EXCLI Journal 2006;5:191-208 – ISSN 1611-2156
Received: 2. November 2006, accepted: 11. November 2006, published: 20. November 2006

Original article:

INTERACTIONS OF AROMATIC ISOCYANATES WITH N-ACETYL-L-CYSTEINE UNDER PHYSIOLOGICAL CONDITIONS: FORMATION OF CONJUGATES, UREAS AND AMINES

Werner Mormann [1]*, Rosario Lucas Vaquero [1], Klaus Seel [2]

[1]Universität Siegen FB 8 Chemie/Biologie, Laboratorium für Makromolekulare Chemie, Adolf-Reichwein-Str. 2, 57076 Siegen, Germany; [2]formerly: Bayer AG, Polyurethane Division, 51368 Leverkusen, Germany. *Corresponding author (Email address: mormann@chemie.uni-siegen.de, phone: +49 271 7404713)

ABSTRACT

Isocyanates are of toxicological relevance since they are considered to cause occupational asthma. The majority of polyurethanes is based on aromatic diisocyanates (e.g., TDI and MDI), they are used for foams, elastomers, adhesives and coatings. Therefore we studied reactions of p-tolylisocyanate (pTI), 2,4-toluene diisocyanate (TDI) and 4,4'-methylenediphenyl diisocyanate (MDI) with N-acetyl-L-cysteine (AcCys) with different molar ratios in aqueous buffer solutions of pH 5.0 – 7.4. Type and amounts of products formed in these reactions were identified and quantified. Conjugates of AcCys and the aromatic isocyanates have been synthesized and characterized as reference materials. Conjugates and ureas were found to be the main products. The ratio of these two compounds varied with the ratio of AcCys to isocyanate. Approximately 90 % of pTI conjugate were found for the 10 : 1 ratio, approximately 40 % conjugate for the 10 : 5 and around 15 % for the 10 : 15 ratio. For TDI yields of conjugate were comparable. Ureas, apart from minor amounts of TDA-urea could not be determined quantitatively due to formation of oligomeric ureas with different end groups. Minor amounts of MDI-conjugates were found apart from high amounts of insoluble material, which proved to be unreacted MDI encapsulated by oligomeric ureas. The reaction of the –SH group with the isocyanate moiety is independent of the pH of the solution in the range studied. No diamine, i.e. 2,4-TDA or 4,4'-MDA, could be detected in reactions of the diisocyanates 2,4-TDI or 4,4'-MDI. Small amounts of p-toluidine (pTA) were found in the reaction of the mono isocyanate pTI when it was in excess with respect to AcCys. Reactions of the isocyanates with an aqueous buffer solution of pH 6.5 in the absence of AcCys gave ureas as main products, while significant amounts of unreacted diisocyanates remained encapsulated in the mixture. No 2,4-TDA or 4,4'-MDA was detected under these conditions. Again small amounts of pTA were formed from the reaction of pTI with water.

Key words: Aromatic isocyanates, cysteine-S-conjugates, ureas, oligomers,aromatic amines

INTRODUCTION

Aromatic isocyanates are highly reactive compounds that undergo nucleophilic attack by a variety of functional groups (e.g. NH_2, NH, OH, SH or COOH) (Oertel, 1993; Brochhagen, 1991; Saunders and Frisch, 1962). As most of these functional groups are also present in aqueous biological systems, aromatic as well as aliphatic isocyanates can either interact with biomolecules or undergo non-enzymatic hydrolysis. If, for example, an aqueous medium contains reactive biological compounds such as amino acids, peptides and proteins, competing reactions of isocyanates with water and the functional

group of the biomolecules can be assumed (Brown et al., 1987; International Isocyanate Institute, 1999; Mráz and Bousková, 1999; Schuetze et al., 1995; Zhong et al., 2001). Products formed may be ureas, polyureas, amines and/or conjugates ("bioureas", "biothiocarba-mates"). Most of the work published to date has been devoted to 2,4-TDI or 4,4'-MDI. Practically no experimental data are available on the type and amount of reaction products formed under physiological conditions.

Reactions of aromatic mono- and diisocyanates in aqueous solutions of glycine (Gly) under simulated physiological pH conditions have been studied by Seel (Seel and Kuck, 2001). During *in vitro* reactions of 4,4'-MDI or 2,4-TDI in aqueous, buffered solutions of glycine they found that Gly_2MDI or Gly_2TDI as well as the ureas (mainly polyureas) of MDI and TDI were the main products formed. No detectable amounts of 4,4'-methylene dianiline (MDA) or 2,4-diaminotoluene (TDA) were generated. However, reaction of monofunctional phenylisocyanate (PhI) with glycine under these conditions led to the generation of minor amounts of amine, in this case aniline, besides the main products GlyPhI and diphenylurea.

Glutathione (GSH) S-conjugation appears to be one of the most salient mechanisms involved in isocyanate metabolism (Zhong et al., 2001; Day et al., 1997;Pauluhn et al., 2006; Reisser et al., 2002). Inhalation is a predominant route of exposure (Sabbioni et al., 1997). Several attempts have been made to investigate the chemistry of these rather complex systems; so far they do not provide detailed quantitative information on the individual compounds formed.

Investigation of reactions of MDI and TDI in aqueous solutions is challenging as both di-isocyanates easily react with water while being poorly soluble. By consequence reaction takes place at the interface between two immiscible phases. The ratio of interfacial area and weight (volume) of isocyanates depends on the size of particles (droplets) being in contact with body fluids. Simplified reactions mimicking physiological conditions are necessary to understand the whole scope of isocyanate-tissue interaction.

Although it is generally accepted that the simple hydrolysis products of MDI and TDI, MDA and TDA, are not major metabolites, the question whether and to what extend they are formed still needs to be answered (Reisser et al., 2002; Deutsche Forschungsgemeinschaft, 1992; Deutsche Forschungsgemeinschaft, 1999; Bundesan-stalt fuer Arbeitsschutz und Arbeits-medizin, 2000). Especially for TDI it was recognized that the question of the TDA formation by hydrolysis of TDI needs to be answered to allow for a quantitative assessment of cancer risk after inhalation of TDI at the workplace (Deutsche Forschungsgemeinschaft, 1999).

In the present work N-acetyl-L-cysteine (AcCys), the simplest model compound of a thiofunctional biomolecule, was chosen to study the interactions of sulfhydryl groups containing peptides with aromatic isocyanates. Competing reactions of the isocyanate with thiol and with the aqueous medium are expected to occur.

The objective of this investigation was to identify and quantify the chemical products formed when TDI and MDI react under heterogeneous conditions with aqueous solutions of AcCys. The reaction conditions chosen were those that could be encountered when isocyanates come into contact with biological fluids, during skin contact or inhalation, which corresponds to pH values between 5.0 and 7.4.

Monofunctional pTI was included in this study as a model substance. Its advantage, in comparison to diisocyanates, is that no polymerization reactions will occur in an aqueous environment, thus neither complex mixtures of conjugates nor polymeric ureas

are likely to be formed, and reference compounds are readily available.

MATERIALS AND METHODS

Chemicals

2,4-toluene diisocyanate (2,4-TDI, Desmodur® T100), CAS-No. 584-84-9 and 4,4'-methylenediphenyl diisocyanate (4,4'-MDI, Desmodur® 44M), CAS-No. 101–68–8 were obtained from Bayer AG, Germany; *p*-tolylisocyanate (pTI), CAS-No. 622-58-2 and N-acetyl-L-cysteine (AcCys), purity >99%; CAS-No. 61-91-1 from Fluka; 2,4-diaminotoluene (2,4-TDA), CAS-No. 95-80-7 and 4,4'-methylene dianiline (4,4'-MDA), CAS-No. 101-77-9 from Bayer AG; p-toluidine (pTA),CAS-No.106-49-0 from Fluka, purity >99%; N,N'-bis-(3-amino-4-methylphenyl)urea (4,4'-TDA-urea), CAS-No. 101086-45-0 and N,N'-di-p-tolylurea (pT-urea) CAS-No. 621-00-1 from Bayer AG.

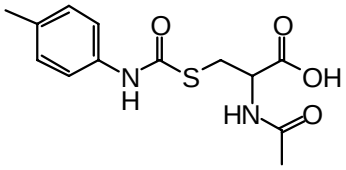
Solvents: Dimethylacetamide (DMAc) was distilled first over phosphorus pentoxide then over MDI. pTI, 2,4-TDI, and 4,4'-MDI were distilled in vacuo prior to use.

Synthesis of conjugates (general procedure)

20 mmol of pTI (10 mmol of 2,4-TDI, or 4,4'-MDI), 22 mmol of N-acetyl-L-cysteine (33 mmol AcCys and 2 mg DABCO for 2,4-TDI) in 12 - 15 ml of dry DMAc were stirred for 2 h at 40 °C (18 h, in the case of 2,4-TDI). As no precipitate was formed the solvent DMAc was removed in vacuo. Work-up procedures are described below for the individual conjugates.

p-Tolylisocyanate-N-acetyl-L-cysteine-conjugate (pTIAcCys) ;S-(4-tolylaminocarbonyl)-N-acetyl-L-cysteine

The residue obtained after removal of DMAc was purified by extraction with boiling diethylether in a Soxhlet apparatus. Both the extracted part and the remaining residue had the same composition and gave a sharp melting point. They consisted of an adduct which contained 1 mol of DMAc per mol of conjugate.



Yield: 4.03 g (68 %)

m.p.: DSC:115°C (190°C decomposition), microscope: 111-113 °C

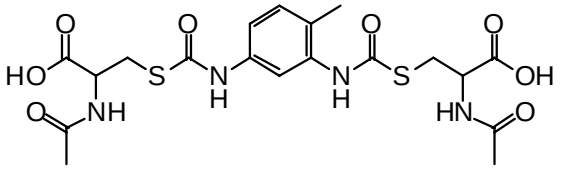
^{1}H-NMR$_{(DMSO-d6)}$: 1.84 (s, 3H, C**H**$_3$-CO); 2.21 (s, 3H, C**H**$_3$-Ph); 3.09 (dd, 1H, -C**H**$_2$-S); 3.37 (dd, 1H, -C**H**$_2$-S); 4.37 (m, 1H, -C**H**-CH$_2$); 7.10 (d, 2H, arom.); 7.35 (d, 2H, arom.); 8.32 (d, 1H, -N**H**-CO-CH$_3$); 10.24 (s, 1H, -N**H**-Ph)

^{13}C-NMR: 0.67 (**C**H$_3$-Ph); 22.64 (**C**H$_3$-CO); 30.57 (-**C**H$_2$-S); 52.11(-**C**H-CH$_2$); 119.33, 129.55, 132.71, 136.71 (arom.); 164.03 (-**C**O-S); 169.70 (-**C**O-CH$_3$); 172.262 (-**C**OOH)

MS m/z (%): 297.1 (100, [M+1]$^+$), 253 (23), 164 (48)

2,4-Diisocyanatotoluene-N-acetyl-L-cysteine-conjugate (TDI (AcCys)$_2$) 2,4-bis-[S-(N-acetyl-L-cysteinyl)-carbonylamino]-toluene

The highly viscous residue, obtained after removal of DMAc, was stirred several times with cold water containing 1 ml 2 N hydrochloric acid in 100 ml water and dried over phosphorous pentoxide.



Yield: 2.0 g (50 %)

m.p.: DSC: 130°C (203°C decomposition), microscope: 134.5-135.5°C

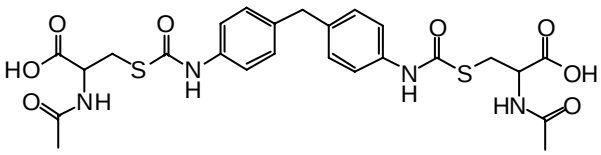
^{1}H-NMR$_{(DMSO-d6)}$: 1.84 (s, 6H, C**H**$_3$-CO); 2.11 (s, 3H, C**H**$_3$-Ph); 3.02 (dd, 2H, -C**H**$_2$-S); 3.32 (dd, 2H, -C**H**$_2$-S); 4.35 (m, 2H, -C**H**-CH$_2$); 7.13, 7.21, 7.35 (m, 3H, arom.); 8.30 (d, 2H, -N**H**-CO-CH$_3$); 9.76 (s, 1H, -N**H**-Ph); 10.35 (s, 1H, -N**H**-Ph)

^{13}C-NMR: 17.61 (**C**H_3-Ph); 22.72 (**C**H_3-CO); 30.95 (-**C**H_2-S); 52.63 (-**C**H-CH_2); 117.1, 122.2, 130.9, 136.4, 137.2 (arom.); 164.26 (**C**O-S); 169.82 (-**C**O-CH_3); 172.24 (-**C**OOH)

MS m/z (%): 501.1 (100, $[M+1]^+$), 482.9 (3), 371.9 (2), 163.9 (7)

4,4'-Diisocyanatodiphenylmethane-N-acetyl-L-cysteine-conjugate (MDI(AcCys)$_2$) Bis[4-S-(N-acetyl-L-cysteinyl)carbonylaminophenyl]-methane

MDI(AcCys)$_2$ was treated three times with boiling chloroform, filtered off and dried at 60 °C in vacuo.



Yield: 2.7 g = 51%

m.p.: DSC: 174 °C (202 °C decomposition), microscope: 158-160 °C

^{1}H-NMR$_{(DMSO-d6)}$: 1.84 (s, 6H, C**H**$_3$-CO); 3.04 (dd, 2H, C**H**$_2$-S); 3.35 (dd, 2H, -C**H**$_2$-S); 3.79 (s, 2H, -C**H**$_2$-Ph); 4.37 (m, 2H, -C**H**-S); 7.12 (d, 4H, arom.); 7.38 (d, 4H, arom.); 8.29 (d, 2H, -N**H**-CO-CH_3); 10.28 (s, 2H, -N**H**-Ph); 12.66 (-COO**H**).

^{13}C-NMR: 22.72 (**C**H_3-CO); 30.96 (-**C**H_2-S); 52.58 (-**C**H_2-S-); 79.48 (-**C**H_2-Ph), 119.6, 129.4, 136.7, 137.2 (arom.); 164.16 (-**C**O-S); (169.80 (-**C**O-CH_3); 172.29 (-**C**OOH)

MS m/z (%): 577.1 (100, $[M+1]^+$), 558.9 (52), 388 (24), 163.9 (8)

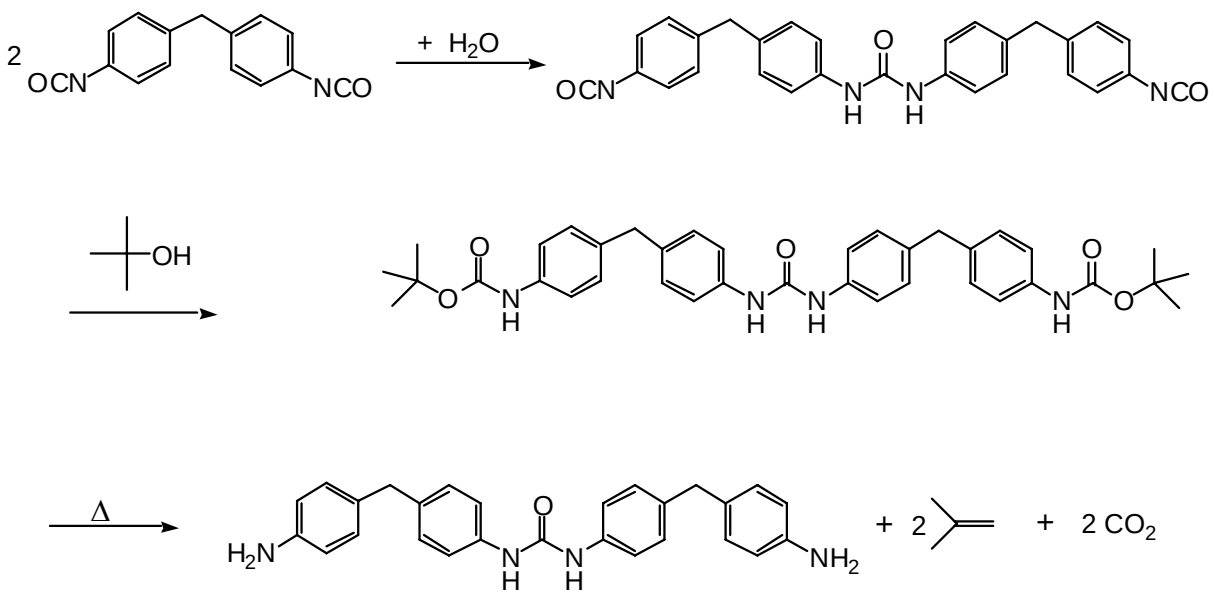
Synthesis of MDA-urea

MDA-urea was synthesized according to the reaction sequence shown in scheme 1. To a solution of 25 g of 4,4'-MDI in 100 mL toluene in a 250 mL flask were added 27.27 g of ethyl acetate saturated with water (3,3 weight percent) and the mixture was stirred at room temperature for 20 h. The diisocyanato urea, which precipitated as white crystals from the solution, was isolated by filtration, washed with toluene and dried in vacuum.

5.71 g of the MDI-urea were dissolved in 20 mL dimethylacetamide, 18 mL *tert*-butanol (15 fold excess) and one drop of dibutyltindilaurate as catalyst were added. When the reaction was complete, the isocyanate absorption in the infrared spectrum had disappeared, excess *tert*-butanol was distilled off, several crystals of p-toluene sulfonic acid were added and the mixture was heated to reflux until no more gas was evolved. The precipitate formed was filtered off and dried in vacuum. According to the proton nmr-spectrum it was MDA-urea with less than 4 % of residual *tert*-butyl urethane groups. This material was used for the calibration of the HPLC.

Stock and buffer solutions

Stock solutions (0.1 molar) were prepared by dissolving 8.195 g (50 mmol) AcCys in approximately 400 mL aqueous buffer solution in a 500 mL volumetric flask, adjusting the pH with 0.2 molar NaOH and filling to the mark with the appropriate buffer solution. Four different solutions that covered an alkalinity or acidity range from pH 7.4 to pH 5.0 were prepared as phosphate buffers with pH 7.4, pH 6.5, pH 5.7 and pH 5.0 respectively. After addition of AcCys the pH was adjusted to the theoretical value by addition of NaOH solution. All solutions were used within 24 hours.



Scheme 1: Synthesis of MDA-urea.

*Reaction of isocyanates with aqueous N-acetyl-L-cysteine solutions***.**
Depending on the ratio of N-acetyl-L-cysteine to isocyanate groups 50 mL, 25 mL and 10 ml stock solutions (containing 5 mmol, 2.5 mmol or 1 mmol AcCys) in a reaction flask were stirred slowly at 37 °C. To each of the flasks a different quantity of an aromatic isocyanate was added dropwise over approximately 30 seconds through a microliter syringe leading to molar ratio of AcCys : isocyanate groups of 10 : 1, 10 : 5 and 10 : 15 respectively. The exact amount of isocyanate added was determined by weighing the syringe before and after addition of the isocyanate.

pTI and 2,4-TDI are liquids at ambient temperature, 4,4'-MDI was liquefied at 60 °C. After addition of the isocyanate each reaction mixture was stirred at 37 °C for 2 h. The precipitates were filtered off and processed for gravimetric quantification and identification. The filtrates were analysed and quantified by HPLC/UV within 1.5 h after the end of the reaction. Retention times of the reference compounds were used to identify the main reaction products.

Instrumental Analysis
^{1}H- and ^{13}C-nmr-spectra were obtained with a Bruker AC-200FT-spectrometer (200 MHz for proton and 50.3 MHz for ^{13}C-spectra). DMSO-d6 was used as solvent. The signal of the non-deuterated impurities (= 2,49 ppm) was used as internal standard for proton nmr, the signal of the methyl-carbon (= 39.7 ppm) as reference for ^{13}C-spectra.

Mass spectra were made with a Finnigan MAT 8200 double focussing sector field mass spectrometer using the DCI or FAB ionisation method.

Thermal properties were investigated on a Mettler TC 11/15 system with a DSC 30 unit and a TG 50 unit. Experiments were run under nitrogen, heating rate was 10 K/min.

HPLC analyses were performed on an Agilent 1100 Series liquid chromatographic system consisting of a model G1312A binary pump, a G1379A vacuum degasser, a G1313A autosampler, a G1315B diode array detector (UV), and Agilent ChemStation data handling program (Agilent Technologies). The reaction mixtures were chromatographed on a Zorbax 300SB-C18, 150 mm, 4.6 mm i.d reversed phase C18 analytical column.

Injection volumes varied from 0.1 to 100 µL. A binary gradient mixture of water/acetonitrile in a mass ratio of 98:2 containing 0.1 % HCOOH (volume), and acetonitrile with 0.1 % HCOOH (volume) was used. Total flow rate was 1 mL per minute. The gradient used is described in table 1 starting with 98 percent of water, then with a linear gradient to 100 percent of acetonitrile. This gradient was used for all three isocyanates.

Table 1: Gradients for separation of reaction products

Gradient 1 water / acetonitrile			**Gradient 2 water / methanol**		
t/min	% acetonitrile	Flow mL/min	t/min	% methanol	Flow mL/min
0	2	1	0	10	1
4	2	1	10	40	1
15	100	1	15	100	1
17	100	1	20	100	1

For the calibration of 2,4-diaminotoluene (TDA) a different solvent combination and different gradient were used. Solvent A was water and solvent B was methanol. Samples from reactions of TDI were run in either solvent combination. The lowest amounts of amines that could be detected were 0.1 nmol for TDA and MDA, 0.15 nmol for p-toluidine. Retention times of reference compounds are included in table 2.

For calibration, solutions of each of the reference compounds in acetonitrile were prepared and amounts between 0.1 µL and 40 µL were injected. Detector wavelength was 214 nm. Reproducibility was checked by repeated injection and correlation of injection volume with detector response. Impurities in the reference compounds of less than 5 % (TDA-urea, TDI-conjugate) were neglected. For each of the reference materials a linear correlation between detector response (peak area) and concentration was established.

Table 2: Reference compounds and retention times

Reference compound	**Rt / min.**	**Reference compound**	**Rt / min.**
AcCys	3.3	$TDI(AcCys)_2$	8.8; 9.2; (9.6)
$(AcCys)_2$	7.6	2,4-TDA*	5.1
		TDA-urea	8.3
pTIAcCys	11.0	$MDI(AcCys)_2$	10.9
p-toluidine	4.3	4,4'-MDA	4.2
pTI-urea	13.1	MDA-urea	10.0

* gradient 2

RESULTS AND DISCUSSION

Synthesis of conjugates of N-acetyl-L-cysteine with pTI, 2,4-TDI and 4,4'-MDI

Conjugates of aromatic mono- or diisocyanates with N-acetylcysteine have not been described in the literature so far. Only the *p*-tolylisocyanate N-acetyl-L-cysteine conjugate has been reported (Sabbioni et al., 1997). These authors reacted AcCys under aqueous, slightly alkaline conditions with excess pTI, filtered off the insoluble part and obtained the product after acidification and recrystallization from water/ethanol mixtures. Target of their work were potential biomarkers of isocyanates.

In this study, dimethylacetamide (DMAC) was chosen as reaction medium in order to have a homogeneous reaction as both the starting materials and the reaction products were soluble in this organic solvent. Stoichiometric amounts of reactants could be used under these conditions and formation of undesired by-products was reduced. Furthermore the carboxyl group of AcCys does not react with the isocyanate under these non-alkaline conditions. Complete reaction was achieved in 2 hours except for 2,4-TDI where the isocyanate group in the 2-position reacts rather slowly. The use of excess AcCys and of a crystal of DABCO (1,4-diazabicyclo[2.2.2]octane) as catalyst together with stirring overnight resulted in complete reaction of the isocyanate groups in this case, too. Work up of the crude reaction products caused major difficulties. Precipitation in non-polar liquids or in water/methanol mixtures did not result in crystalline material. Gels formed especially in the case of TDI(AcCys)$_2$; therefore the DMAC was removed in vacuo below 60 °C, although the last traces were difficult to remove.

A crystalline conjugate was obtained from pTI It contained one mol of dimethylacetamide per mol conjugate that could not be removed by recrystallization or in vacuo (cf. NMR-spectrum in fig. 1). Attempts to achieve recrystallization from water/ethanol as reported by Sabbioni (Sabbioni et al., 1997) were not successful because the material dissolved in both.

The TDI-conjugate was obtained as a highly viscous non-crystallizing material after removal of solvent and purified by trituration with slightly acidic water in order to remove excess AcCys. The powder thus obtained was stirred in boiling chloroform, which, however, proved to be unnecessary as no changes in the infrared (IR) and NMR-spectra were observed. The MDI-conjugate was purified by trituration with boiling chloroform. All conjugates were characterized by IR, NMR-, mass-spectroscopy (MS) and thermal analysis (DSC). The analytical data proved that the desired materials had been obtained.

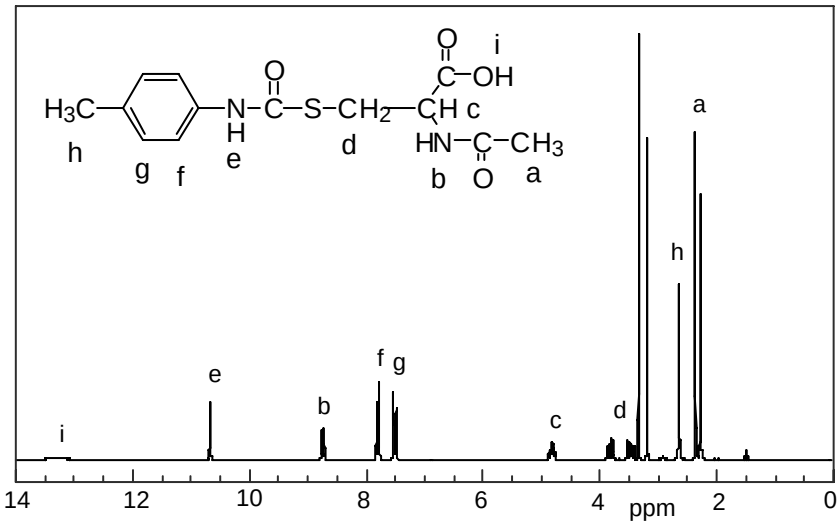


Figure 1: Proton NMR-spectrum of pTI-N-acetyl-L-cysteine conjugate (letters indicating peaks correspond to protons in the chemical formula indicated with those letters).

The conjugates of the three isocyanates are colorless powders. Only the pTI-conjugate gives a sharp melting point when heated under the microscope and a sharp melting endotherm in the DSC-trace. This is probably due to the fact that the other two conjugates could not be recrystallized and had to be isolated by precipitation in a non-solvent. The DSC-trace of MDI(AcCys)$_2$ shows a glass transition of around 110 °C followed by recrystallization and a melting peak at 180 °C. All conjugates have an additional

endotherm around 200 °C which is most likely due to the reverse reaction and to evaporation of volatiles at higher temperature.

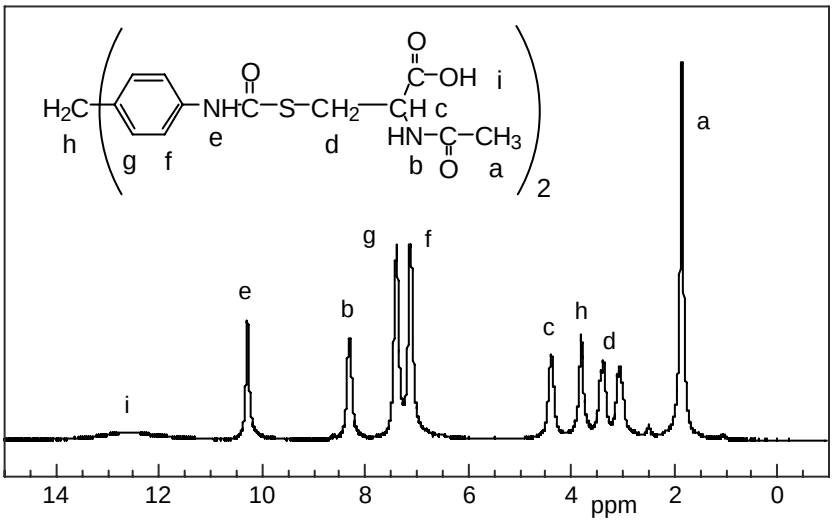


Figure 2: Proton NMR-spectrum of MDI-N-acetyl-L-cysteine conjugate (letters indicating peaks correspond to protons in the chemical formula indicated with those letters).

NMR-spectra of conjugates were made in deuterated dimethylsulfoxide as solvent. They show the characteristic resonances of the protons associated with the acetyl cysteine, plus resonances of the isocyanate part of the molecules. The protons attached to the nitrogen of acetyl cysteine and to the nitrogen of the thiocarbamate have two distinct resonances at 8.8 and 10.8 ppm which are slightly different for the MDI conjugate (cf. figures 1 and 2). From the NMR-spectrum of the pTI conjugate the resonances of DMAC can be identified at 2.2 and 3.3 ppm (fig.1).

The mass spectra confirmed the structure of the conjugates giving correct molecular ions and similar fragmentation patterns for all compounds. The conjugates were stored under nitrogen and were stable over several months.

Reaction of N-acetyl-L-cysteine with aromatic isocyanates under aqueous conditions

N-acetyl-L-cysteine and aromatic isocyanates were reacted in three different molar ratios in aqueous media under different, simulated physiological conditions corresponding to the possible exposure scenarios. Acidity/alkalinity levels from pH 5.0 to pH 7.4 were selected to represent skin contact as well as inhalation conditions. The latter was mirrored by low isocyanate concentrations, i.e. N-acetyl-L-cysteine:isocyanate ratios >1, whereas skin contact was simulated by increased isocyanate levels, e.g. SH:NCO ratios <1.

Experiments were made with 50 mL (ratio 10:1), 25 mL (ratio 10:5) and 10 mL (ratio 10:15) of 0.1 molar stock solution of AcCys in appropriate round bottom flasks. 0.5 mmol, 1.25 mmol, or 1.5 mmol of pTI and equivalent amounts of 2,4-TDI or 4,4'-MDI were added to the stock solutions of pH 5.0, pH 5.7, pH 6.5 and pH 7.4, respectively. The molar ratios are identical to the ratios of the functional groups SH:NCO. Reactions were complete after stirring the mixtures for two hours at 37 °C. Test runs conducted over an extended period of time confirmed these findings. Product profiles and yields remained unchanged.

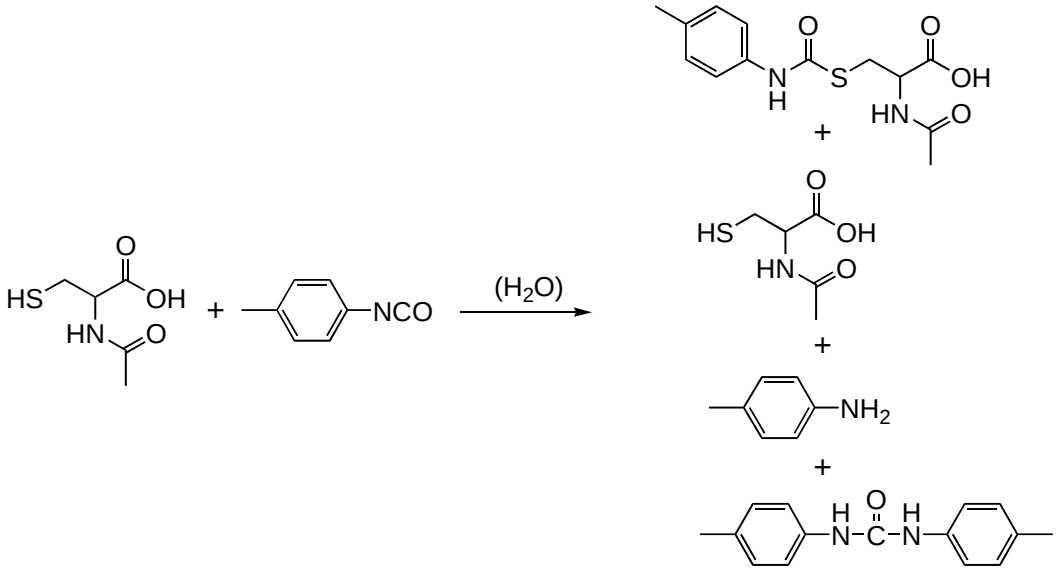
Reactions of p-tolylisocyanate in aqueous N-acetyl-L-cysteine solutions

The reaction of AcCys with pTI is illustrated in scheme 2. Depending on the molar ratios of the reactants residual AcCys will be present, also the conjugate and the urea,

which is formed by reaction of *p*-toluidine, the intermediate hydrolysis product of pTI and unreacted isocyanate. In addition, some of the amine may still be present in the mixture. From the chemistry of isocyanates and from the reactivity of amines it is well known that once an amine has been formed it will react very fast with any isocyanate present in the mixture to form a urea. Hence, only small amounts of amine if any are to be expected.

In all reactions of pTI and AcCys solutions a precipitate was formed either immediately or after several minutes. After filtration and drying an aliquot of the precipitate was dissolved in acetonitrile for HPLC-analysis. According to HPLC the precipitate was practically pure pTI-urea (>96%) with a small amount of the pTI conjugate as an impurity. For practical purposes in the calculation of the fate of pTI the precipitate formed was assumed to be pure urea.

The results of the HPLC-analysis of products generated in the reaction of pTI with AcCys in aqueous buffer solutions are summarized in table 3. Due to the monofunctionality of pTI only products from scheme 1, i.e. N-acetyl-L-cysteine, the conjugate, N,N'-di-p-tolyl urea, and in some cases minor amounts of *p*-toluidine were found in the reaction mixture. Some general trends can be observed: Eighty six mol percent of conjugate or more is formed at SH to NCO ratios of 10:1, 35 to 40 mol percent at 10:5, and between 15 and 19 mol percent at 10:15. As to be expected the amount of precipitated urea increases with increasing isocyanate/AcCys ratio. N,N'-di-p-tolyl urea is the only other product found as long as the reaction mixture contains an excess of AcCys. When there is an excess of AcCys no amine (p-toluidine) is found. With excess isocyanate, small amounts of amine and urea are found in solution.



Scheme 2: Possible products of the reaction of N-acetyl-L-cysteine with pTI in aqueous medium

The product distribution is almost independent of the acidity or alkalinity of the buffer solutions. This is in contrast to results reported for the reactions of glycine and aromatic isocyanates (Seel, 2001). This is to be expected as neither protonation nor deprotonation of AcCys plays a role in the pH range (5.0 to 7.4) used in this study.

Table 3: Compounds formed after the addition of *p*-tolylisocyanate to aqueous N-acetyl-L-cysteine solutions at different pH

N-AcCys / pTI (mol:mol)		pTI reaction products (mol%)				
		Soluble fraction			Insoluble*	Balance
		pTIAcCys	*p*-Toluidine	pTI-urea	pTI-urea	
pH 5.0	10 : 1	86.3	-	-	14.5	100.8
	10 : 5	35.1	-	-	54.3	89.4
	10 : 15	14.8	2.1	0.6	70.4	97.9
pH 5.7	10 : 1	92.5	-	-	13.3	105.8
	10 : 5	39.5	-	-	56.1	95.6
	10 : 15	18.9	1.8	1.1	70.0	91.8
pH 6.5	10 : 1	93.3	-	-	15.1	108.4
	10 : 5	39.6	-	-	55.8	95.4
	10 : 15	14.3	0.3	1.6	76.3	92.5
pH 7.4	10 : 1	92.5	-	-	17.4	109.9
	10 : 5	40.4	-	-	50.1	90.5
	10 : 15	18.0	7.5	2.8	71.5	99.8

*the insoluble fraction consists of pTI-urea contaminated with 3 % of pTIAcCys

The percentage of derivatives found in the analytical investigation is between 90 and 110 %. This error is most likely due to slight losses during the handling of small amounts of solids.

Table 4: Products generated by reaction of p-tolylisocyanate to buffer solution of pH 6.5

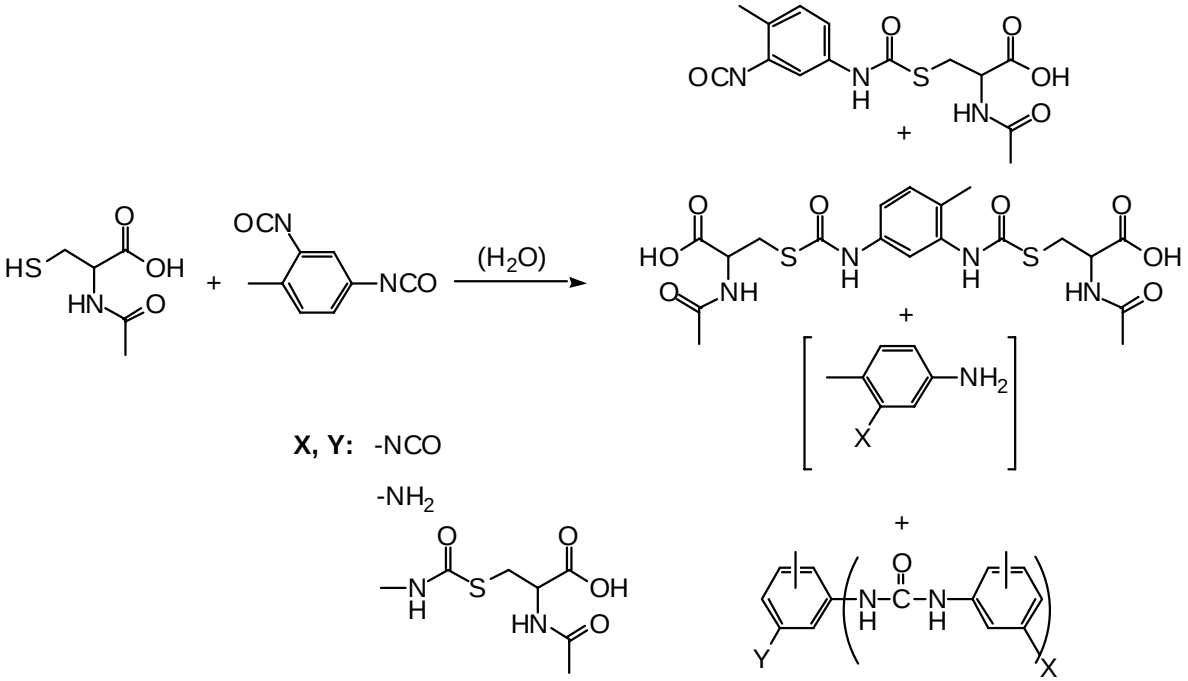
	pTI added mol L^{-1}	*p*-Toluidine (found) mol %	pTI-urea	Balance mol %
pH 6.5	0.0118	2.4	88.1	90.5
	0.0534	1.8	100.0	101.8
	0.1531	1.2	103.8	105.0

The reaction of pTI with water in an aqueous buffer solution of pH 6.5 in the absence of N-acetyl-L-cysteine has also been investigated using the concentrations of isocyanate described above. The aqueous phase was analyzed with HPLC, the results are displayed in table 4. Under these conditions the isocyanate can only react with water. The amine formed as an intermediate of the reaction of the isocyanate with water competes with water for the reaction with residual isocyanate to give either more amine or the urea. The insoluble fraction was identified as N,N'-di-p-tolyl urea. It is worth while noting that the amounts of amine formed at the highest isocyanate concentration (1.2 mol%) are lower but in the range of those that are found in the presence of AcCys (0.3 mol%).

Reactions of 2,4- toluene diisocyanate in aqueous N-acetyl-L-cysteine solutions

2,4-TDI as a diisocyanate can undergo reactions that lead to polymers under suitable conditions. A peculiarity of this diisocyanate is the difference in reactivity of the two isocyanate groups. Depending on the reaction conditions the isocyanate group in the 4-position is more reactive than the isocyanate in the 2-position by a factor of 5 to 10 (Saunders and Frisch, 1962). This is partly due to the steric hindrance of the o-NCO group; another factor is the electronic effect of the isocyanate in the 4-position

which changes from electron withdrawing to electron releasing after transformation to the thiocarbamoyl moiety.



Scheme 3: Possible products of the reaction of N-acetyl-L-cysteine with 2,4-TDI in aqueous medium

Possible products from the reaction of 2,4-TDI with AcCys are shown in scheme 3. The monoadduct of 2,4-TDI and AcCys is the initial reaction product. It can further react to yield the bisadduct or react with water to give the 2-amino monoadduct. The isomer is obtained from the reverse reaction sequence, i.e. hydrolysis of the 4-isocyanate group and reaction of the 2-isocyanate with AcCys. The amines most likely react with further isocyanate to give ureas which have further reactive functional groups, e.g. isocyanate, amine, or the S-(N-acetyl)cysteinylcarbonylamino moiety.

Table 5: Products of 2,4-TDI and aqueous N-acetyl-L-cysteine solutions at different pH

N-AcCys/NCO (mol:mol)		TDI reaction products (mol%)					
		$(AcCys)_2TDI$	TDA	TDA-urea	Insoluble	Mass balance	
pH 5.0	10 : 1	88.8	-	1.9	16.0	106.7	1*
	10 : 5	23.7	-	0.8	84.8	109.5	3*
	10 : 15	11.5	-	0.4	91.4	103.3	6*
pH 5.7	10 : 1	92.5	-	1.2	13.0	106.8	0*
	10 : 5	45.0	-	1.3	54.5	100.8	3*
	10 : 15	22.4	-	2.2	47.0	71.6	40*
pH 6.5	10 : 1	75.9	-	2.0	26.5	104.4	1*
	10 : 5	29.8	-	1.7	68.9	100.4	3*
	10 : 15	12.3	-	0.5	74.9	87.7	6*
pH 7.4	10 : 1	92.5	-	0.05	16.6	109.1	2*
	10 : 5	45.3	-	0.2	50.9	96.4	1*
	10 : 15	16.1	-	4.5	32.1	52.7	44*

*area percent of unidentified peaks

Oligomers (higher homologues) may be formed from all of these compounds in the presence of suitable reaction partners. Isocyanate groups react with the amine, with water, and with AcCys. S-(N-acetyl)cysteinylcarbonylamino groups can undergo solvolysis or aminolysis, while the amine reacts with the isocyanate to give a urea group, all with the incorporation of at least one new aromatic ring. These higher oligomers are unlikely to be formed, and if so, only in very small quantities. This is due to the poor solubility of all these oligomeric products, except for the conjugate in aqueous media.

Experiments were carried out with the NCO/SH ratios described for pTI. The reaction products are included in table 5. Apart from the $(AcCys)_2$TDI and minor amounts of TDA-urea, a number of very small unidentified peaks were observed in the HPLC-chromatograms, probably by traces of higher homologues. A representative example of an HPLC-chromatogram is shown in fig. 3. In two of the experiments with excess isocyanate groups (pH 5.7 and pH 7.4), approximately 40 percent (by area) of the soluble fraction result from unknown components (fig. 3). At the same time the amount of insolubles is significantly lower than in those experiments with lower area percents of unidentified peaks. We believe that the mono adduct is formed in significant quantities in experiments with excess isocyanate groups. Hydrolysis of the isocyanate group in the mono adducts yields the corresponding amino mono adducts which can further react to produce insoluble ureas.

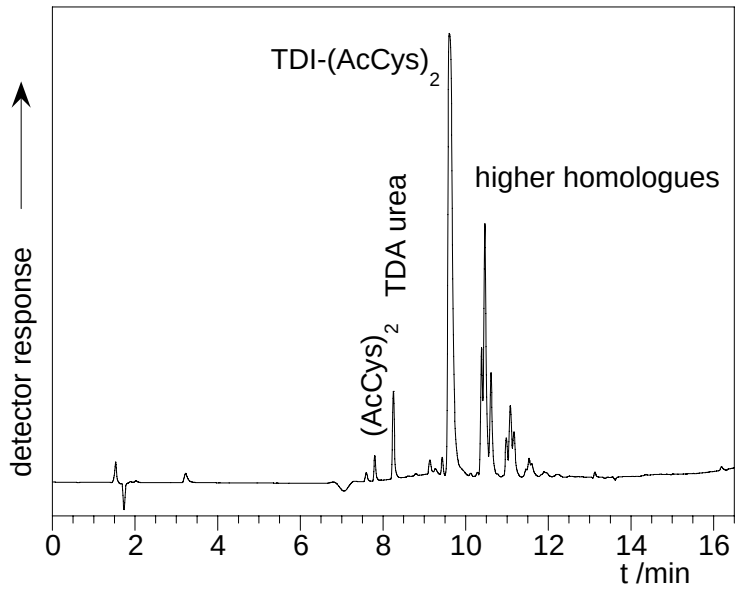


Figure 3: HPLC-chromatogram of mixture from reaction between TDI and N-acetyl-L-cysteine (pH 5.7; ratio NCO/SH 15/10)

Apart from the level of unidentified peaks, the results of the reaction of 2,4-TDI with AcCys are comparable to those of pTI with the exception that the amine, in this case 2,4-diaminotoluene, could not be detected in any of the experiments even when samples were concentrated by a factor of 10. Based on the HPLC detection limit of 0.1 nmol TDA or MDA, injection of 40 µL solution, the minimum detectable conentration of diamines is 2.5 µmol/L, e.g., 0.35 ppm for TDA and 0.5 ppm for MDA; for injection of 1 µL solution it is 100 µmol/L, e.g., 14 ppm for TDA and 20 ppm for MDA. Based on injection volumes of 20 µL and isocyanate concentrations of 5 to 75 mmol/L, the amount of aromatic diamines must be less than 0.01 % of the weighed in amount of diisocyanate.

Again there is no influence of pH, for the reasons explained already in the reaction of

AcCys with pTI. The amount of conjugate formed decreases with increasing isocyanate to AcCys ratios, i.e., 75 to 90 percent for 1:10, 25 to 45 percent for 5:0 and 12 to 22 percent for 15:10. As with pTi at least for the 5:10 ratio, high amounts of conjugate in solution are accompanied by low amounts of solids and vice versa. This trend is less pronounced for the 10:15 ratio, which may be due to the incomplete mass balance at pH 5.7 and 7.4.

Table 6: Reaction products from addition of 2,4-TDI to aqueous buffer solution of pH 6.5

	TDI added mol L^{-1}	TDA found mol %	TDA-urea found mol %	Balance mol %
	0.0057	-	78.5	78.5
pH 6.5	0.0245	-	116.3	116.3
	0.0779	-	96.0	96.0

Reaction of 2,4-TDI with aqueous buffer solution of pH 6.5 in the absence of AcCys gave TDA-urea as product and again no amine (TDA) regardless of the concentration of the diisocyanate as shown from data in table 6.

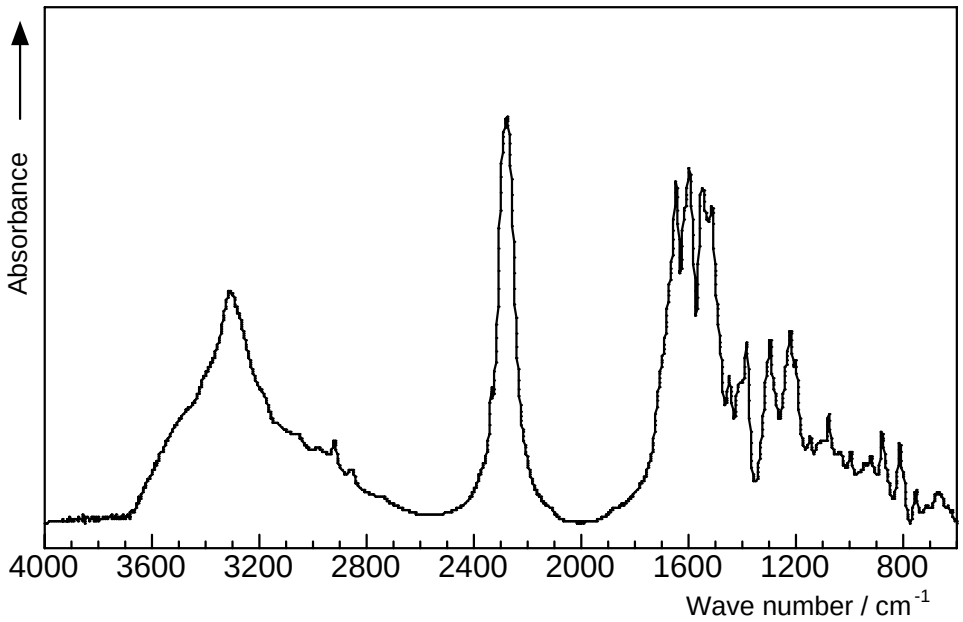


Figure 4: Infrared spectrum of precipitate from reaction of 2,4-TDI with AcCys (pH 5, ratio 15-10) with strong isocyanate absorption at 2270 cm^{-1}.

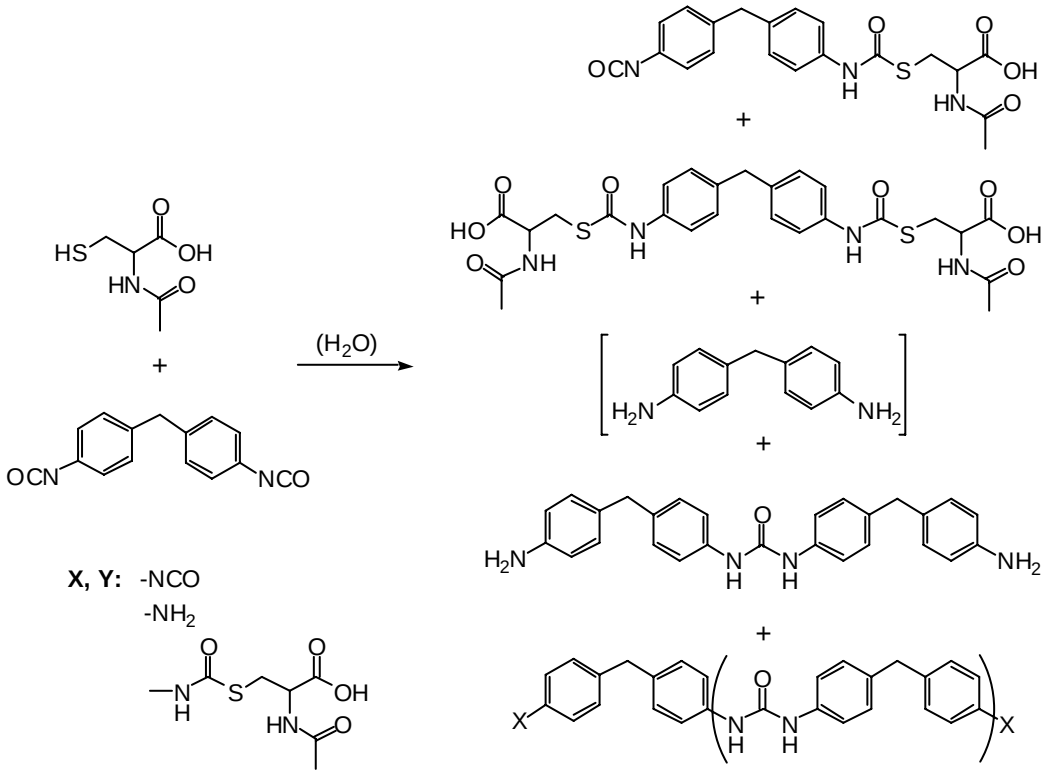
The difficulty of ensuring "homogeneous" conditions in the reaction of diisocyanates with AcCys in aqueous buffer solutions can be seen from the infrared spectrum of the insoluble fraction obtained from the reaction of TDI with AcCys in the ratio 15:10 at pH 5.0. As shown in fig. 4 apart from carbonyl groups (1650 and 1700 cm^{-1}) originating from ureas and thiourethanes, isocyanate groups (absorption around 2270 cm^{-1}) are still present in the solid material. This is probably due to the fact that the surface of the droplets of the isocyanate is covered by a layer of insoluble reaction products, e.g. urea, which can further react from within to create a "protective" layer which prevents diffusion and further reaction of the occluded diisocyanate.

Reactions of 4,4'-methylenediphenyl diisocyanate in aqueous N-acetyl-L-cysteine solutions

4,4'-MDI, also a diisocyanate, can undergo reactions that are similar to those of TDI. Hence also polymeric or oligomeric products are formed under certain conditions. The two isocyanate groups of MDI have equal reactivity. There is no steric hindrance of the isocyanate groups in 4,4'-MDI and no electronic effect of one group on the other because of the central methylene group. The products one can expect from the reaction of MDI with N-acetyl-L-cysteine in aqueous reaction medium are shown in scheme 4.

Formation of the monoadduct with MDI is less likely than with TDI because of the equal reactivity of the isocyanate groups. Hence the main products to be expected are the conjugate of AcCys and functional ureas. The main functionality of these ureas will be amino or S-(N-acetyl)cysteinylcarbonylamino groups. Formation of free diamine is even less likely than in the corresponding reactions with TDI.

Reactions were made as described for the other isocyanates. It was difficult, however, to meet exactly the intended N-acetyl-L-cysteine/isocyanate ratios. 4,4'-MDI is a solid melting at 39.5 °C. Hence, molten MDI was transferred to a preheated micro syringe and injected as a liquid into the reaction medium. Amounts needed were injected at once which affected the distribution of MDI in the reaction medium. Furthermore, when MDI was added to the AcCys buffer solution it solidified almost immediately. This resulted in a precipitate that consisted to a large extent of unreacted MDI covered with MDA-urea or other insoluble diffusion inhibiting compounds. This can be seen from the IR-spectrum (fig. 5) where the isocyanate absorption at 2270 cm^{-1} is very strong whilst carbonyl bands in the range between 1750 and 1650 cm^{-1} are almost absent. The precipitate was not completely soluble in acetonitrile, hence only the soluble part, probably MDI, has been "extracted" which overemphasizes the MDI content. It should also be noted that the spectrum is taken from a mixture with an AcCys to NCO groups ratio of 10:1.



Scheme 4: Possible products of the reaction of N-acetyl-L-cysteine with 4,4'-MDI in aqueous medium

Reaction products in this series were analyzed as described for pTI and 2,4-TDI. Calculation of solids is based on the assumption that they consist only of MDA-urea. This results in percentages which are too high because amine instead of isocyanate and urea vs. two isocyanate groups means a loss in molar mass of 26. Molar mass of MDA-urea is 422 and, the molar mass of two molecules of MDI is 500 or a difference of nearly 20 %. This explains why the balance of products found is usually above 100 percent. The results of the reactions are included in table 7.

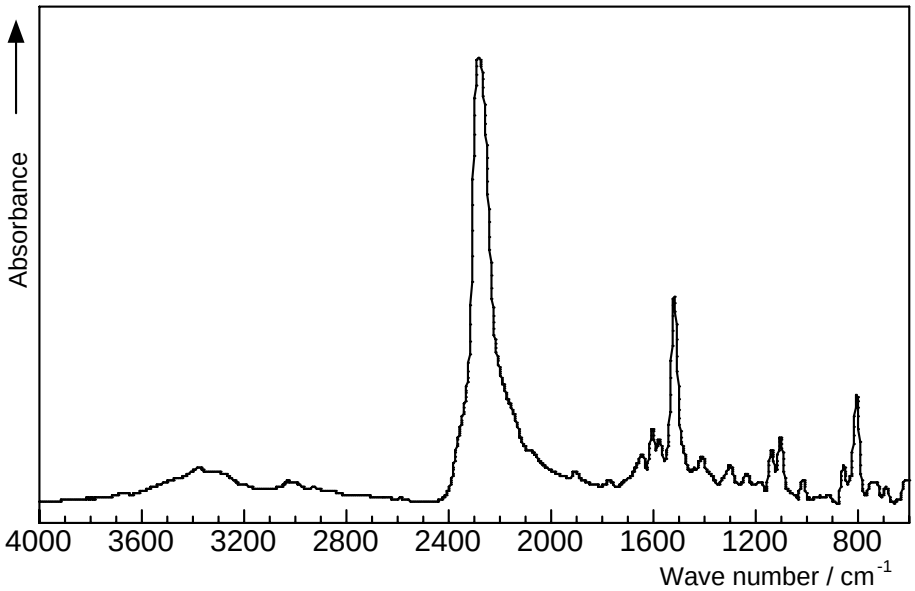


Figure 5: Infrared spectrum of the precipitate from reaction of AcCys with 4,4'-MDI (pH 6.5, ratio 10:1) with strong isocyanate at 2270 and weak urea carbonyl absorption (1650 cm^{-1})

From table 7 it can be seen that, apart from the reaction at pH 7.4, only small amounts of the conjugate MDI$(AcCys)_2$ are formed. The trend observed for the other isocyanates i.e. the decrease of the amount of conjugate with increasing amount of isocyanate cannot be seen in this case since it is more or less reversed. This can be explained by the fact that introduction of a larger amount of MDI with a micro syringe increases the probability of formation of more and smaller droplets.

Table 7: Products from addition of 4,4'-MDI to N-acetyl-L-cysteine solutions at different pH

AcCys/NCO (mol:mol)		**MDI consumption (mol%)**				
		$(AcCys_2)$MDI	**MDA**	**MDA-urea**	**Insoluble***	**Balance**
pH 5.0	10 : 1	1.3	-	-	112	113.3
	10 : 5	1.2	-	-	108.4	109.6
	10 : 15	4.1	-	-	105.8	109.9
pH 5.7	10 : 1	1.3	-	-	103.8	105.1
	10 : 5	3.3	-	-	109.2	112.5
	10 : 15	3.9	-	-	101.3	105.2
pH 6.5	10 : 1	4.8	-	-	103.1	107.9
	10 : 5	5.5	-	-	104.1	109.6
	10 : 15	4.8	-	-	104.6	109.4
pH 7.4	10 : 1	21.3	-	-	77.4	98.7
	10 : 5	20.8	-	-	92.7	113.5
	10 : 15	19.3	-	-	90.8	110.1

*insoluble fraction consists mostly of unreacted MDI covered by urea

Neither the diamine MDA nor MDA-urea was found in the chromatograms of the soluble fraction. Only N-acetyl-L-cysteine, small amounts of its dimeric cystine derivative and the conjugate could be identified. Formation of cystine derivatives occurred in the presence of air (oxygen) in particular with high excess of cysteine and at higher pH. It was not important in reactions of the other isocyanates.

The reaction of MDI with aqueous buffer at pH 6.5 in the absence of AcCys gave similar results. Again more than 100 % of MDI-reaction products were found because the major part of MDI was encapsulated and thus prevented from further reaction. No MDA could be detected and only traces of MDA-urea were seen in the chromatograms.

CONCLUSIONS

Organic isocyanates are hydrophobic and show very low solubility in water. Consequently they are present as small droplets or solid particles which react heterogeneously at the interface. In the case of pTI no insoluble protective layer is formed that acts as a barrier for AcCys or water. Diisocyanates obviously do form an insoluble layer which prevents the core from being attacked by the possible reagents. TDI does this only at low AcCys/NCO ratios (cf. fig. 4), and MDI under all conditions, probably facilitated by solidification.

Products likely to be formed are the conjugates, amines and urea; all three are well defined for monofunctional pTI, while a homologous series of ureas with amino-, cysteine- or both as end groups are to be expected from the diisocyanates. Amounts of conjugates formed depend on the nature of the isocyanate and on the SH to NCO ratio. For pTI and TDI approximately 90 % of the conjugates are formed if there is a 10 fold excess of AcCys (SH to NCO ratios of 10:1) going down to approximately 20 % if isocyanate is in excess (SH to NCO ratios of 10:15). Insoluble products for pTI and TDI reactions are the ureas, in the case of TDI with amino end groups. These increase with decreasing SH:NCO ratios, which is to be expected.

The product distribution is almost independent of the acidity or alkalinity of the buffer solutions. This is to be expected as neither protonation nor deprotonation of the thiol group in AcCys plays an important role in the pH interval from 5.0 to 7.4, which has been used in this study. MDI does not fit into this scenario because of encapsulation of MDI by an (oligo)urea layer.

Amines could be detected only in mixtures of reactions with pTI. TDA or MDA were not detected even in mixtures that were concentrated by a factor of 10. Taking into account the detection limits and injected amounts, percentage of diamines formed must be less than 0.01 % of initial isocyanate concentration. Due to the bifunctionality there are two possible reactions that prevent formation of diamines: Reaction of the amino group with another isocyanate or reaction of the remaining isocyanate group with AcCys or with an amino group, both of which give insoluble products. This does not exclude the presence of free amino end groups in some of the higher oligomers.

The highest possible concentration of amine (assuming that half of the isocyanate reacts to amine and then competes for the urea formation) is 0.075 molar and that of water 55.6 molar. The most favourable ratio of amine to water is 1:740. Under real conditions it is by far less favourable. Assuming the relative rate of reaction of amine with isocyanate compared to that of water with isocyanate is in the ratio of 1000:1 then both reactions have approximately the same probability of occurring. This reasoning explains why the concentration of amine found is highest for the lowest isocyanate concentration.

Neither the diamine MDA nor MDA-urea was found in the chromatograms of the soluble fraction. Only N-acetyl-L-cysteine, small amounts of its dimeric cystine derivative and the conjugate could be identified. Formation of cystine derivatives occurred in the presence of air (oxygen) in particular with high excess of cysteine and at higher pH. It was not important in reactions of the other isocyanates.

Acknowledgments: This study was sponsored by the International Isocyanate Institute, USA. The conclusions are those of the authors and not necessarily those of the Institute.

REFERENCES

Brochhagen FK. Isocyanates, in: O. Hutzinger (Ed.), The Handbook of Environmental Chemistry, Vol. 3, Part G, Springer Verlag, Berlin, 1991

Brown WE, Green AH, Cedel TE, Cairns J. Biochemistry of protein-isocyanate interactions: A comparison of the effects of aryl vs. alkyl isocyanates. Environmental Health Perspectives 1987; 72: 5 – 11

Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (2000): Begründung zur Bewertung von Stoffen, Tätigkeiten und Verfahren als krebserzeugend, erbgutverändernd oder fortpflanzungsgefährdend. 4,4'-Methylendiphenyldiisocyanat (MDI) (CAS-Nr. 101-68-8) und technisches (sog. "Polymeres") MDI (pMDI) (CAS-Nr. 9016-87-9) als atembare Aerosole. Ausgabe: Oktober 2000 (26 p.) Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, P. O. 17020, 44061 Dortmund, Germany. http://www.baua.de/de/Themen-von-A-Z/Gefahrstoffe/TRGS/Begruendungen-905-906.html_nnn=true

Day BW, Jin R, Basalyga DM, Kramarik JA, Karol MH. Formation, solvolysis and transcarbamoylation reactions of Bis(S-glutathionyl)adducts of 2,4- and 2.6-TDI. Chem. Res. Toxicol. 1997; 10: 424-431

Deutsche Forschungsgemeinschaft, Commission for the investigation of Health Hazards of Chemical Compounds in the Work Area, H. Greim, D. Henschler (eds.) Occupational Toxicants, Critical Data Evaluation for MAK Values and Classification of Carcinogens: 4,4'-Methylene diphenyl isocyanate (MDI) and "polymeric MDI" (PMDI), Vol. 4 (1992) 65 - 95 ISBN-10: 3-527-27025-6, VCH Weinheim (1992)

Deutsche Forschungsgemeinschaft, Commission for the investigation of Health Hazards of Chemical Compounds in the Work Area, Greim, Helmut (ed.) Occupational Toxicants, Critical Data Evaluation for MAK Values and Classification of Carcinogens: Toluylen-diisocyanate, Vol. 12, Supplement 28 VCH Weinheim (1999) ISBN-10: 3-527-27043-4

International Isocyanate Institute project AM-AB-80. 1999. Investigations of the chemistry of TDI in biological materials. Study conducted by Kennedy AL and Brown WE. Contractor: Carnegie Mellon University, Department of Biological Sciences, 4400 Fifth Avenue, Pittsburgh, Pennsylvania, USA

Mráz J, Bousková S. 2,4-tolulenediisocyanate and hexamethylene-diisocyanate adducts with blood proteins: Assessment of reactivity of amino acid residues in vitro. Chem Biol Interact 1999; 173-186

Oertel G. Polyurethane, in: G.W. Becker, D. Braun (Eds.), Kunststoff Handbuch, Band 7, Polyurethane, Carl Hanser Verlag, München, Wien, 1993

Pauluhn J, BrownWE, Hext P, Leibold E, Leng G. Analysis of biomarkers in rats and dogs exposed to polymeric methylenediphenyl diisocyanate (pMDI) and its glutathione adduct. Toxicology 2006; 222: 202 - 212.

Reisser M, Schmidt BF, Brown WE. Synthesis, characterization, and solvolysis of mono- and bis-S-(glutathionyl)adducts of methylene-bis-phenylisocyanate (MDI). Chem. Res. Toxicol. 2002; 15: 1235 – 1241.

Sabbioni G, Lamb J, Farmer PB, Sepai O. Reactions of 4-methylphenyl isocyanate with amino acids. Biomarkers 1997; 2: 223-232.

Saunders JH, Frisch KC. High Polymers, Vol. XVI, Part 1, Polyurethanes, Chemistry and Technology, Interscience Publishers, New York, 1962.

Schuetze D, Sepai O, Lewalter J, Miksche L, D. Henschler D, Sabbioni G. Biomonitoring of workers exposed to 4,4'-MDA and 4,4'-MDI. Carcinogenesis 1995; 16: 573-582

Seel K, Kuck M. Reactions of TDI, MDI and PhI in aqueous glycine solutions under physiological pH conditions: Identification and quantification of reaction products, unpublished Bayer report (2001)

Zhong BZ, Depree GJ, Siegel PD. Differentiation of the mechanism of micronuclei induced by cysteine and glutathione conjugates of MDI from that of 4,4'-MDA. Mutation Research 2001; 497: 29-37.