar−H), 7.17 (m, 1H, Ar−H), 4.40 (m, 2H, SCH_2CH), 3.86 (s, 6H, CO_2CH_3), 3.59 (dd, $J = 15.1$ Hz and $J = 4.6$ Hz) and 3.5 (dd, $J = 15.1$ Hz and $J = 6.2$ Hz, 2H, SCH_2CH), 2.22 (s, 6H, CO_2CH_3). ^{13}C NMR: δ 169.1 (s, CO_2CH_3) 165.8 (s, SCO), 138.0 (s, Ar−C), 137.0 (s, Ar−C), 132.0 (d, Ar−C), 131.1 (s, Ar−C), 120.1 (d, Ar−C), 119.6 (d, Ar−C), 54.8 (s, SCH_2CH), 54.2 (s, CO_2CH_3), 30.8 (s, SCH_2CH), 17.5 (s, CH_3). ESI-MS: *m*/*z* 445.1 $[M + H]^+$, 223.1 $[M + 2H]^{2+}$.

Verification of HCl Salt. MDI−CME (50.49 mg, 0.110 mmol) or HDI−CME (44.24 mg, 0.118 mmol) in H_2O (3 mL) was allowed to dissociate and hydrolyze for 2 h. It was then filtered and put in silver nitrate (2 mL, 2%). A white precipitate formed immediately, which was collected, dried, and weighed. A 1:2 thiocarbamate: chloride ratio was found. This test was done to determine if the thiocarbamate is in the form of a hydrochloride salt. (The CME used is in the form of an HCl salt) Because the chloride ion is still present as dNCO−CME·HCl, two HCl molecules were included in the formular weight calculation of the thiocarbamate.

Reaction of the MDI and MDI−CME with Methylamine. MDI and MDI−CME were added dropwise into 1 mL aliquots of methylamine/pH 9.4, 0.1 M borate buffer at a molar ratio of 1:10 and 1:100 MDI:methylamine or MDI−CME:methylamine. The white precipitate formed in the reaction of methylamine, and MDI was collected and analyzed using NMR and IR as previously described to confirm the urea linkage. The urea has a C═O stretch at 1634 cm^{-1} for IR and a peak at 156 ppm in the ^{13}C NMR spectrum corresponding to the C on the urea. The reaction products were separated by HPLC as described above.

Reaction of MDI and MDI−CME with HSA. MDI−CME (1.69, 3.37, 8.43, or 16.85 mg) in 500 μL of MeOH was added dropwise into 5 mL of HSA (5 mg/mL phosphate-buffered saline, PBS) yielding HSA/MDI−CME ratios of 1:10, 1:20, 1:50, and 1:100, respectively. Similarly, MDI (in dry acetone) was added to HSA in ratios of 1:1, 1:5, 1:10, and 1:20. These were stirred at room temperature for 3.5 h. Controls of 5 mL of HSA, HSA with 0.5 mL of acetone, and HSA/acetone were run in parallel. All samples and controls were centrifuged at 900*g* for 45 min, the supernatant fluid was collected and dialyzed with a 14000 kDa molecular mass cutoff tubing against PBS (0.1 M, pH 7.4, 4 °C). The MDI/MDI−CME samples and standards were again centrifuged at 900*g* for 30 min. The amount of protein in each sample was determined using Coomassie Blue dye (Bio-Rad, Hercules, CA). The amount of free primary amine in each sample was determined using picrylsulfonic acid (Sigma Chemical Co.). Picrylsulfonic acid (25 μL of 200 μL/mL, 0.1 M borate buffer, pH 9.3) was added to a 1 mL sample diluted in borate buffer and the absorbance was monitored at 420 nm (*23*). Glycine (1.25−10 μg/mL) and HSA (5−500 μg/mL) standards were used. All protein controls and samples were stored at 4 °C until analysis.

Kinetic Monitoring of Hydrolysis of Dithiocarbamates under Aqueous Conditions. Stock solutions of the bis-thiocarbamates (1 mg/mL methanol) were prepared immediately prior to each experiment using methanol. Standard curves for chemical kinetic studies were made by incubating 6.26−100 μg/mL bis-thiocarbamate for MDI−CME, 6.26−75 μg/mL for HDI−CME, and 0.75−6.0 μg/mL for TDI−CME with 0.1 mg/mL (DTNB)/0.1 M Na_2HPO_4 (pH 9, 37 °C) for 45 min (1 mL total volume). The DTNB−thiol reaction product formed was monitored at its maximum absorbance of 412 nm. Hydrolysis kinetics of each bis-thiocarbamate were determined at pH 10, 9, 8, 7.4, and 5 using 25 μg/mL MDI−CME, 25 μg/mL HDI−CME, and 6 μg/mL TDI−CME in 0.1 mg/mL DTNB/0.1 M Na_2HPO_4 (1 mL total volume). Absorbance at 412 nm was monitored over time while the temperature was held constant at 310, 298, or 277 K. Each experiment was run in triplicate.

Controls Run for Hydrolysis. Methylamine, MDI, methylenedianiline (MDA), MDI−CME, and phosphate buffer had no detectable absorbance at 412 nm and, hence, did not interfere with absorbance readings. Ten microliters of 5 mg/mL methylamine (100-fold molar excess) was included with MDI−CME to effect inhibition of reformation of thiocarbamate following hydrolysis. The absorbance was then monitored for 30 min alongside a sample made using the procedure described above for kinetic monitoring of hydrolysis. No difference was noted with or without methylamine suggesting that reformation of thiocarbamates following hydrolysis was not significant and did not interfere with kinetic measurements under the present experimental conditions. A saturated glycine buffer (3 M, pH 7.4) was used in place of phosphate buffer to evaluate the potential for the phosphate to act as a nucleophile. dNCOs were not observed to conjugate to glycine, which is consistent with previous observations and reports (*23*).

Results

Hydrolysis kinetics were followed spectrophotometrically when the thiolate or thiol (protonated under acidic conditions) reduced DTNB to afford the highly absorbing chromophore, 2-mercaptonitrobenzoic acid (MBA), and a nonabsorbing (around 412 nm) mixed disulfide R′SSNB (where R′ = CME). For every 2 mol of MBA produced, 1 mol of the thiocarbamate adduct had to undergo hydrolysis assuming that the reactions were all going to completion.

The change in absorbance of the MBA with time was hence used to calculate the amount of adduct remaining at time *t*, $[RDI-CME]_t$, by using the following equation

$$[RDI - CME]_t = [RDI - CME]_0 - \frac{A_{MBAt}}{2\xi} \qquad (E1)$$

where $[RDI-CME]_0$ is the initial concentration of the bis-thiocarbamate, A_{MBAt} is the absorbance of the chromophore MBA at time *t*, and ξ (14150 M^{-1} cm^{-1}) is its absorptivity coefficient (*24*).

Kinetic studies were based on the working hypothesis that base/nucleophile-catalyzed substitutions and eliminations were the major reaction pathways. Reaction R2a outlines a base-catalyzed bimolecular substitution reaction (BAc2) whereas R2b gives the E1cB reaction. The notation # in the reactions R2a−c signifies that the compound is a labile intermediate. Where the pH afforded the availability of nucleophiles (Nu^-) in the solution, there was the possibility of either the BAc2 or the E1cB mechanism depending on the thiol and the organic functional group. Where the pH was lower than 7, the hydrolysis was possibly effected through an acid-catalyzed elimination of the 2 mol of CME from the adduct (reaction R2c):

$$R-[NHC(O)SR']_2 + 2Nu^- \rightarrow R-[NHC(O)Nu]_2^{\#} + 2R'S^- \quad (R2a)$$

$$R-[NHC(O)SR']_2 + 2Nu^- \rightarrow R-[^{\ominus}NC(O)SR']_2^{\#} + 2NuH \rightarrow R-(NCO)_2 + 2R'S^- \quad (R2b)$$

$$R-[NHC(O)SR']_2 + 2H^+ \rightarrow R-[NHC(O)S^+HR']_2 \rightarrow R(NCO)_2^{\#} + 2R'SH + 2H^+ \quad (R2c)$$

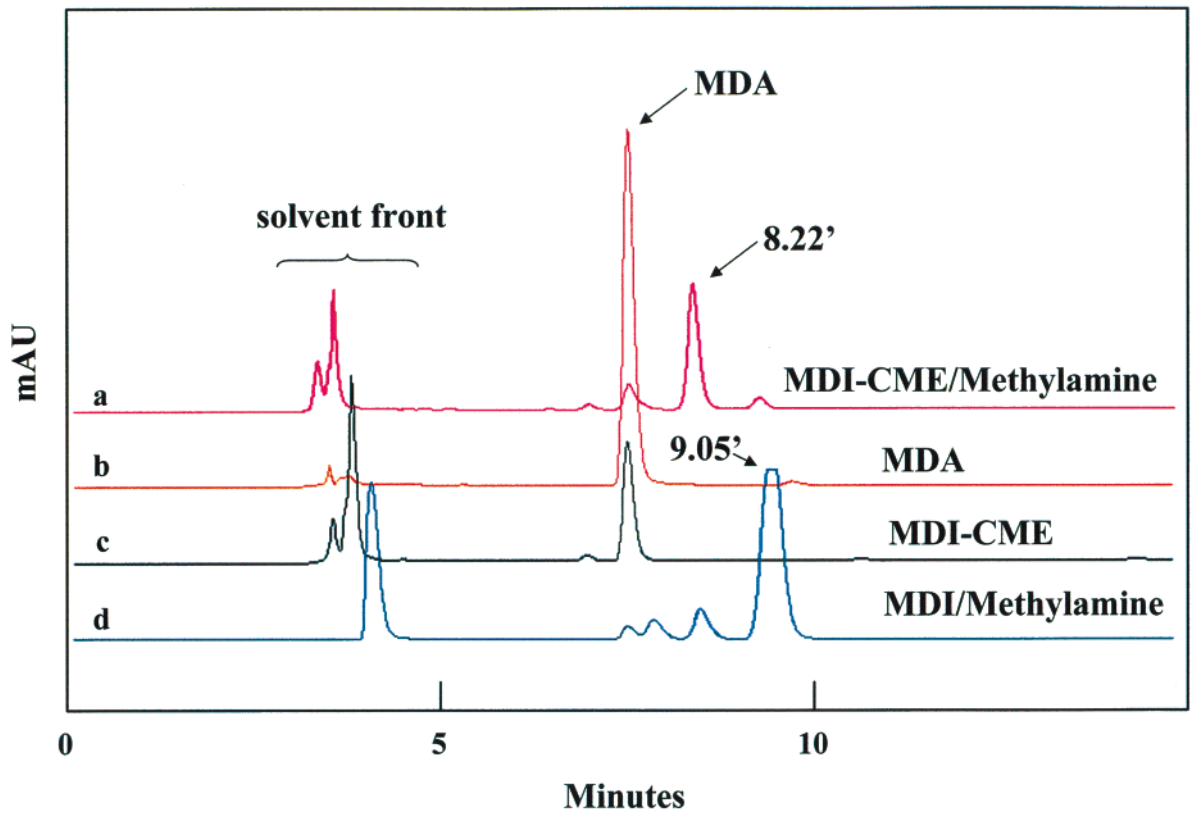
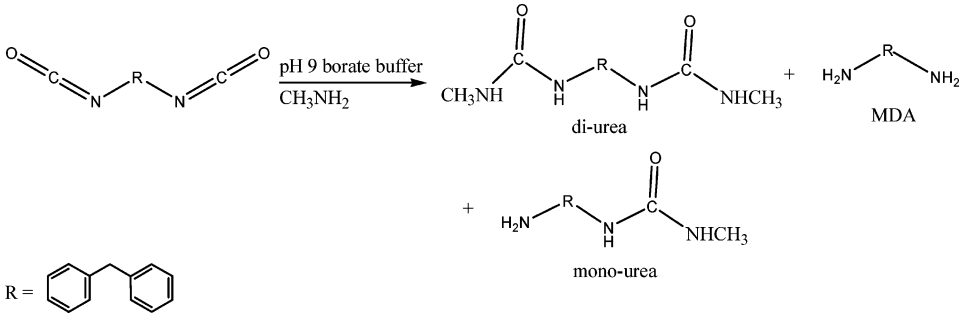
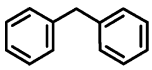


Figure 1. HPLC UV chromatogram overlay of MDI−CME/methylamine (a), MDA (b), methylene diphenyl CME (MDI−CME) (c) and MDI/methylamine (d) (to see if the isocyanate reacts with free amines). The eluents were monitored at 247 nm. Note the presence of MDA for the MDI−CME trace.

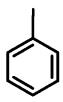
Scheme 1. Formation of the Monourea, Diurea, and Dianiline in the Reaction between an Isocyanate and an Amine



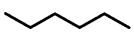
These are generalized equations where R can be any of the following organic moieties.



methylene diphenyl

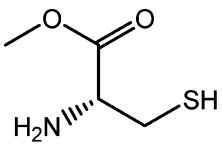


toluene



hexamethylene

R′SH is the CME.

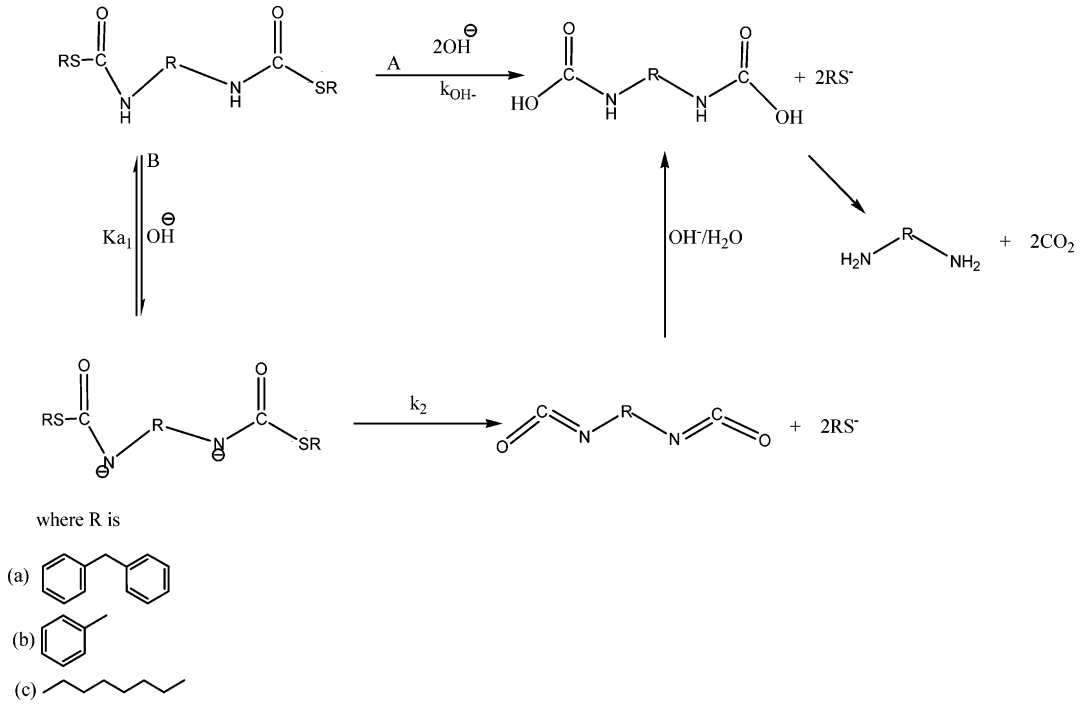


cysteine methylester.

BAc2 or E1cB? Reaction of Hydrolysis Products with Primary Amines: dNCO vs Bis-thiocarbamate. Experimental evidence showed both the BAc2 and the E1cB mechanisms as being effective at pH greater than 7, the E1cB more so than the BAc2. MDI and its corresponding cysteine methyl ester bis-thiocarbamate were reacted with methylamine under alkaline conditions (borate buffer, pH 9). The reaction products were separated and monitored by HPLC UV detection (Figure 1). Each product was confirmed by mass spectrometry, IR analysis, and NMR. An MDA standard was also analyzed by HPLC. MDI's reaction with methylamine produced three identifiable reaction products: MDA, a monourea, and a diurea (Scheme 1).

The diurea (Figure 1, chromatogram d, elution at 9.05 min) was the predominant product in excess methylamine. Small amounts of the monourea and MDA were also present. The MDI−CME was able to hydrolyze in the borate buffer in the absence of methylamine to give the complete hydrolysis product (MDA) (Figure 1, chromatogram c), which eluted at the same time as the control compound MDA (Figure 1, chromatogram

Scheme 2. Proposed BAc2 Mechanism (A) and E1cB Mechanism (B) of the Hydrolysis of the dNCO−CME Conjugates (RDI−CME) in the Presence of OH− or Nucleophilic Ions



b). The reaction of MDI−CME to methylamine produced predominantly the monourea (Figure 1, chromatogram a, elution at 8.22 min); however, some MDA and diurea were apparent. The scheme that had been initially proposed (Scheme 2) was supported by the superimposed chromatograms shown in Figure 1. Regardless of the mechanism by which the hydrolysis occurred, the end products in water were the same.

Thus, the carbamic acid, which is very labile and decomposes to the amine by releasing carbon dioxide, is common to both of the nucleophilic-based mechanisms. Figure 1 (chromatogram a) was a set of experiments that were carried out to distinguish between the BAc2 mechanism and the E1cB mechanism. Incubation of the reaction solution with methylamine was done to probe the nature of the intermediate that is formed when the hydrolysis occurs. The presence of an isocyanate as an intermediate enabled the formation of the urea products, which result from the methylamine isocyanate reaction and identification of the E1cB mechanism as the major route in the hydrolysis, as predicted by Scheme 2. As a control, a parent dNCO (MDI) reacted with methylamine gave products with the same elution profile (Figure 1, chromatogram d). The elution peak at 9.05 min is attributed to the formation of urea moieties on both ends of the adduct, whereas the peak at 8.22 min was consistent with the formation of the urea on one end of the adduct. In both cases, the formation of the urea product(s), either on one or both ends, of the bis-adduct indicated the generation of the isocyanate intermediate. This is strong evidence that the E1cB is a prevalent mechanism. The BAc2 mechanism could not be ruled out completely as the chromatogram (a) in Figure 1 showed that there was formation of the carbamic acid with subsequent decomposition to the diamine. This is consistent with what is proposed in Scheme 2 (pathway A). The reaction between amine groups and isocyanates is a well-known rapid reaction (*25*). Thus, an E1cB mechanism is able to avail the isocyanate intermediate, which then rapidly reacts with the methylamine before it can hydrolyze to the highly unstable carbamic acid. Even though this is a very rapid reaction, there was still some isocyanate that was able to hydrolyze to the carbamic acid. On the other hand, a BAc2 pathway (resulting in MDA or the monourea) will readily furnish the carbamic acid, which is too unstable to react with any methylamine present. Carbamic acids are short-lived in solution, and they have been known to be too unstable to react with any amines present in solution (*26*).

Table 1. Percent Loss of Primary Amines from the HSA after Reaction with MDI−CME[a]

ratio of MDI or MDI−CME to HSA	MDI−HSA	MDI−CME−HSA
1:1	11.42	not measured
5:1	27.69	not measured
10:1	53.78	19.95
20:1	69.33	15.64
50:1	not measured	15.03
100:1	not measured	14.7

[a] Picrylsulfonic acid was used to determine the amount of free primary amines on the HSA.

HSA was used to further confirm the E1cB pathway as a dominant, but not the sole, mechanism. The MDI−CME reacted with the HSA in PBS (pH 7.4). Table 1 shows that a 19.95% loss of amines on the HSA is attributed to MDI−CME relative to the 53.78% that was attributed to the free isocyanate (MDI) at 10:1 dNCO/HSA molar ratios. The loss of primary amines was a good indicator that the intermediate resulting from the hydrolysis of the MDI−CME was an isocyanate (because of urea formation), and this was further confirmation that the E1cB mechanism was a major pathway through which the dissociation was occurring. The limited loss of amine reactivity is possibly indicative of one end of the adduct conjugating with the protein leaving the other end to hydrolyze to the amine suggesting the potential for the BAc2 mechansim. Conjugation of MDI−CME to HSA was confirmed by a shift in migration in polyacrylamide gel electrophoresis experiments and reverse phase HPLC (data not shown).

Kinetics. Maintaining the temperature at 298 K and varying the pH enabled the determination of the reaction rates as the pH changed. Rapid hydrolysis in the initial stages is shown in Figure 2i−iii. The HDI−CME shows a slow dissociation rate

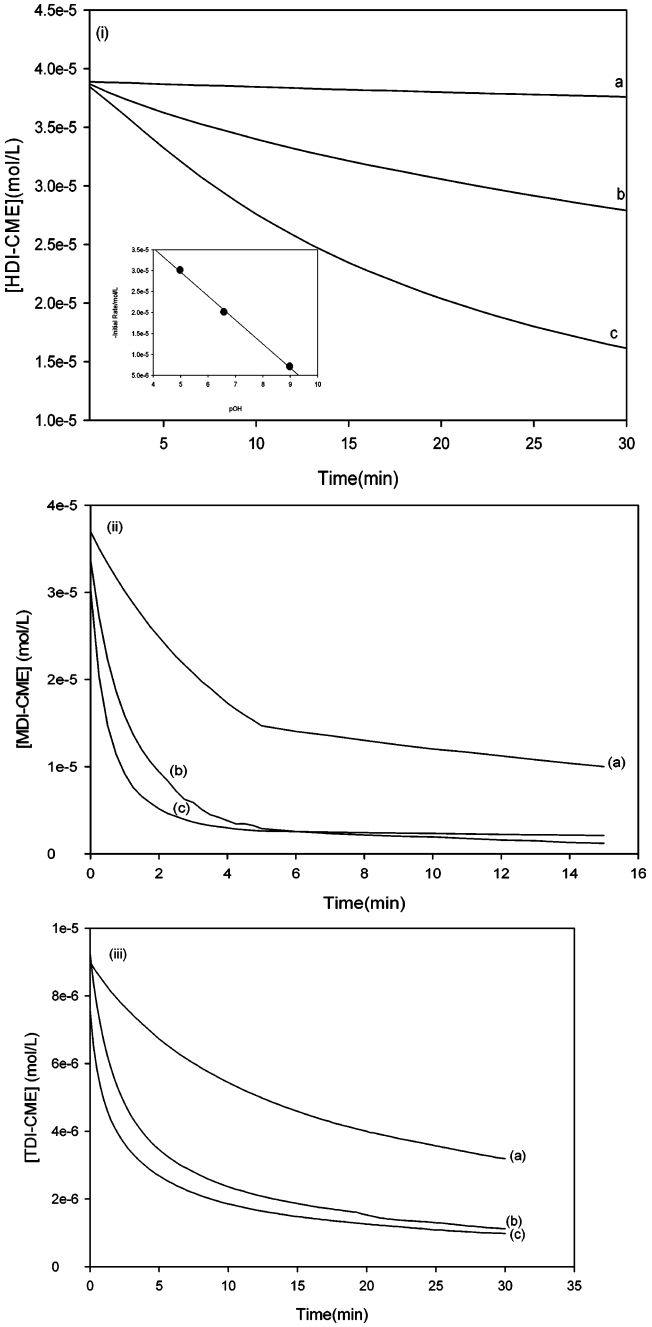


Figure 2. Kinetics of the hydrolysis for the three adducts at constant temperature (298 K) and changing pH where a, b, and c correspond to pH 5, pH 7.4, and pH 9, respectively. (i) HDI−CME and the dependence of the initial rate on the OH^- concentration (insert), (ii) MDI−CME, and (iii) TDI−CME. The aromatic adducts are more labile relative to the aliphatic adduct.

relative to the MDI−CME and TDI−CME. The distinct increase in the rate of the dissociation as the pH rises is apparent with the HDI−CME (see Figure 2i).

The kinetics for the aromatic adducts are comparable for pH 7.4 and pH 9. At these pH values, the hydrolysis is affected by the same mechanism, where the nucleophiles present in solution are H_2O, OH^-, and buffer-associated nucleophilic species. The reaction dynamics for the aromatic adducts were complex and exhibited a shift in the kinetics after the first 5 min. The MDI−CME hydrolysis slowed after the first 5 min, where the second-order rate constant dropped from 5.77×10^4 to 3.54×10^4 M^{-1} min^{-1} (Table 2).

Utilization of the integrated rate equation for second-order kinetics gave Figure 3. The aliphatic adduct gave a good second-order fit for all of the pH variations (Figure 3i). There was a noticeable deviation from second-order kinetics when the R group was aromatic (Figure 3ii). The second-order fit is in agreement with a combination of the three mechanisms (BAc2, E1cB, and E2), which are all second-order processes (*27*). In acidic conditions, the dynamics of the hydrolysis shifted with the possible protonation of the electron-rich sulfur atom of the CME. This resulted in a positively charged intermediate, which decomposed to furnish the thiol. Reactions R3i, ii, and iii outline the initial part of the hydrolysis, which produces the CME (R′SH). For pH 7.4 and above:

$$\mathrm{RDI-(SR')_2 + 2OH^- \xrightarrow{k_{OH^-}} 2R'S^- + RDI-(OH)_2} \quad \text{(R3i)}$$

$$\mathrm{RDI-(SR')_2 + 2H_2O \xrightarrow{k_{H_2O}} 2R'S^- + RDI-(O^+H_2)_2} \quad \text{(R3ii)}$$

and for pH < 7, the following pathway applies

$$\mathrm{RDI-(SR')_2 + 2H \xrightarrow{k_{H^+}} RDI-(S^+HR')_2 \rightarrow 2R'SH + RDI^{2+}} \quad \text{(R3iii)}$$

Table 2. Values of the Initial Rates and Overall Rate Constants for the Hydrolysis of the Conjugates at 298.15 K

	initial rates (mol/L min)			second-order plot rate constants (M^{-1} min^{-1})		
pH	HDI−CME	MDI−CME	TDI−CME	HDI−CME	MDI−CME (after 5 min)	TDI−CME
5	5×10^{-8}	7×10^{-6}	7×10^{-7}	30.47	7607.8 (2971.3)	6655.4
7.4	7×10^{-7}	2×10^{-5}	3×10^{-6}	335.92	57753 (35401)	24911
9	1×10^{-6}	3×10^{-5}	4×10^{-6}	1263.7	78909 (9214.8)	27778

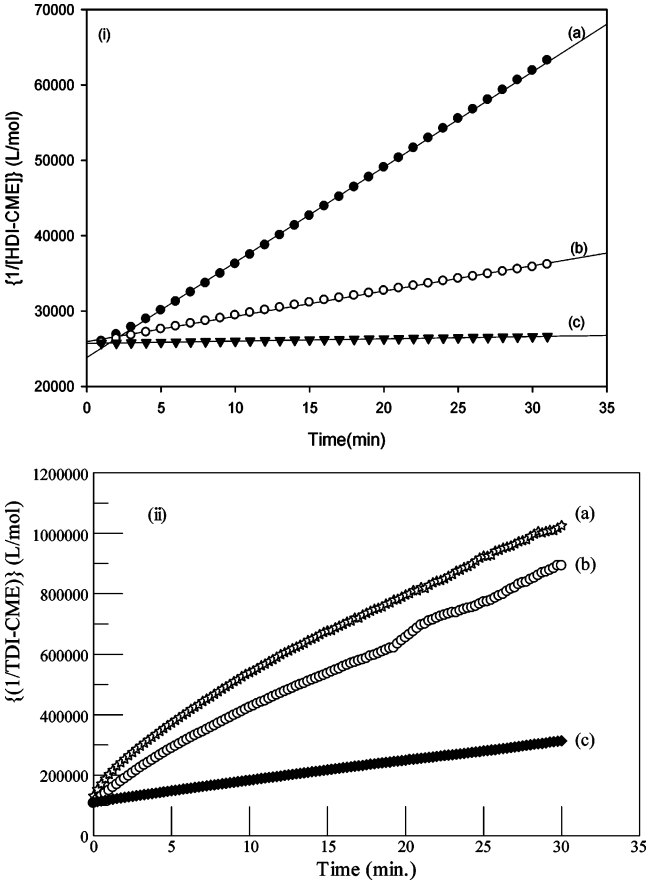


Figure 3. Second-order plots for the adducts (i) HDI−CME and (ii) TDI−CME observed at the different pH values (a, pH 5; b, pH 7.4; and c, pH 9). Note that for the aromatic bis-thiocarbamates two distinct dissociation rates are evident at pH 5 and pH 7.4, suggesting that both thiocarbamates on the molecule dissociate at different rates.

It was imperative to determine whether the hydrolysis was general acid/base- or specific acid/base-catalyzed. When the buffer was switched from the 0.1 M sodium phosphate buffer to glycine buffer (3 M, pH 7.4), a remarkable drop in the initial reaction rate was observed (phosphate, 7×10^{-6} M s^{-1}; glycine, 1.75×10^{-6} M s^{-1}). The phosphate anion was therefore a competing nucleophile in the system, and this showed that the hydrolysis is general base-catalyzed. The participation of buffers such as the hydrogen carbonate and phosphate buffer in the hydrolysis of thiocarbamate adducts and dNCOs has been reported (*28*). The fact that the buffer was reacting prompted the inclusion of the rate constant that was contributed by the phosphate anion. Equation R3iv introduces the participation of the phosphate anion in the overall kinetics of the system.

$$\mathrm{RDI-(SR')_2} + 2\mathrm{Nu^-} \xrightarrow{k_{\mathrm{Nu^-}}} 2\mathrm{R'S^-} + \mathrm{RDI-(Nu)_2} \quad \text{(R3iv)}$$

The overall reaction rate can be best represented by combining the different reacting species from equations R3i−iv in the system.

$$\frac{-\mathrm{d[RDI-(SR')_2]}}{\mathrm{d}t} = [\mathrm{RDI-(SR')_2}]\{k_{\mathrm{OH^-}}[\mathrm{OH^-}] + k_{\mathrm{H_2O}}[\mathrm{H_2O}] + k_{\mathrm{H^+}}[\mathrm{H^+}] + k_{\mathrm{Nu^-}}[\mathrm{Nu^-}]\} \quad \text{(E2)}$$

leading to the following equations

$$\frac{-\mathrm{d[RDI-(SR')_2]}}{[\mathrm{RDI-(SR')_2}]} = \{k_{\mathrm{OH^-}}[\mathrm{OH^-}] + k_{\mathrm{H_2O}}[\mathrm{H_2O}] + k_{\mathrm{H^+}}[\mathrm{H^+}] + k_{\mathrm{Nu^-}}[\mathrm{Nu^-}]\}\,\mathrm{d}t \quad \text{(E3)}$$

$$[\mathrm{RDI-(SR')_2}]_t = [\mathrm{RDI-(SR')_2}]_0\, e^{-k_{\mathrm{obs}}t} \quad \text{(E4)}$$

where

$$k_{\mathrm{obs}} = k_{\mathrm{H_2O}}([\mathrm{H^+}][\mathrm{OH^-}]/Kw) + k_{\mathrm{Nu^-}}[\mathrm{Nu^-}] + k_{\mathrm{OH^-}}(Kw/[\mathrm{H^+}]) + k_{\mathrm{H^+}}[\mathrm{H^+}] \quad \text{(E5)}$$

Invoking logarithms and applying that to eq E5 yields the following equation

$$-\log(k_{\mathrm{obs}}) = \mathrm{pH} - \log(k_t) \quad \text{(E6)}$$

where

$$k_t = \{k_{\mathrm{H^+}} + k_{\mathrm{OH^-}} + k_{\mathrm{H_2O}} + k_{\mathrm{Nu^-}} + [\mathrm{OH^-}] + [\mathrm{Nu^-}]\} \quad \text{(E7)}$$

A plot of the overall rate constants ($-k_{\mathrm{obs}}$) against the pH yields a linear plot for the MDI−CME adduct with a slope of 0.4068.

The values of the second-order rate constants (k_{obs}) given in Table 2 are indicative of the fact that the aromatic adducts will hydrolyze quickly in aqueous media and more so when the conditions are alkaline. To confirm the bimolecular nature of the reactions, a plot of the initial rates against the pOH for the adducts gave linear plots (Figure 2i insert) with corresponding rate constants (HDI−CME, 2.40×10^{-7}mol L^{-1} min^{-1}; MDI−CME, 5.72×10^{-6} mol L^{-1} min^{-1}; and TDI−CME, 8.35×10^{-7} mol L^{-1} min^{-1}). This indicates that the reaction is first-order with respect to [OH^-].

The half-lives given in Table 3 show that the order of stability of the thiocarbamates is HDI−CME > TDI−CME > MDI−CME where the TDI−CME is about nine times more stable than the MDI−CME at pH 7.4.

Table 3. Values of the Half Lives ($t_{1/2}$) for the Thiocarbamate Adducts at Different pH Values

	pseudo-first-order half-life (min)		
pH	MDI−CME	TDI−CME	HDI−CME
9	0.416	4.33	20.6
7.4	0.513	4.74	76.9
5	3.55	8.6	844

Table 4. Values of the Rate Constants for the Hydrolysis of the Adducts at pH 7.4 for the Various Temperatures

	second-order plot rate constants (M^{-1} min^{-1})		
temperature (K)	HDI−CME	MDI−CME	TDI−CME
277.15	43.178	1081.2	48622
298.15	335.92	7024.1	96633
310.15	1154.5	8121.7	446763

Table 5. Activation Energies for the Hydrolysis of Various Conjugates

conjugate	E_a (kJ/mol)
HDI−CME	70.604
TDI−CME	46.09282
MDI−CME	44.50152

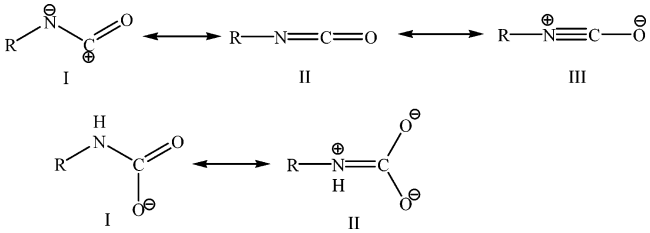
Effect of Temperature. When the temperature was varied from 277.15 to 310.15 K with the pH fixed at 7.4, the rate increased with temperature. The HDI−CME showed that it was the most stable of the three adducts as the temperature was changed. Table 4 lists the second-order rate constants for the three adducts at the different temperatures. Plotting $1/T$ against $\ln(k)$ gave the Arrhenius plots (eq E8) where A is the Arrhenius constant, R is the gas constant, and E_a is the activation energy. The data gave a reasonably good linear fit (data not shown) for HDI−CME, and there were slight deviations from linearity, possibly due to competing hydrolysis mechanisms for the aromatic adducts.

$$\ln(k) = A - \frac{E_a}{RT} \quad \text{(E8)}$$

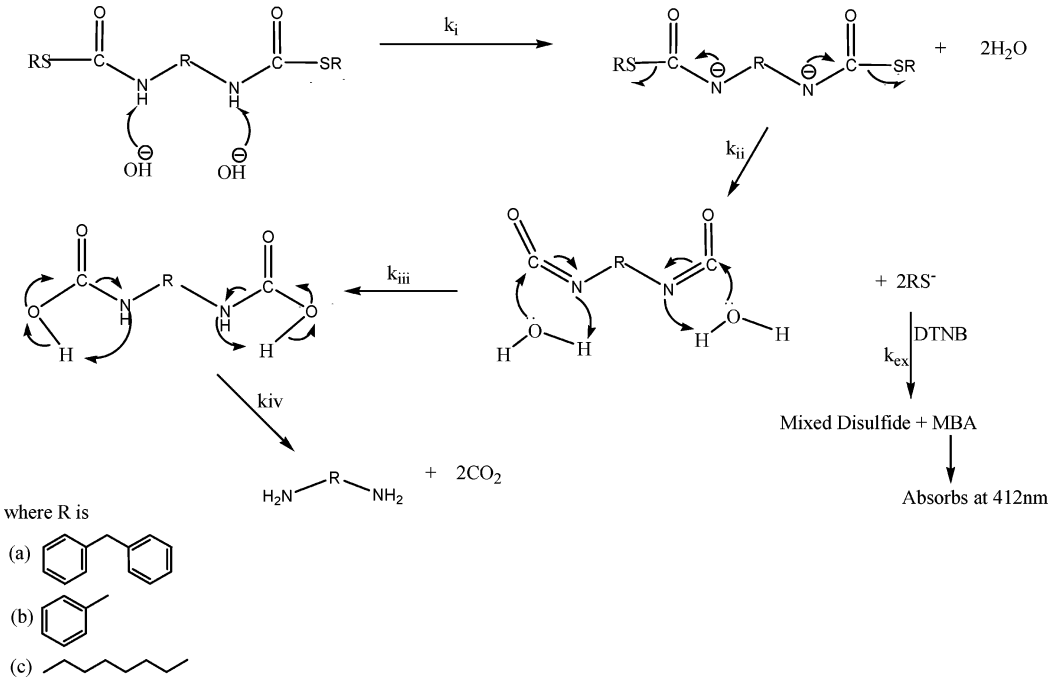
The temperature dependence plots and the corresponding activation energy values demonstrate that the aliphatic adduct is the most stable, with the highest E_a (Table 5). The activation energies of the aromatic adducts were comparable. These results are consistent with those of pH dependence with respect to the relative stability of these compounds.

Discussion and Conclusions

An inspection of the resonance structures of the isocyanate and the carbamic acid suggests that the isocyanate is relatively stable and an incoming electron donor such as an amine is more likely to attack the positively charged carbon center as the canonical form I of the isocyanate. The canonical forms of the carbamic acid are unstable relative to the isocyanate.



Scheme 3. Outline of the Major Steps in the E1cB Mechanism of the Hydrolysis of the Bis-thiocarbamates[a]



[a] The rate constant k_i is the rate determining constant.

A Mulliken population analysis reported for an isocyanate (*29*) gave a charge of +0.51 on the carbon atom. Important factors such as the polar protic solvent (H_2O/MeOH), the production of the thiolate anion (CME^-), which is a poor leaving group (LG) by virtue of its relatively high thiol pK_a value (pK_a = 6.6) (*30*), the lack of steric hindrance on the secondary amine of the bis-adducts, and the strength and size of the attacking nucleophiles (which are the small H_2O, $H_2PO_4^-$, and OH^- for pH 7 and above) all favor the E1cB mechanism of the hydrolysis as opposed to the BAc2. From Scheme 3, the rate-determining and important step would be the slow elimination of the thiolate ion in the second step (k_{ii}) to give the isocyanate. The first step, which is rapid, is a bimolecular process. The fact that the amine is a secondary amine with an acidic proton that can be abstracted favors an E1cB pathway where the conjugate base has the three canonical forms that stabilize it relative to the carbamic acid.

The first and second steps, if reversible, should have reversible rate constants that are infinitely small. Once the thiolate ion is released, it will rapidly react with the DTNB. The rate constant $k_{ex} \gg k_{ii} > k_i$ enables the rapid removal of the thiolate ion before it can attack the carbonyl to effect the reverse reaction, thus promoting the forward reactions of steps i and ii. The production of the amines introduces a complication to the dynamics of the system since it is also a nucleophile, which can participate in the reaction. After the initial stages of the hydrolysis, deviations from second-order would set in as the amine is produced.

At pH values lower than 7, the H^+ ions will begin to contribute to the hydrolysis as the nucleophiles are protonated. With H^+ as the attacking electrophile, the mechanism of the hydrolysis of the adducts changes from being a nucleophilic substitution (Scheme 3) to an electrophilic addition in the first step, which will be followed by the elimination of the R′SH in the second step. As in Scheme 3, the presence of DTNB ensures the quick removal of the RSH thereby minimizing the possibility of reverse reactions in both steps.

Ellman's reagent in 0.1 M phosphate buffer was used to examine the hydrolysis kinetics at all temperatures and pH values employed and eliminate any possible side reactions involving CME, including the regeneration of the thiocarbamate. This represented an isotonic system differing from blood in that the tonicity comes entirely from the phosphate, whereas the major contributor in blood is NaCl. The phosphate ion concentration is approximately 100× that of blood. This buffer was chosen because (i) it would not react with −N═C═O, (ii) it would adequately maintain [OH^-] over the entire pH range throughout the reaction time, and (iii) phosphate is a major contributor to the buffering capacity of biological systems.

The rate of thiocarbamate hydrolysis is pH-dependent and the overall kinetics of reaction appeared to be second-order. Deviations from the second-order kinetics were apparent with the aromatic adducts, and these could be attributed to solvent participation. A solvent that has hydrogen bonding will introduce a drift from the order of the reaction (*31*). Even though the acidic hydrogens on the amines of the adducts were both equally accessible to the nucleophiles, the nucleophilic attack was not a synchronous reaction. There was the possibility of one proton being abstracted first followed by the other. This tended to introduce consecutiveness in the reaction rates. The fact that the nucleophilic substitution is general base-catalyzed leads to a concerted nucleophilic attack on the carbonyl centers at pH values above 7, and this may introduce deviations from the simple second-order model. This trend was observed with the MDI−CME and TDI−CME.

The possibility of one thiocarbamate dissociating on two separate molecules and then the two molecules forming a dimer was considered. If this took place, then the second rate observed would be that of a larger molecule. This rate could be skewed by the hydrolysis of the thiocarbamates on this dimer. This possibility was ruled out by adding methylamine to the reaction mixture. The methylamine reacts with the free NCO to form a urea, preventing any side reactions/polymerization. With this addition, the rates of hydrolysis do not change significantly, suggesting that the isocyanate groups did not polymerize/react before completely hydrolyzing the second thiocarbamate functionality. No precipitation was observed in any of the reaction

mixtures, further suggesting that polymerization did not take place.

These rates are not the absolute rates of hydrolysis of the thiocarbamate. They are a good indicator of the relative stability of the thiocarbamates under physiological conditions. Previous studies on the hydrolysis rate of a *bis*-glutathione−TDI adduct were measured and treated with pseudo-first-order kinetics in the initial phase of the reactions and with apparent pseudo-second-order kinetics for the entire course of the reaction (*20*, *21*).

The mono- and bis-thiocarbamates may be important intermediates in the pathogenesis of dNCO asthma and/or other dNCO-mediated toxicities. At minimum, they prevent hydrolysis or direct reaction of the dNCO at the point of entry in the body. This may allow transport across cellular membranes. Alternatively, bis-thiocarbamates may have some protective effect since at least part of the hydrolysis proceeds through the BAc2 mechanism resulting in nonprotein reactive intermediates and products. In our previous studies (*32*) of Chinese hamster fibroblasts (V79) exposed to an MDI−GSH (relatively insoluble) adduct, we noted a dose-dependent increased micronuclei (MN) formation and number of cells in metaphase. Intracellular precipitants were also noted. MDI added directly to the cell culture did not result in increased MN, probably due to rapid hydrolysis and polymerization in the culture medium. MDI−GSH-induced MN had a significant increase in the number of anti-kineticore antibody labeled MN. These results suggest that MDI−GSH MN induction was mediated through disruption of microtubules (*32*). The ultimate asthmogenic hapten−protein complex may preferentially form through the thiocarbamate intermediate, rather than directly by reaction with the dNCO. Our present findings suggest that the hydrolysis of the bis-thiocarbamate in the presence of excess primary amine is more likely to form a monourea (i.e., like a monoisocyanate), while the dNCO readily forms diureas. This suggests less cross-linking of proteins and that a bis-thiocarbamate-derived haptenized protein would present with a free amine at one of the dNCO−hapten positions as observed in the present study. It has been noted that the monoisocyanate, phenyl isocyanate, was an order of magnitude more potent than TDI as both a contact (type IV) and a humoral (type I) sensitizer in a murine model (*33*). Phenyl isocyanate was also found to produce chronic airway inflammation in a rat model that had features characteristic of asthma (*34*).

In the body, cysteine is thought to be the most reactive endogenous thiol. When reacted with an isocyanate, a thiocarbamate is formed, which is labile under physiological conditions. Under laboratory conditions, thiocarbamate formed by the reaction of MDI and cysteine had poor solubility in water (or any organic solvent tested) and therefore could not be used to determine hydrolysis rates with the methods outlined here. The bis-thiocarbamate formed with glutathione was also very insoluble. However, when suspended in aqueous buffer, both show evolution of free thiol. The MDI−CME thiocarbamate is a very similar thiocarbamate, which is soluble in both water and methanol. Because of its solubility characteristics, it was used to determine the relative stability of the products under physiological conditions.

The rate of formation of the thiocarbamates under physiological conditions is unknown but has been reported to be among the most rapid dNCO reactions (*35*). The bis-thiocarbamate products formed, while subject to hydrolysis, are considerably more stable than the parent dNCO. The bis-thiocarbamate could be potentially carried from the lung to a distal site, whereupon hydrolysis would occur, releasing a free NCO that could react with another nucleophile. Evidence has been found that TDI can travel past the lung and bind to (intracellular) hemoglobin (*19*). Because the rates of hydrolysis of the different NCOs vary widely, their reactivity in the body may also vary. dNCO-derived bis-thiocarbamates have only recently been suggested as potential intermediates or mediators of dNCO-induced diseases (*20*). The work done here creates a basis of comparison for the different dNCO molecules that can be used to further research the ability of protected NCOs (such as thiocarbamates) to dissociate and react with other biomolecules at sites distal to the portal of entry.

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References

(1) Brown, W. E., Green, A. H., Cedel, T. E., and Cairns, J. (1987) Biochemistry of protein-isocyanate interactions: A comparison of the effects of aryl vs alkyl isocyanates. *Environ. Health Perspect. 72*, 5−11.
(2) Swensson, A., Holmquist, C. E., and Lundgren, K. D. (1955) Injury to the respiratory tract by isocyanates used in making lacquers. *Br. J. Ind. Med. 12* (1), 50−53.
(3) Upjohn Company (1970) *Urethanes: Engineering, Medical Control and Toxicologic Considerations*, Technical Bulletin No. 105, Upjohn Company, Kalamazoo, MI.
(4) Fisher, A. A. (1967) *Contact Dermatitis*, pp 134−135, Lea and Febiger, Philadelphia, PA.
(5) Charles, J., Bernstein, A., Jones, B., Jones, D. J., Edwards, J. H., Seal, R. M., and Seaton, A. (1976) Hypersensitivity pneumonitis after exposure to isocyanates. *Thorax 31* (2), 127−136.
(6) Fink, J. N., and Schlueter, D. P. (1978) Bathtub refinisher's lung: An unusual response to toluene diisocyanate. *Am. Rev. Respir. Dis. 118* (5), 955−959.
(7) Selden, A. I., Belin, L., and Wass, U. (1989) Isocyanate exposure and hypersensitivity pneumonitis—Report of a probable case and prevalence of specific immunoglobulin G antibodies among exposed individuals. *Scand. J. Work Environ. Health 15* (3), 234−237.
(8) Malo, J. L., Cartier, A., Desjardins, A., Van de, W. R., and Vandenplas, O. (1995) Occupational asthma caused by oak wood dust. *Chest 108* (3), 856−858.
(9) Fabri, G. (1997) The prevention of occupational asthma in industries. *J. Invest. Allerg. Clin. Immunol. 7* (5), 377−379.
(10) Rosenman, K. D., Reilly, M. J., and Kalinowski, D. J. (1997) A state-based surveillance system for work-related asthma. *J. Occup. Environ. Med. 39* (5), 415−425.
(11) Wisnewski, A. V., Meredith H. S., Cartier, A., Liu, Q., Liu, J., Chen, L., and Redlich, C. A. (2004) Isocyanate vapor-induced antigenicity of human albumin. *J. Allergy Clin. Immunol. 113* (6), 1178−1184.
(12) Karol, M. H., Tollerud, D. J., Campbell, T. P., Fabbri, L., Mastrelli, P., Saetta, M., and Mapp, C. E. (1994) Predictive value of airways hyperresponsiveness and to circulating IgE for identifying types of responses to toluene diisocyanates inhalation challenge. *Am. J. Respir. Crit. Care Med. 149* (3 Part 1), 611−615.
(13) Baur, X., Marek, W., Ammon, J., Czuppon, A. B., Marczynski, B., Raulf-Heimsoth, M., Roemmelt, H., and Fruhmann, G. (1994) Respiratory and other hazards of isocyanates. *Int. Arch. Occup. Environ. Health 66* (3), 141−152.
(14) Matheson, J. M., Lemus, R., Lange, R. W., Karol, M. H., and Luster, M. I. (2002) Role of tumor necrosis factor in toluene diisocyanate asthma. *Am. J. Respir. Cell Mol. Biol. 27* (4), 396−405.
(15) Choi, J. H., Nahm, D. H., Kim, S. H., Kim, Y. S., Suh, C. H., Park, H. S., and Ahn, S. W. (2004) Increased levels of IgG to cytokeratin 19 in sera of patients with toluene diisocyanate-induced asthma. *Ann. Allergy, Asthma, Immunol. 93* (3), 293−298.
(16) Jin, R. Z., and Karol, M. H. (1988) Diisocyanate antigens that detect specific antibodies in exposed workers and guinea pigs. *Chem. Res. Toxicol. 1* (5), 288−293.
(17) Aul, D. J., Bhaumik, A., Kennedy, A. L., Brown, W. E., Lesage, J., and Malo, J. L. (1999) Specific IgG response to monomeric and polymeric diphenylmethane diisocyanate conjugates in subjects with

respiratory reactions to isocyanates. *J. Allergy Clin. Immunol. 103* (5 Part 1), 749−755.
(18) Wisnewski, A. V., Lemus, R., Karol, M. H., and Redlich, C. A. (1999) Isocyanate-conjugated human lung epithelial cell proteins: A link between exposure and asthma? *J. Allergy Clin. Immunol. 104* (2 Part 1), 341−347.
(19) Lange, R. W., Day, B. W., Lemus, R., Tyurin, V. A., Kagan, V. E., and Karol, M. H. (1999) Intracellular S-glutathionyl adducts in murine lung and human bronchoepithelial cells after exposure to diisocyanatotoluene. *Chem. Res. Toxicol. 12* (10), 931−936.
(20) Day, B. W., Jin, R., Basalyga, D. M., Kramarik, J. A., and Karol, M. H. (1997) Formation, solvolysis, and transcarbamoylation reactions of bis(S-glutationyl) adducts of 2,4- and 2,6-diisocyanatotoluene. *Chem. Res. Toxicol. 10*, 424−431.
(21) Reisser, M., Schmidt, B. F., and Brown, W. E. (2002) Synthesis, characterization, and solvolysis of mono- and bis-S-(glutathionyl) adducts of methylene-bis-(phenylisocyanate) (MDI). *Chem. Res. Toxicol. 15* (10), 1235−1241.
(22) Snyder, S. L., and Sobocinski, P. Z. (1975) An improved 2,4,6-trinitrobenzenesulfonic acid method for the determination of amines. *Anal. Biochem. 64* (1), 284−288.
(23) Mraz, J., and Bouskova, S. (1999) 2,4-toluenediisocyanate and hexamethylene-diisocyanate adducts with blood proteins: Assessment of reactivity of amino acid residues in vitro. *Chem.-Biol. Interact. 117* (2), 173−186.
(24) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5′-Dithiobis(2-nitrobenzoic acid)—A reexamination. *Anal. Biochem. 94* (1), 75−81.
(25) Baker, J. W., and Gaunt, J. (1949) The mechanism of the reaction of the aryl isocyanates with alcohols and amines. Part IV. *J. Chem. Soc. 19*, 24−27.
(26) Aresta, M., Ballivet-Tkatchenko, D., Belli Dell'Amico, D., Boschi D., Calderazzo, F., Labella, L., Bonnet, M. C., Faure, R., and Marchetti, F. (2000) Isolation and structural determination of two derivatives of the elusive carbamic acid. *Chem. Commun. 13*, 1099−1101.
(27) Baillie, T. A., and Slatter, J. G. (1991) Glutathione: A vehicle for the transport of chemically reactive metabolites in vivo. *Acc. Chem. Res. 24*, 264−270.
(28) Berode, M., Testa, B., and Savolainen, H. (1991) Bicarbonate-catalyzed hydrolysis of hexamethylene diisocyanate to 1,6-diaminohexane. *Toxicol. Lett. 56* (1−2), 173−178.
(29) Raspoet, G., Nguyen, M. T., McGarraghy, M., and Hegarty, A. F. (1998) Experimental and theoretical evidence for a concerted catalysis by water clusters in the hydrolysis of isocyanates. *J. Org. Chem. 63* (20), 6867−6877.
(30) Burner, U., Jantschko, W., and Obinger, C. (1999) Kinetics of oxidation of aliphatic and aromatic thiols by myeloperoxidase compounds I and II. *FEBS Lett. 443* (3), 290−296.
(31) Ephraim, S., Woodward, A. E., and Mesrobian, R. B. (1958) Kinetic studies of the reaction of phenyl isocyanate with alcohols in various solvents. *J. Am. Chem. Soc. 80*, 1326−1328.
(32) Zhong, B. Z., Depree, G. J., and Siegel, P. D. (2001) Differentiation of the mechanism of micronuclei induced by cysteine and glutathione conjugates of methylenedi-p-phenyl diisocyanate from that of 4,4′-methylenedianiline. *Mutat. Res. 497* (1−2), 29−37.
(33) Karol, M. H., and Baillie, T. A. (1996) Phenyl isocyanate is a potent chemical sensitizer. *Toxicology 4* (2), 157−161.
(34) Pauluhn, J., Rungeler, W., and Mohr, U. (1995) Phenyl isocyanate-induced asthma in rats following a 2-week exposure period. *Fundam. Appl. Toxicol. 24* (2), 217−228.
(35) Lantz, R. C., Lemus, R., Lange, R. W., and Karol, M. H. (2001) Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. *Toxicol. Sci. 60* (2), 348−355.

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