Isocyanate-Specific Hemoglobin Adduct in Rats Exposed to 4,4′-Methylenediphenyl Diisocyanate

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4,4′-Methylenediphenyl diisocyanate (MDI) is the most important of the isocyanates used as intermediates in the chemical industry. Among the main types of damage after exposure to low levels of MDI are lung sensitization and asthma. Protein adducts of MDI might be involved in the etiology of sensitization reactions. It is therefore necessary to have sensitive and specific methods for monitoring the isocyanate exposure of workers. To date, urine metabolites or protein adducts have been used as biomarkers in workers exposed to MDI. However, with these methods it is not possible to determine if the biomarkers result from exposure to MDI or to the parent aromatic amine 4,4′-methylenedianiline (MDA). This work presents a procedure for quantitating isocyanate-specific hemoglobin adducts. Blood proteins are used as markers of exposure and possibly as markers of dose size for the modifications of macromolecules in the target organs where the disease develops. For the quantitation of hemoglobin adducts, N^1-[4-(4-isocyanatobenzyl)phenyl]acetamide (AcMDI) was reacted with the tripeptide valyl-glycyl-glycine and with valine yielding *N*-[4-(4-acetylaminobenzyl)phenyl]carbamoyl]valyl-glycyl-glycine and *N*-[4-[4-(acetylaminobenzyl)phenyl]carbamoyl]valine, respectively. *N*-[4-[4-(Acetylamino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]carbamoyl]valine was synthesized from valine, as was N^1-[4-(4-isocyanato-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide, for use as an internal standard. These adducts were cleaved in 2 M HCl to yield the corresponding hydantoins, 3-[4-(4-aminobenzyl)phenyl]-5-isopropyl-1,3-imidazoline-2,4-dione (MDA-Val-Hyd) and 3-[4-(4-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]-5-isopropyl-1,3-imidazoline-2,4-dione, respectively. In globin of rats exposed to MDI, MDA-Val-Hyd could be found in a dose-dependent manner. The adduct was identified by HPLC/MS/MS and quantified by GC/MS after derivatization with heptafluorobutyric anhydride. The amount of MDA-Val-Hyd found after acid hydrolysis of globin at 100 °C is about 12 times larger than the sum of *N*-acetyl-4,4′-methylenedianiline **(**AcMDA) and MDA obtained from mild base hydrolysis of hemoglobin. The MDA-Val-Hyd is an isocyanate-specific adduct. MDA and AcMDA released after mild base hydrolyses result most likely from a sulfinamide adduct which is a typical adduct of arylamines. According to these results, higher amounts of isocyanate adducts than arylamine adducts should be expected in workers exposed to isocyanates.

Introduction

4,4′-Methylenediphenyl diisocyanate (MDI)[1] is the most important isocyanate intermediate in the manufacture of polyurethanes, dyes, pigments, and adhesives (*1*). High concentrations of isocyanates are a potent respiratory irritant. Among the main types of damage after low levels of MDI exposures are lung sensitization and asthma (*1*−*3*). The sensitization properties of MDI are well-documented. The corresponding aromatic amine of MDI, 4,4′-methylenedianiline (MDA), is carcinogenic in animal experiments (*4*). Arylisocyanates and arylamines can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects (*5*, *6*). Protein adducts are believed to be involved in the etiology of sensitization reactions (*7*). An established method for biomonitoring exposed people is the identification of adducts with biomolecules (*8*). Blood protein adducts have been widely used as dosimeters for modifications of macromolecules in the target organs where the disease develops. To improve the methods of risk assessment for workers exposed to MDI, it is important to develop dosimeters to establish if the toxic, reactive intermediate is MDI or a metabolite of MDA. Aromatic amines are metabolized to highly reactive *N*-hydroxy arylamines (*9*) by mixed function monooxygenases. *N*-Hydroxy arylamines can be further metabolized to *N*-sulfonyloxy arylamines, *N*-acetoxy arylamines, or *N*-hydroxy arylamine *N*-glucuronides. These highly reactive intermediates are respon-

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[1] Abbreviations: MDI, 4,4′-methylenediphenyl diisocyanate or 1-isocyanato-4-(4-isocyanatobenzyl)benzene; MDA, 4,4′-methylenedianiline; TDI, toluene diisocyanate; AcMDI, N^1-[4-(4-isocyanatobenzyl)phenyl]-acetamide; AcMDA, N^1-[4-(4-aminobenzyl)phenyl]acetamide or *N*-acetyl-4,4′-methylenedianiline; Ac[$^2H_4$]MDA, N^1-[4-(4-amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide; AcMDA-Val, *N*-[4-[4-(acetylaminobenzyl)phenyl]carbamoyl]valine; Ac[$^2H_4$]MDA-Val, *N*-[4-[4-(acetylamino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]carbamoyl]valine; AcMDA-Val-Gly-Gly, *N*-[4-(4-acetylaminobenzyl)phenyl]carbamoyl]valyl-glycyl-glycine; MDA-Val-Hyd, 3-[4-(4-aminobenzyl)phenyl]-5-isopropyl-1,3-imidazoline-2,4-dione.

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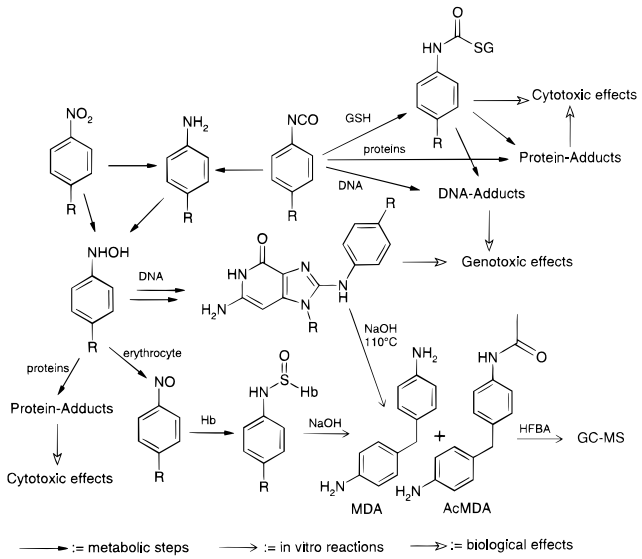

Figure 1. Reactions of arylamines and isocyanates with proteins and DNA.

sible for the genotoxic and cytotoxic effects of this class of compounds. In exposed animals, arylamines such as 4-aminobiphenyl (*10*), a human bladder carcinogen, are known to form adducts with DNA, with tissue proteins, and with the blood proteins albumin and hemoglobin in a dose-dependent manner. In contrast, isocyanates do not need any further activation to react with biomolecules (Figure 1). Important vehicles for isocyanates are their reaction products with glutathione (*11*). The glutathione adducts release the isocyanate moiety to react with other nucleophiles, e.g., proteins. Therefore, glutathione adducts are thought to be responsible for the transport of isocyanate to reactive sites away from the site of isocyanate uptake.

MDA binds to DNA (*12*), and ca. 0.044% of the dose binds to hemoglobin (*13*). The methods of identification of such adducts are well-established (*14*−*17*). Arylamine-specific adducts are of the sulfinamide type (*18*, *19*). The chemical structure of aromatic isocyanate adducts found in vivo is unknown.

Several applications have been described for the derivatization of proteins with isocyanates and/or isothiocyanates. Stark and Smith (*20*) applied carbamylation with potassium cyanate to the N-terminal analysis of proteins. Carbamylation of amino acids with phenyl isothiocyanate has been used for Edman degradation and sequence analysis of proteins (*21*). Törnqvist et al. (*22*) used pentafluorophenyl isothiocyanate to analyze the N-terminally alkylated valines of hemoglobin. Virtually all functional groups on proteins can react with isocyanates (*23*, *24*), but under physiological conditions, the potential sites of reaction are restricted: (i) the α-amino group of the N-terminal amino acids, (ii) the sulfhydryl group of cysteine (*11*, *25*, *26*), (iii) the hydroxyl groups of tyrosine (*27*) and especially serine (*28*), (iv) the ϵ-amino group of lysine, and (v) the imidazole ring of histidine. Although the pK_a of lysine is around 10.5, especially reactive lysines have been located in proteins (*29*, *30*). In vivo adducts of lysine have been found with acetaldehyde (*31*), glycated proteins (*32*), and aflatoxin B1 (*33*). Carbamylation of cysteine has been noticed as an artifact in peptide mapping of hemoglobins in the presence of urea (*34*). Carbamylated hemoglobin is formed by the reaction of hemoglobin with cyanate, a product of in vivo urea dissociation. It is found in high levels in patients with renal failure and may be useful in their clinical evaluation (*35*).

The following questions are important for the toxiological evaluation of isocyanate exposure. (i) What is more important for the toxicity of isocyanates, the isocyanate or the metabolically released corresponding amine? (ii) What kind of adducts are formed after isocyanate exposure, isocyanate- and/or arylamine-specific adducts? (iii) Are *N*-hydroxy arylamines biologically available after the exposure to aryl isocyanates? (iv) Which isocyanate adducts are most immunogenic? Several attempts have been undertaken to answer these questions. Human serum albumin was modified in vitro with 4-methylphenyl isocyanate to produce an antigen with which to assay immunoglobulin IgE of workers exposed to toluene diisocyanate (TDI) (*36*−*38*). These adducts have never been chemically characterized. Recently, Day et al. (*39*) performed ion-spray MS analysis on the Hb isolated from guinea pigs exposed by inhalation to 2,4-TDI. They found carbamylation products with both the α- and *β*-chains in which one of the two original isocyanato groups had been hydrolyzed to the amine. In addition, Day et al. found an amine−nitroso adduct on the α-chain in the in vivo sample. These results indicate that at least one of the isocyanato moieties (or a masked derivative) of 2,4-TDI survived passage through the lung, into the serum, and through the erythrocyte membrane to form adducts with hemoglobin that were stable to dialysis, gel filtration, and reversed phase HPLC separation under acidic conditions. Carbamylation of the N-terminal valine of hemoglobin with methyl isocyanate in rats and rabbits has been demonstrated in vitro and in vivo by gas chromatography. N-Methylcarbamylated hemoglobin, converted by cyclization into 3-methyl-5-isopropylhydantoin, has been quantified by gas chromatography in the range of 0.06−2 nmol (*40*, *41*).

To biomonitor workers exposed to isocyanate, several research groups have hydrolyzed urine, plasma, albumin, and hemoglobin under acidic and basic conditions and quantified the released parent arylamine with GC/MS. The chemical structure of the adducts prior to cleavage is, however, unknown. Putative adducts of isocyanates with biomolecules have to be synthesized to establish the reacting intermediate, aryl isocyanate or arylamine. Therefore, for this work, a putative reaction product of AcMDI with hemoglobin was synthesized. AcMDI was reacted with valine and a tripeptide with valine as the N-terminal amino acid. The presence of the N-terminal adduct with valine was investigated in rats exposed to MDI.

Materials and Methods

Caution: *The aromatic amines used in this work are potentially carcinogenic. Isocyanates are strong irritants. Triphosgene is severely irritating to the eyes and skin. Avoid contact and inhalation. All these compounds should be handled with protective clothing in a well-ventilated fume hood.*

Chemicals. 4,4′-Methylenedianiline, [$^2H_6$]DMSO, dry dioxane, 4-methylaniline, trifluoroacetic acid, $NaHCO_3$ (analytical grade), 37% HCl TraceSelect, and triphosgene were purchased from Fluka (Neu-Ulm, Germany). Water for HPLC analysis, diethyl ether (analytical grade), *tert*-butyl methyl ether (TBME) SupraSolv for trace analysis, methanol for trace analysis, and NaCl (analytical grade) were from Merck (Darmstadt, Ger-

many). Decane (>99%) and ethanol for spectroscopy were from Sigma-Aldrich (Deisenhofen, Germany). Methanol for HPLC analysis and toluene for residue analysis were from Promochem (Weser, Germany). Heptafluorobutyric anhydride was from Supelco (Deisenhofen, Germany) Valyl-glycyl-glycine was from Bachem (Heidelberg, Germany). L-Valine was from Serva (Heidelberg, Germany). AcMDA [N^1-[4-(4-aminobenzyl)phenyl]-acetamide] was synthesized according to ref *13*. N^1-[4-(4-Amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]acetamide (Ac[$^2H_4$]-MDA) was synthesized as described previously (*42*).

Instrumentation. NMR spectra were recorded on a Bruker AC 250 instrument with [$^2H_6$]DMSO as the solvent and as the internal standard. The degree of substitution of the C atoms was determined using the distortionless enhancement by polarization transfer (DEPT) method. The raw NMR data were processed with the program MestRe-C (Magnetic Resonance Companion, J. C. Cobas, J. Cruces, and F. J. Sordina, Departamente de Quimica Organica, Universidad de Santiago de Compostela, 15706 Santjago de Compostela, Spain). HPLC analyses were performed on a Hewlett-Packard 1100 system with a quaternary HPLC pump and a photodiode array detector. Gas chromatography/mass spectrometry (GC/MS) analyses were carried out on a Hewlett-Packard gas chromatograph (HP 5890II) interfaced with a mass spectrometer (HP 5989A).

Isolation of Globin. The animal experiments were performed at the Fraunhofer-Institut in Hannover and have been described previously (*43*). The animals were exposed for 3 months for 17 h per 5 days a week with MDI concentrations of 0, 0.26, 0.7, and 2.06 mg/m^3. The rats were sacrificed 1 day after their last day of exposure. The animals were anesthetized with pentobarbital, and blood was taken by heart puncture. For the analysis of protein adducts, 1−3 mL of blood was obtained, and centrifuged for 5 min at 2000*g*. After removal of the plasma, the red blood cells were washed three times with equal volumes of a 0.9% sodium chloride solution. For complete hemolysis, the erythrocytes were diluted with 4 volumes of water and left at room temperature for 30 min. Cell debris was removed by centrifugation (10 min at 4000*g*). Globin was precipitated from the clear red supernatant by adding 4 volumes of cold 0.2% HCl in acetone at 0 °C. The precipitate was resuspended and isolated by centrifugation after the following washing sequence: ethanol/water (8:2), ethanol, ethanol/diethyl ether (1:3), and diethyl ether. Globin was dried over silica gel in a desiccator, and all samples were stored at −20 °C.

Synthesis of N^1-[4-(4-Isocyanatobenzyl)phenyl]acetamide (AcMDI). Triphosgene (415 mg, 1.4 mmol) was added to a solution of AcMDA (480 mg, 2.00 mmol) in dry dioxane (20 mL) and the mixture stirred for 3 h at 80 °C under nitrogen. The reaction mixture was used without further cleanup for the next steps. According to HPLC analyses, all AcMDA was consumed under these conditions. HPLC analyses were performed on a Lichrospher RP18 (125 mm × 4 mm, 5 μm) column with a 20 min, 30 to 80% MeOH gradient in 50 mM ammonium formate [flow rate of 1 mL/min, t_R(AcMDA) = 9.9 min, t_R(AcMDI) = 13.0 min] or with a 20 min, 30 to 80% MeOH gradient in 0.01% trifluoroacetic acid [flow rate of 1 mL/min, t_R(AcMDA) = 6.4 min, t_R(AcMDI) = 13.2 min].

Synthesis of Ureas from AcMDI and Amino Acids. Procedure 1. Amino acid (1 mmol) or tripeptide (1 mmol) was dissolved in 0.25 M $NaHCO_3$ (20 mL). The solution was stirred, and a hot solution (80 °C) of freshly prepared AcMDI (1 mmol) in dioxane (10 mL) was added dropwise. $NaHCO_3$ was added to maintain the pH at ca. 8.0. After 3 h, the reaction mixture was cooled with ice and the ice solution was filtered. The filtrate was evaporated to a final volume of ca. 15 mL, carefully acidified to pH 2 with 2 M HCl, and extracted with ethyl acetate (3 × 50 mL). The organic phase was extracted with a saturated $NaHCO_3$ solution (3 × 20 mL). The pooled aqueous phases were acidified, saturated with NaCl, and extracted with ethyl acetate (3 × 50 mL). After the sample had been dried over $MgSO_4$, the ethyl acetate was evaporated at reduced pressure. The residue was recrystallized from ethanol after adding a few drops of water.

***N*-[4-[4-(Acetylaminobenzyl)phenyl]carbamoyl]valine (AcMDA-Val).** L-Valine (117 mg, 1 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded AcMDA-Val (138.0 mg, 36%) as white crystals. ^{1}H NMR ([$^2H_6$]-DMSO, 250 MHz): δ 0.86 (d, J = 6.8 Hz, 3H, CHCH_3), 0.90 (d, J = 6.8 Hz, 3H, CHCH_3), 1.97 (s, 3H, COCH_3), 2.08 (m, 1H, CHCH$_3$), 3.75 (s, 2H, CH_2), 4.12 (dd, J = 4.9, 8.7 Hz, 1H, NHCH), 6.32 (d, J = 8.7 Hz, 1H, NHCH), 7.03 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.45 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 8.45 Hz, 2H), 8.54 (s, 1H, Ph-NH), 9.94 (s, 1H, Ph-NHCONH), 12.68 (s, 1H, COOH). ^{13}C NMR ([$^2H_6$]DMSO, 63 MHz): δ 18.0 (CHCH$_3$), 19.7 (CHCH$_3$), 24.3 (COCH$_3$), 30.7 (CHCH$_3$), 40.4 (CH$_2$), 58.0 (NHCH), 118.7 (CH), 120.2 (CH), 129.4 (CH), 129.6 (CH), 135.4 (C), 137.3 (C), 137.5 (C), 138.3 (C), 156.0 (NHCONH), 169.8 (COCH$_3$), 174.7 (COOH). UV (methanol): λ_{max} = 253 nm. HPLC/MS/MS [LCQ-Duo (Thermoquest, San Jose, CA), positive ESI]: 384 [M + H]$^+$, MS of 384 = 241 [AcMDA + H]$^+$, MS of 384 = 366 (M − H_2O), MS of 366 = 338. HPLC analyses were performed on a Lichrospher RP18 (125 mm × 4 mm, 5 μm) column with a 20 min, 30 to 80% MeOH gradient in 50 mM ammonium formate [flow rate of 1 mL/min, t_R(AcMDA-Val) = 11.2 min] or a 20 min, 30 to 80% MeOH gradient in 0.01% trifluoroacetic acid [flow rate of 1 mL/min, t_R(AcMDA-Val) = 14.3 min].

***N*-[4-[4-(Acetylamino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]carbamoyl]valine (Ac[$^2H_4$]MDA-Val).** The deuterated adduct was synthesized according to the procedure given above for the undeuterated compound. The purity and identity of the compound were checked by HPLC with a photodiode array detector and by HPLC/MS/MS (LCQ-Duo, positive ESI): 388 [M + H]$^+$, MS of 388 = 245 [Ac[$^2H_4$]MDA + H]$^+$.

***N*-[4-[4-(Acetylaminobenzyl)phenyl]carbamoyl]valyl-glycyl-glycine (AcMDA-Val-Gly-Gly).** Val-Gly-Gly (115.5 mg, 0.5 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded AcMDA-Val-Gly-Gly (85 mg, 34%) as a white solid. ^{1}H NMR ([$^2H_6$]DMSO, 200 MHz): δ 0.86 (d, J = 6.8 Hz, 3H, CHCH_3), 0.90 (d, J = 6.8 Hz, 3H, CHCH_3), 1.97 (s, 3H, COCH_3), 2.08 (m, 1H, CHCH$_3$), 3.75 (s, 6H, CH_2), 4.06 (dd, J = 5.5, 8.0 Hz, 1H, NHCH), 6.55 (d, J = 8.0 Hz, 1H, NHCH), 7.03 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.45 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 8.45 Hz, 2H), 8.16 (t, J = 5.8 Hz, 1H, NHCH$_2$), 8.29 (t, J = 5.9 Hz, 1H, NHCH$_2$), 8.90 (s, 1H, Ph-NH), 9.94 (s, 1H, Ph-NHCONH), 12.68 (s, 1H, COOH). ^{13}C NMR ([$^2H_6$]DMSO, 50 MHz): δ 17.7 (CHCH$_3$), 19.3 (CHCH$_3$), 23.9 (COCH$_3$), 30.6 (CHCH$_3$), 40.2, 40.4 (CH$_2$, CH$_2$N), 41.7 (CH$_2$N), 58.1 (NHCH), 117.5 (CH), 119.1 (CH), 128.6 (CH), 128.8 (CH), 133.9 (C), 136.1 (C), 137.3 (C), 138.4 (C), 155.3 (NH-CONH), 168.0 169.1, 171.0, 172.1 (2NHCO, COOH, NHCOCH$_3$). UV (methanol): λ_{max} = 253 nm. HPLC/MS/MS (LCQ-Duo, positive ESI): 498 [M + H]$^+$, MS of 498 = 366 (M − Gly-Gly), MS of 366 = 338. HPLC analyses were performed on a Lichrospher RP18 (125 mm × 4 mm, 5 μm) column with a 20 min, 30 to 80% MeOH gradient in 50 mM ammonium formate [flow rate of 1 mL/min, t_R(AcMDA-Val-Gly-Gly) = 12.0 min] or a 20 min, 30 to 80% MeOH gradient in 0.01% trifluoroacetic acid [flow rate of 1 mL/min, t_R(AcMDA-Val-Gly-Gly) = 12.3 min].

3-[4-(4-Aminobenzyl)phenyl]-5-isopropyl-1,3-imidazoline-2,4-dione (MDA-Val-Hyd). AcMDA-Val (38 mg, 0.1 mmol) was dissolved in 0.3 mL of methanol and the mixture heated for 90 min at 90 °C in 5 M HCl (5 mL). The reaction mixture was cooled to room temperature and extracted with ethyl acetate (3 × 5 mL). The water phase was made basic by addition of $NaHCO_3$ and extracted with ethyl acetate (3 × 6 mL). The ethyl acetate extract was dried over Na_2SO_4, filtered, and evaporated to dryness. A white solid was obtained, MDA-Val-Hyd (23 mg, 70%). ^{1}H NMR ([$^2H_6$]DMSO, 250 MHz): δ 0.86 (d, J = 6.7 Hz, 3H, CHCH_3), 1.05 (d, J = 6.7 Hz, 3H, CHCH_3), 2.13 (m, 1H, CHCH$_3$), 3.78 (s, 2H, CH$_2$), 4.11 (d, J = 3.6 Hz, 1H, CHNH), 4.9 (s, 2H, NH$_2$), 6.49 (d, J = 8.2 Hz, 2H), 6.87 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.3 Hz, 2H), 7.25(d, J = 8.3 Hz, 2H), 8.52 (s, 1H, NH). ^{13}C NMR ([$^2H_6$]DMSO, 63 MHz): δ 15.7 (CH$_3$), 18.4 (CH$_3$), 29.8 (CHCH$_3$), 39.9 (CH$_2$), 61.2 (CHNH), 113.9 (CH),

126.4 (CH), 127.6 (C), 128.6 (CH), 129.0 (CH), 129.5 (C), 142.1 (C), 146.7 (C), 156.0 (NCONH), 172.7 (CON). HPLC/MS/MS (LCQ-Duo, positive ESI): 324 $[M + 1]^+$, MS of 324 = 231 (M − aniline), MS of 231 = 203, MS of 203 = 132. GC/MS of the HFBA derivative (for the GC conditions, see below) was carried out. EI (70 eV): *m/z* (rel intensity) 520 (1.2) $[M + 1]^+$, 519 (15) $[M^+]$, 420 (5), 378 (5), 302 (30), 165 (20), 132 (100). NCI (methane): *m/z* (rel intensity) 500 (26), 499 (100) $[M - HF]^-$, 413 (15), 359 (5).

3-[4-(4-Amino-3,5-dideuteriobenzyl)-2,6-dideuteriophenyl]-5-isopropyl-1,3-imidazoline-2,4-dione ($[^2H_4]$MDA-Val-Hyd). The reaction was performed with 10 μg of Ac$[^2H_4]$MDI-Val as described below. The product was characterized by GC/MS after derivatization with HFBA. EI (70 eV): *m/z* (rel intensity) 524 (7) $[M + 1]^+$, 523 (32) $[M^+]$, 424 (15), 382 (9), 304 (38), 134 (100). NCI (methane): *m/z* (rel intensity) 504 (28), 503 (100) $[M - HF]^-$, 502 (12), 417 (20), 363 (10).

Gas Chromatographic Analysis and Calibration Line of the Hydantoin MDA-Val-Hyd. AcMDA-Val-Gly-Gly (0, 0.01, 0.02, 0.100, and 0.201 nmol), Ac$[^2H_4]$MDA-Val (10.3 pmol) in methanol (10 μL), and 4-methylaniline (10 μg) in methanol (10 μL) were added to 2 M HCl (1 mL), and the mixture was heated for 2 h at 100 °C in 2 M HCl (1 mL) in a reagent tube with a Teflon-lined screw cap. The experiments were performed in triplicate. The hydrolysate was adjusted to pH 9 with $NaHCO_3$, and then extracted with 3:1 TBME/toluene (2 mL). The organic layer was passed through a pipet filled with anhydrous Na_2SO_4 (1 g). The Na_2SO_4 was rinsed with 3:1 TBME/toluene (1.5 mL). The dried organic phase was collected in a tapered tube. Decane (10 μL) was added as a keeper to the organic phase prior to evaporation with the speed evacuator. The residue was taken up in ethyl acetate (2 × 75 μL) and derivatized with heptafluorobutyric anhydride (HFBA) (2 μL). After 10 min at room temperature, the derivatization was stopped by adding a methanol solution (50 μL) of 4-methylaniline (10 μg). After evaporation to dryness, the residue was taken up in ethyl acetate (10 μL). The extracts were analyzed on a fused silica capillary column [Rtx-5MS (Restek, Bellefonte, PA), 0.25 mm i.d., 10 m long, 0.25 μm film thickness] attached to a methyl-deactivated tubing precolumn (Supelco, 0.25 mm, 1 m long) with a Hewlett-Packard chromatograph (model 5890II) coupled to a mass spectrometer as a detector (HP 5989A). An aliquot (2 μL) was injected on-column with a fused silica syringe at 80 °C. The transfer line temperature was set at 260 °C. The oven temperature was kept for 1 min at 80 °C and then increased at a rate of 50 °C/min to 300 °C. The compounds were identified and quantified using negative chemical ionization mass spectrometry, with methane as the reactant gas (1.5 Torr, ion source temperature of 250 °C, electron energy of 200 eV) and SIM of *m/z* = 570 and 574 up to 5 min, and then SIM of *m/z* = 499 and 503 (dwell time of 150 ms). The derivatized internal standard $[^2H_4]$MDA-Val-Hyd-HFBA and the MDA-Val-Hyd-HFBA eluted at 5.5 min. The calibration line was generated from the amount of MDA-Val-Gly-Gly (*x*) expressed in nanomoles and the peak ratio (*y*) of MDA-Val-Hyd-HFBA and $[^2H_4]$MDA-Val-Hyd-HFBA: $y = 102.9x + 0.06$, $r^2 = 0.999$. $[^2H_4]$-MDA-$(HFBA)_2$ and MDA-$(HFBA)_2$ eluted at 3.8 min and were quantified with an external calibration curve.

Hydrolysis of Globin and GC/MS Analyses. Rat globin (40−50 mg) in 2 mL of 2 M HCl was spiked with Ac$[^2H_4]$MDA-Val (10 pmol) in methanol (10 μL) and 4-methylaniline (10 μg) in methanol (10 μL) and the mixture heated for 2 h at 100 °C. The samples were then processed as described above.

Hydrolysis of Globin and HPLC/MS/MS Analyses. Rat globin (40−50 mg) in 2 mL of 2 M HCl was spiked with Ac$[^2H_4]$-MDI-Val (20 pmol) in methanol (10 μL) and 4-fluoroaniline (1 μg) and the mixture heated for 2 h at 100 °C. The samples were then processed as described for the GC/MS analyses, except the derivatization with HFBA was omitted. The samples were separated on a Prontosil C-18 reversed phase column (Bischoff, Leonberg, Germany, 125 mm × 2 mm, 5 μm) with a 20 min, 30 to 80% methanol gradient in 0.1% formic acid and a flow rate of 0.3 mL/min. The detector was a quadrupole ion trap tandem mass spectrometer (LCQ-Duo, Thermoquest). The MS parameters were optimized in the electrospray ionization mode (ESI). Positive ions were detected. Parameter optimization was carried out with a 4 pg/μL solution and a flow rate of 0.3 mL/min. The signal abundance of *m/z* 324 $[M + H]^+$ was maximized with the autotune program. The other parameters were set as follows: capillary temperature of 220 °C, sheath gas flow of 60 (arbitrary units), and auxiliary gas flow of 20. MS/MS spectra were obtained by ion trap collision-induced decay. When 20% (arbitrary units) energy was applied, the following daughter ions were obtained for the parent ions at *m/z* 324 and 328 (rel intensity): 324 = 231 and for 328 = 231 (60), 232 (100), 233 (22).

Hydrolysis of Globin and Analysis by HPLC with Electrochemical Detection. Rat globin (40−50 mg) was heated for 2 h at 100 °C in 2 M HCl (1 mL) in a reagent tube with a Teflon-lined screw cap. The reaction solution was cooled, and 4-fluoroaniline (1 μg) was added as a carrier. The solution was neutralized with $NaHCO_3$ and extracted with 3:1 toluene/TBME (2 mL). The mixture was vortexed for 1 min, frozen in liquid nitrogen, thawed in a water bath, and centrifuged for 10 min. The organic layer was passed through a pipet filled with 100 mg of deactivated Al_2O_3 and 100 mg of anhydrous Na_2SO_4. [Al_2O_3 was dried overnight at 150 °C. After the Al_2O_3 had been cooled in a desiccator containing silica gel, 15% (v/w) water was added.] The pasteur pipet was then rinsed with TBME (2 mL). The eluates were collected in a tapered tube. After addition of DMSO (25 μL) as keeper, the solvents were evaporated on a speed vac at 40 °C for ca. 30 min under vacuum. To evaporate all toluene, methanol was added and the evaporation procedure was continued for 10−15 min. Water was added to the residue to obtain a final volume of 100 μL. An aliquot (5 μL) of this mixture was separated on a Lichrospher select B (125 mm × 4 mm, 5 μm) column with 60:20:20 10 mM sodium phosphate (pH 7.1)/acetonitrile/methanol and a flow rate of 1 mL/min. The peaks were detected with an electrochemical detector (Coulochem ESA, analytical cell 5010) at a voltage of 0.8 V and a gain of 20 nA. MDA-Val-Hyd eluted at 10.5 min under the given conditions.

Results and Discussion

Synthesis of Isocyanate Adducts. The urea derivatives with the free amino group of valine and Val-Gly-Gly were synthesized according to the scheme depicted in Figure 2. AcMDI was generated from AcMDA and triphosgene in dioxane, and used without prior purification for the reactions. AcMDA-Val and AcMDA-Val-Gly-Gly were synthesized by adding AcMDI to valine and/or Val-Gly-Gly in carbonate buffer at room temperature. The products were obtained in satisfactory yields (ca. 35%). These products were characterized by NMR, UV, and MS. The deuterated derivative Ac$[^2H_4]$MDA-Val was synthesized from Ac$[^2H_4]$MDI and valine, and was characterized by HPLC, UV, and MS. Treatment of these adducts in acid at temperatures above 80 °C yields the hydantoins MDA-Val-Hyd and $[^2H_4]$MDA-Val-Hyd. For characterization by NMR, the hydantoin MDA-Val-Hyd was synthesized on a larger scale with a yield of 70%. In addition, MDA-Val-Hyd was analyzed by GC/MS after derivatization with HFBA in the negative chemical ionization mode. The major fragment (*m/z* = 499) of MDA-Val-Hyd-HFBA resulted from the loss of HF. The same procedure was followed for the synthesis of $[^2H_4]$-MDA-Val-Hyd. The major fragment of $[^2H_4]$MDA-Val-Hyd-HFBA was at *m/z* 503. The derivatization agent HFBA must be eliminated before GC/MS analysis, e.g., by addition of methanol with an excess of an amine or

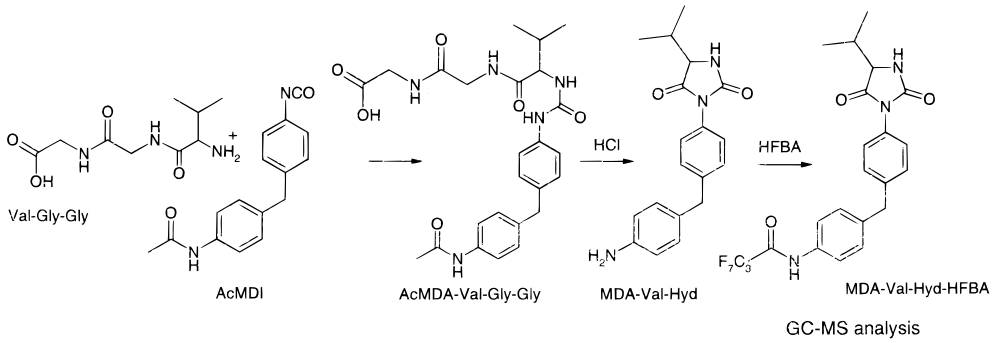

Figure 2. Synthesis of AcMDA-Val-Gly-Gly and MDA-Val-Hyd.

by washing with phosphate buffer. Without such precautions, only derivatized MDA, MDA-$(HFBA)_2$, will be detected and not the desired product MDA-Val-Hyd-HFBA. The same effect has been observed for the HFBA derivatization of AcMDA (*34*). Therefore, the fragments of MDA-$(HFBA)_2$ and [D_4]MDA-$(HFBA)_2$ at *m/z* 570 and 574, respectively, should be monitored. On a molar basis, the levels of the amines were lower than 3% of those of the corresponding hydantoins MDA-Val-Hyd and [$^2H_4$]-MDA-Val-Hyd.

The MS data of the other compounds were obtained using a HPLC/MS/MS instrument equipped with an electrospray ionization (ESI) probe. For all compounds, only the positive ion mode of ESI gave sufficient sensitivity. The protonated molecular ion was seen for all compounds, e.g., *m/z* 324 for MDA-Val-Hyd, 384 for AcMDA-Val, and 498 for AcMDA-Val-Gly-Gly. For the deuterated compound Ac[$^2H_4$]MDA-Val (*m/z* 388), no molecular ions for the potential contaminating undeuterated compounds were observed. Signals in the NMR spectra of the AcMDA adducts were assigned according to other AcMDA derivatives published previously (*12*, *13*). The interpretation of the peaks has been introduced in Materials and Methods.

Analysis of the Hydantoin MDA-Val-Hyd. Several methods were tested to quantify the MDA-Val-Hyd which is cleaved from the MDI adduct with the N-terminal valine of hemoglobin. Standard MDA-Val-Hyd was analyzed by GC/MS and by HPLC with UV detection (data not shown), by HPLC with electrochemical detection, and by HPLC/MS/MS. The same detection methods were then tested by processing globin as described for the in vivo samples in the presence of the synthesized standard AcMDA-Val-Gly-Gly. The detection limit for synthetic MDA-Val-Hyd using isocratic HPLC conditions was 2 pmol by UV at a wavelength of 200 nm, 0.1 pmol by electrochemical detection at 0.8 V, and 0.1 pmol by HPLC/MS/MS. Except for the HPLC/MS/MS method, the HPLC methods were not specific enough when globin samples were analyzed. The background signals from the globin extracts obscured the signal of MDA-Val-Hyd. For the GC/MS analyses, MDA-Val-Hyd required derivatization with HFBA for low levels to be detected in the negative chemical ionization mode. Gas chromatography of MDA-Val-Hyd-HFBA was tried with several different columns and injection modes. The best sensitivities were obtained using a short 10 m Rtx-5MS column and on-column injection with a fused capillary syringe. The detection limit with standard was 0.2 pmol. After ca. five injections, a piece of the retention gap had to be removed, since the sensitivity drops drastically. The same detection limits are reached from globin extracts. The method used for the quantification of the rat samples was GC/MS. However, the method of choice is probably the quantification by HPLC/MS/MS, since the GC/MS method is very labor-intensive and impossible to automate. In this work, the HPLC/MS/MS apparatus was not available in the laboratory. Therefore, the method was used only for confirmation of the compounds found in vivo.

Identification of Isocyanate Adducts with Hemoglobin. In a long-term experiment designed to determine the carcinogenic and toxic effects of MDI, rats were exposed chronically for 3 and 12 months to 0.0 (control), 0.26, 0.70, and 2.06 mg of MDI/m^3 as aerosols (*35*). Globin from these rats was isolated and analyzed for detection of isocyanate-specific adducts. Globin was treated in the presence of the internal standard Ac[$^2H_4$]MDA-Val with acid. The resulting hydantoins MDA-Val-Hyd and [$^2H_4$]-MDA-Val-Hyd were extracted in organic solvents, derivatized with HFBA, and quantified by GC/MS in the negative chemical ionization detection mode (Figure 3). The hydantoin found in vivo was quantified against a calibration curve obtained from the hydrolysis of different amounts of AcMDA-Val-Gly-Gly against Ac[$^2H_4$]MDA-Val. The calibration line was linear over a range of 0−0.2 nmol of AcMDA-Val-Gly-Gly. The adduct was found in a dose-dependent manner. The increase in the amount of adduct was linear for the two lowest doses. For the highest dose, the increase was less than expected from a linear increase. For structural confirmation, the hydantoins were analyzed by HPLC/MS/MS prior to derivatization (Figure 4). The chromatographic retention time and the mass fragment were the same for the adduct found in vivo and the standard compound. As for the GC/MS analyses, small amounts of MDA-Val-Hyd were found in the control rats. The analyses of control and rat samples showed that there is no interference at the elution time of MDA-Val-Hyd. Further evidence of the adduct found in vivo was obtained by the analyses of the globin hydrolysates by HPLC with electrochemical detection (Figure 5). At MDA-Val-Hyd's retention time, a peak was present in exposed animals but not in control animals.

The amount of isocyanate-specific adduct was compared to the amount of arylamine-specific adducts from MDA and AcMDA released after mild base hydrolysis (*43*) of hemoglobin (Figures 1 and 6) from the same rats. The levels of MDA and AcMDA increase in a similar way as that of the isocyanate-specific adduct MDA-Val-Hyd (Figure 6). As represented in Figure 6, the amount of isocyanate-specific adduct was 12 (±5) times greater than that of the arylamine-specific adducts MDA and AcMDA released after mild base hydrolysis. From these data, two possible pathways can be postulated. The arylamine-

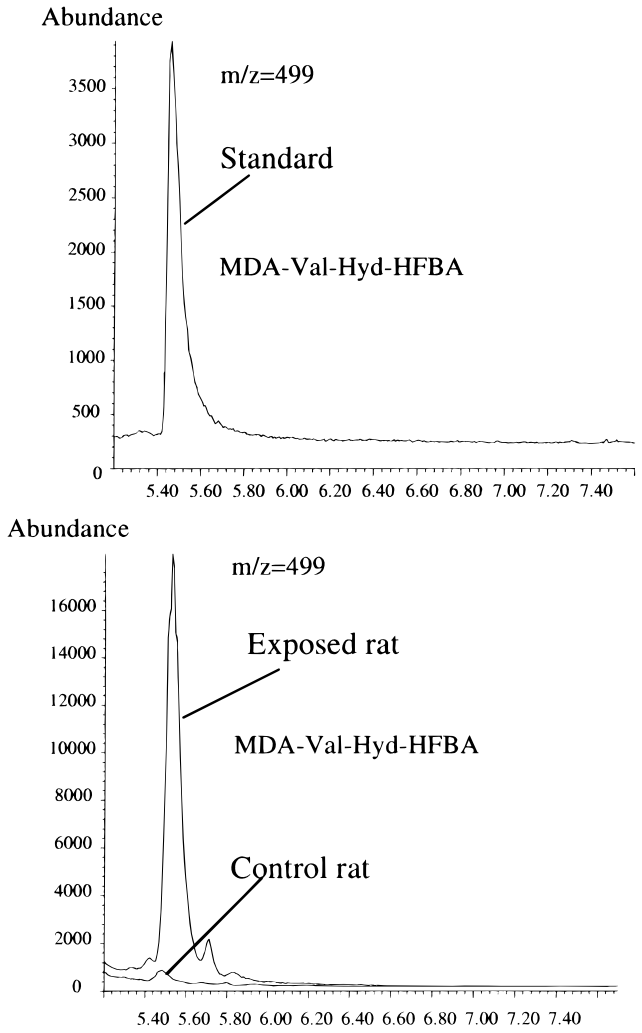

Figure 3. GC/MS with NCI detection of HFBA-derivatized MDA-Val-Hyd: (A) standard solution of MDA-Val-Hyd and (B) $^1/_5$ of total extract of control rat globin and $^1/_5$ of total extract of rat globin of the highest dose.

specific adducts result after MDI hydrolyzes to MDA, which is further oxidized in the liver to its *N*-hydroxy arylamine and subsequently to the nitroso compound in the erythrocytes. The nitroso derivative of MDA and/or AcMDA yields the sulfinamide adducts, which are acid and base labile. The same compounds, MDA and AcMDA, were released after mild base hydrolysis from hemoglobin of rats and humans exposed to MDA. The isocyanate-specific adducts result via the direct reaction of the isocyanate groups with hemoglobin in the erythrocytes or indirectly via the reaction of MDI with glutathione to (Figure 1), as postulated for methyl isocyanate, the resulting thiocarbamate, which releases the isocyanate in the erythrocyte to obtain the adducts with the reactive N-terminal amino acid of hemoglobin.

Conclusions

To distinguish between typical arylamine and isocyanate adducts, procedures for identifying the isocyanate-specific adduct with the N-terminal valine of hemoglobin were developed. For the identification of arylamine-specific adducts, measurement of MDA and AcMDA released after mild base hydrolysis of hemoglobin by established methods suffices (*42*, *43*). Such mild conditions will release arylamines from the typical sulfinamide

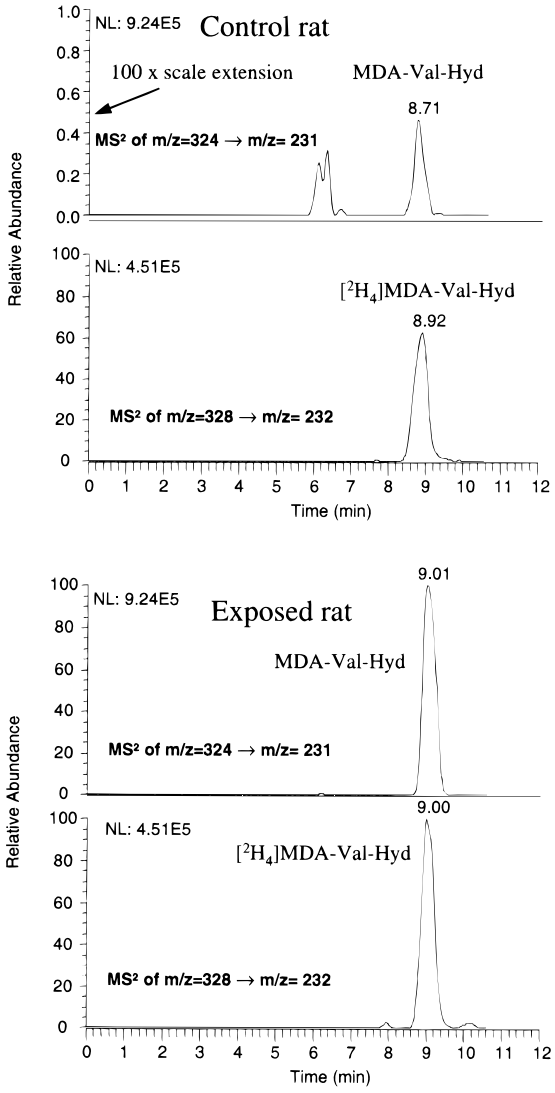

Figure 4. HPLC/MS/MS of hydantoin MDA-Val-Hyd obtained from globin extracts of a control rat and an exposed rat (0.7 mg of MDI/m^3). The HPLC and instrument conditions are given in Materials and Methods.

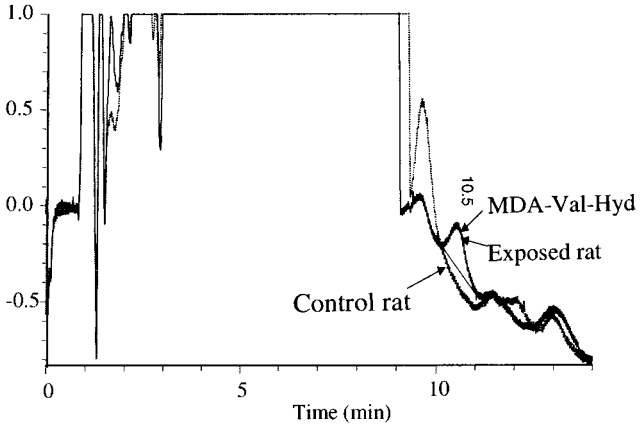

Figure 5. Globin analysis of an exposed and a control rat. Aliquots ($^1/_{20}$) of the extracts of a control rat and of an exposed rat (2.06 mg of MDI/m^3) were analyzed by HPLC with electrochemical detection at 0.8 V. The baseline had to be reset to scale by pressing the autozero key of the detector at 9 min to determine the signal of MDA-Val-Hyd at 10.5 min.

adducts formed by arylamines with the cysteine of hemoglobin. However, mild base hydrolysis can also release isocyanate adducts with tyrosine and cysteine (*44*–

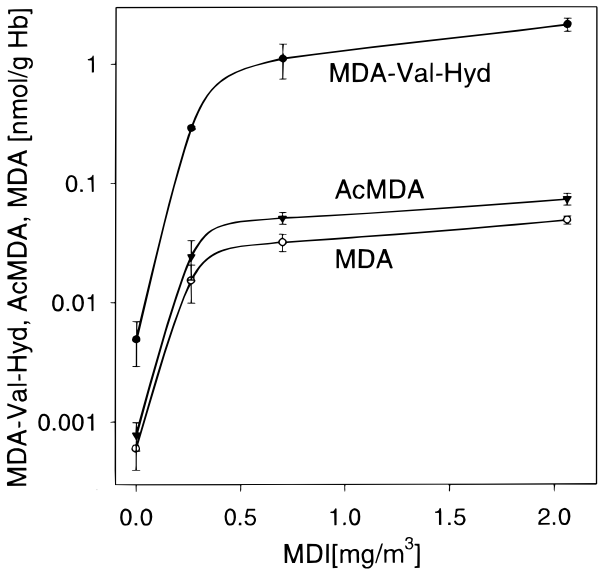

Figure 6. MDA-Val-Hyd levels in MDI-exposed rats at three dose levels and a control group. The adduct levels of the isocyanate-specific adduct MDA-Val-Hyd released from globin after acid hydrolysis are compared with those of the arylamine-specific adducts released after mild basic hydrolysis from hemoglobin (original data from ref *43*).

46). The presence of AcMDA after mild base hydrolysis shows that these adducts were generated from MDA. We consider it unlikely that only one isocyanate group of MDI is hydrolyzed to the amine and then acetylated, while the other isocyanate group could reach the erythrocytes intact and form a covalent bond with hemoglobin. However, since *N*-acetyltransferases (*47*) have been found in erythrocytes, it should be investigated whether the *N*-acetyl group can also be introduced in the diamine when the diamine is bound on the hemoglobin.

MDI probably forms further adducts with hemoglobin; these have yet to be detected, and their detection will likely require different methods. In addition, adducts with albumin (*7*) should be present, since this is believed to be an important step in the sensitization mechanism of people exposed to isocyanate. This will the topic of future research. With the method presented here, it should be possible to determine levels of isocyanate-specific adducts in people exposed to MDI. From the data of the rat experiment, we suggest that the arylamine hydrolysis of MDI to MDA is probably not a major metabolic pathway in rats.

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