N-Methylcarbamoylated Valine of Hemoglobin in Humans after Exposure to *N,N*-Dimethylformamide: Evidence for the Formation of Methyl Isocyanate?

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N,*N*-Dimethylformamide (DMF) is reported to cause testicular germ-cell tumors in exposed workers. The reports, however, are not in line with results obtained in animal and in vitro experiments, where DMF was shown not to be mutagenic and also not to be carcinogenic. Considerable interest raised on the formation of a reactive intermediate, presumably methyl isocyanate (MIC), during metabolism of DMF in humans over the last years. We report the formation of *N*-methylcarbamoylated valine of hemoglobin (Hb) in blood samples from workers exposed to DMF in the polyacrylic fiber industry. *N*-Methylcarbamoylated Hb was formed by the reaction of MIC with Hb. For this purpose, Hb adducts were monitored by means of a modified Edman degradation involving the release of the N-terminal valine adduct in form of 3-methyl-5-isopropylhydantoin (MIH). For internal standardization of the method, 3-ethyl-5-isopropylhydantoin (EIH) was used. Separation and analysis of MIH and EIH were carried out by gas chromatography and mass spectrometry with electron impact ionization (GC/EI-MS). Hb adducts in form of MIH were quantified in blood samples from exposed persons in concentrations between 26.1 and 412.0 nmol of MIH/g of globin. The observed adducts were proven to be identical to those derived from the in situ reaction between Hb and MIC. Taken together with the fact that only *N*-methylcarbamoylated Hb can undergo ring closure to the corresponding hydantoin, the reaction is indirect evidence for the occurrence of MIC in vivo. The formation of MIC directly in the cell and its possible distribution through the human body may lead to critical effects after exposure to DMF. Adducts were determined not to be totally specific for exposure to DMF since an identical adduct was also found in blood samples from the general population. However, concentrations were lower by a factor of about 100. The sources for background adducts are currently unknown.

Introduction

N,*N*-Dimethylformamide (DMF)[1] is one of the most important industrial solvents with worldwide production estimated to be ∼250 000 tons/year. DMF is commonly used in the manufacture of films, fibers, coatings, and for polyurethane lacquers. Human exposure to DMF occurs through inhalation and dermal absorption (*1*). The main toxic effects reported were hepatotoxicity and developmental toxicity (*2*, *3*). Walrath et al. (*4*) reported no increase in the incidence of cancer in humans in one epidemiological study. In several other reports, however, testicular germ-cell tumors (*5*, *6*) were identified among workers who had been exposed to DMF. The results were discussed in detail (*7*−*9*) because no carcinogenic effects of DMF were observed in animal studies (*10*) and also because the majority of various in vitro test systems yielded negative results (*11*). Moreover, multiple exposures to other carcinogens (e.g., aromatic amines and chromium) could not be completely excluded at the workplace (*12*). Therefore, whether DMF is carcinogenic is a question that is not yet solved.

However, Mráz and colleagues reported a fundamental difference in the metabolism of DMF between humans and animals (*13*, *14*). *N*-Acetyl-*S*-(*N*-methylcarbamoyl)-cysteine (AMCC) was found to be a major metabolite in humans but less in rodents. On the basis of the urinary excretion of AMCC and its precursor *N*-acetyl-*S*-(*N*-methylcarbamoyl)glutathione, the formation of a reactive intermediate, presumably methyl isocyanate (MIC), was suggested during the metabolism of DMF (*15*, *16*). MIC may be generated either by oxidation of the formyl carbon or *N*-oxidation of *N*-methylformamide (NMF) (*17*) (Figure 1). Recently, Midorikawa et al. (*18*) reported the formation of carbon-centered or nitrogen-centered radicals during the degradation of DMF. Radicals were also suggested by Gescher (*17*) to be highly reactive intermediates during the suspected formation of MIC. The formation of MIC itself was not described yet, however. Beside the study by Midorikawa et al., only few articles could be traced in the literature, which investigate the mechanistic basis of DMF toxicity (*19*−*21*).

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[1] Abbreviations: AMCC, *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine; DMF, *N*,*N*-dimethylformamide; EIC, ethyl isocyanate; EIH, 3-ethyl-5-isopropylhydantoin; MIC, methyl isocyanate; MIH, 3-methyl-5-isopropylhydantoin; MITC, methylisothiocyanate; NEVal, *N*-ethylcarbamoyl-D/L-valine; NMF, *N*-methylformamide; NMVal, *N*-methylcarbamoyl-D/L-valine.

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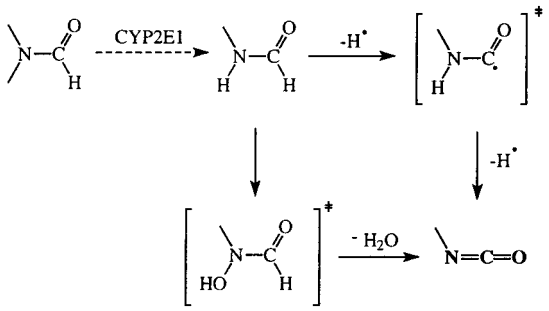

Figure 1. Suggested formation of methyl isocyanate during the metabolism of DMF in humans according to Gescher (*17*) via oxidative demethylation to *N*-methylformamide subsequently followed by C- or N-oxidation.

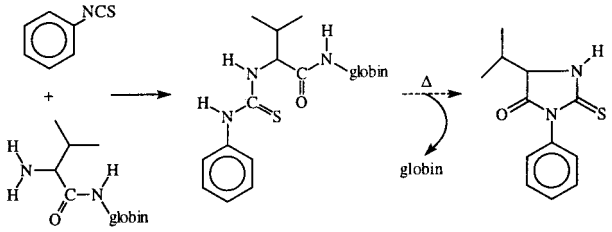

Figure 2. Regular Edman degradation: formation of *N*-phenylthiocarbamoylated valine of globin and subsequent ring closure to 3-phenyl-5-isopropylthiohydantoin by heating.

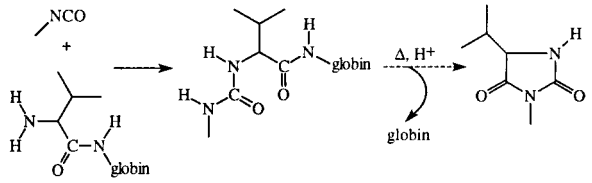

Figure 3. Formation of *N*-methylcarbamoylated valine of globin after reaction of methyl isocyanate and globin. Addition of concentrated acids and heating leads to the formation of 3-methyl-5-isopropylhydantoin.

Because MIC has electrophilic properties, it should react with nucleophilic sites of macromolecules, e.g., with amino groups in hemoglobin (*22*). From the literature it is well-known that the N-terminal valine of hemoglobin (Hb) can be split off in form of its corresponding thiohydantoin by a regular Edman degradation. For this purpose, phenyl isothiocyanate is used as derivatizing reagent (Figure 2). The reaction was modified by Törnqvist et al. (*23*) using pentafluorophenyl isothiocyanate to monitor cancer initiators (especially alkylating substances) through Hb adducts at the N-terminal valine (*N*-alkyl Edman). In her studies, the resulting thiohydantoins were finally analyzed by GC/MS. Nowadays the *N*-alkyl Edman method is of widespread use to determine the internal dose of alkylating substances in blood samples from both, rodents and humans (*24*−*28*).

From a chemical point of view isocyanates are capable to undergo identical reactions such as isothiocyanates. Therefore, thiocarbamoylation of valine by phenyl isothiocyanate can be taken over from carbamoylation of valine by isocyanates in vivo. As a consequence, exposure to isocyanates can be assessed by the formation of hydantoins (*29*). Using this procedure, 3-methyl-5-isopropylhydantoin (MIH) could be analyzed in blood samples from victims exposed to MIC after the Bhopal incident in 1984 (*30*). The formation of MIH, however, can be also used as an indirect evidence for the formation of MIC after exposure to DMF. Ring closure to MIH is not possible without prior *N*-methylcarbamoylation of the N-terminal valine by MIC (Figure 3). This hypothesis was tested in our studies by analyzing blood samples from workers exposed to DMF in the synthetic fiber industry. The results were compared to those derived from (1) in situ reaction of MIC with Hb and (2) from control persons who were not exposed to DMF or MIC.

Experimental Procedures

Caution: *MIC and ethyl isocyanate (EIC) are severe eye, skin, and mucous membrane irritants and sensitizers. Most deaths are a result of lung tissue damage (acute toxicity). Both substances are dangerous, when exposed to heat or flame oxidizers. Mutations were seen in S. typhimurium and mouse lymphocytes with exposure to MIC* (*31*).

Chemicals. The chemicals used for standard synthesis and for GC/MS were obtained by Merck KgaA (Darmstadt, Germany) and Sigma/Aldrich GmbH (Deisenhofen, Germany) and are regularly found in a laboratory. MIC was synthesized according to Kaushik et al. (*32*).

FT-NMR Spectroscopy. ^{1}H NMR, 400 MHz, was performed on a JEOL GX400 spectrometer. TMS was used as internal reference and chemical shifts (δ) are given in parts per million relative to TMS. Samples were prepared in D_2O (δ 4.80 ppm). 2D-COSY ^{1}H NMR and 2D-HETCOR ^{1}H/^{13}C NMR was carried out to verify coupling. ^{13}C NMR, 100 MHz, was carried out on a JEOL PS100 spectrometer. The solvent signals derived from acetone-d_6 (δ 29.95 ppm) were used for internal standardization. ^{13}C NMR signals were assigned using the distortionless enhancement by polarization transfer (DEPT) method to distinguish between CH, CH_2, and CH_3 signals. All ^{1}H and ^{13}C NMR spectra were recorded at 23 °C, with a pulse delay time of 6.0 s. A 2.2 and 1.0 s delay was used for 2D-COSY and 2D-HETCOR, respectively. The raw NMR data was processed with the program Nuts (Acorn NMR Inc., Livermore, CA). The identity of the standards was also investigated by FAB-MS. The spectra were recorded on a Micromass ZabSpec spectrometer using a primary beam of Cs^+. The matrix was 3-nitrobenzyl alcohol.

GC/MS Instrumentation. GC/MS analysis was performed on a GC HP 5890 Series II fitted with a mass selective detector HP 5972 and a split/splitless injector system HP 7673 operating in the splitless mode. The inlet purge off time was set to 1.0 min. The injector temperature was set to 280 °C. The transfer line, ion source, and quadrupole were maintained at 280, 200, and 100 °C, respectively. The ion source pressure was $\sim 3 \times 10^{-6}$ Torr. EI with an electron energy of 70 eV was chosen as the ionization mode at an electron multiplier voltage of 2100 V. Samples analyzed by GC/EI-MS were injected on a capillary column with the stationary phase HP-35MS (35%-diphenyl−65%-dimethylarylene siloxane copolymer), 60 m with an i.d. of 0.25 mm and a phase film thickness of 0.25 μm. Helium was used as carrier gas at a constant flow of 1.0 mL/min. 1 μL of the sample was injected. The initial column temperature at 70 °C was held for 1 min and then raised to 160 °C at 8 °C/min. The temperature was held for 17 min. Finally, ramp rate 2 raised the temperature to 270 °C at 25 °C/min and kept it constant for 10 min. Beside GC/MS-EI, the instrument was also operated in PCI mode. Methane served as the ionizing gas (pressure = 1.0 Torr). Samples analyzed by GC/PCI-MS were injected on a capillary column with the stationary phase HP Innowax [100% poly(ethylene glycol), cross-linked], 60 m with an i.d. of 0.32 mm and a phase film thickness of 0.25 μm. Helium was used as carrier gas. The initial column temperature of 120 °C was programmed to rise to 180 °C at 10 °C/min, held for 25 min and then increased to 240 °C at 20 °C/min. The final temperature was held for 25 min.

Synthesis of 3-Methyl-5-Isopropylhydantoin. MIH was synthesized similar to procedures already described by Ram-

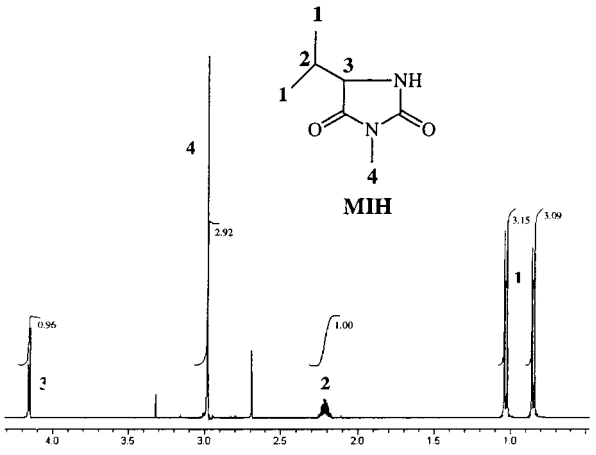

Figure 4. ^{1}H NMR spectra (D_2O) of 3-methyl-5-isopropylhydantoin. Due to hindered rotation of the C5−C6 bond, methyl groups have different shifts. The integration values are normalized to hydrogen 2 (relative value: 1.00).

achandran et al. (*33*) and Venkateswaran et al. (*34*). A total of 936 mg of d/L-valine (8 mmol) were dissolved in 20 mL of double-distilled water, and pH 9 was adjusted by addition of a 1 M Na_2O_2 solution. Then 456 mg of MIC (430 μL, 8 mmol) were directly added. After stirring for 2 h at RT, 80 mL of concentrated HAc/HCl (1:1, v/v) was added, and the solution was refluxed for 15 min. After evaporation to dryness using a vacuum rotator at 80 °C, a viscous substance was obtained. Recrystallation in ethyl acetate yielded 999.6 mg (80.1%) MIH. ^{1}H NMR (Figure 4): δ 0.86 (d, CH_3, $^3J_{1-2} = 6.83$ Hz); 1.04 (d, CH_3, $^3J_{1-2} = 6.83$ Hz); 2.23 (m, CH); 2.99 (s, NCH_3) and 4.17 (d, NCH, $^3J_{3-2} = 3.66$ Hz). ^{13}C NMR: δ 15.3 (*C*H_3), 18.4 (*C*H_3); 24.5 (*C*H); 30.2 (N*C*H_3); 63.2 (N*C*H); 160.2 (N*C*O); and 177.8 (N*C*ON). FAB-MS: *m*/*z* 157 [M + 1] and 289 [M + Cs^+].

Synthesis of 3-Ethyl-5-isopropylhydantoin. EIH was synthesized according to the procedure described for MIH. However, EIC was used for *N*-ethylcarbamoylation. Nevertheless, the outcome was different. Two products were obtained that may be assigned to EIH and the ring-opened product *N*-ethylcarbamoylvaline (NEVal). The ratio was found to be about 1:1.

^{1}H NMR Signal Assignment for EIH and NEVal. Since chemical structures of MIH and EIH are similar and NMR spectra were recorded under identical conditions, ^{1}H NMR assignments for EIH (with the exception of the ethyl moiety) could be based on comparison with ^{1}H NMR chemical shifts for MIH (Figures 4 and 5). Signals at δ 0.85 and 1.03 ppm (labeled **1**, Figure 5) were assigned to EIH since chemical shifts and coupling constants ($^3J_{1-2}$) were found to be essentially identical to those of MIH (Figure 4). ^{1}H correlations from hydrogen **1** to hydrogens **2** (δ 2.22 ppm) and **3** (δ 4.14 ppm) could be established by 2D-COSY. On the basis of relative integration, δ 3.52 ppm was assigned to the methylene group of the ethyl moiety (**4**) and connection to the methyl group **5** (δ 1.15 ppm) was established by 2D-COSY.

An additional set of signals was observed in the ^{1}H NMR spectrum, reflecting the presence of a second molecule, presumably the open form NEVal (Figure 5). Signals at δ 1.08 and 1.10 ppm were assigned to the methyl moieties of the isopropyl group in NEVal (labeled **1***). Connectivities from **1*** to **2*** and **3*** could be established by 2D-COSY. On the basis of the relative integration and 2D-COSY, δ 3.10 and 1.30 ppm were assigned to **4*** and **5***, respectively. Since **4**, **4***, and **5**, **5*** in EIH and NEVal are only based on relative integration to the respective isopropyl protons, it is possible that these assignments are interchangeable. ^{1}H NMR: δ 0.85 (d, EIH−CH_3, $^3J_{1-2} = 6.83$ Hz); 1.03 (d, EIH−CH_3, $^3J_{1-2} = 6.84$ Hz); 1.08 (d, NEVal-CH_3, $^3J_{1-2} = 7.08$ Hz); 1.10 (d, NEVal-CH_3, $^3J_{1-2} = 7.08$ Hz); 1.15, 1.30 (t, CH_2CH_3);[2] 2.22 (m, EIH−CH); 2.38 (m, NEVal-CH); 3.10, 3.50 (m, NCH_2);[2] 3.99 (d, NEVal-NCH, $^3J_{3-2} = 4.40$ Hz) and 4.14 (d, EIH−NCH, $^3J_{3-2} = 3.42$ Hz).

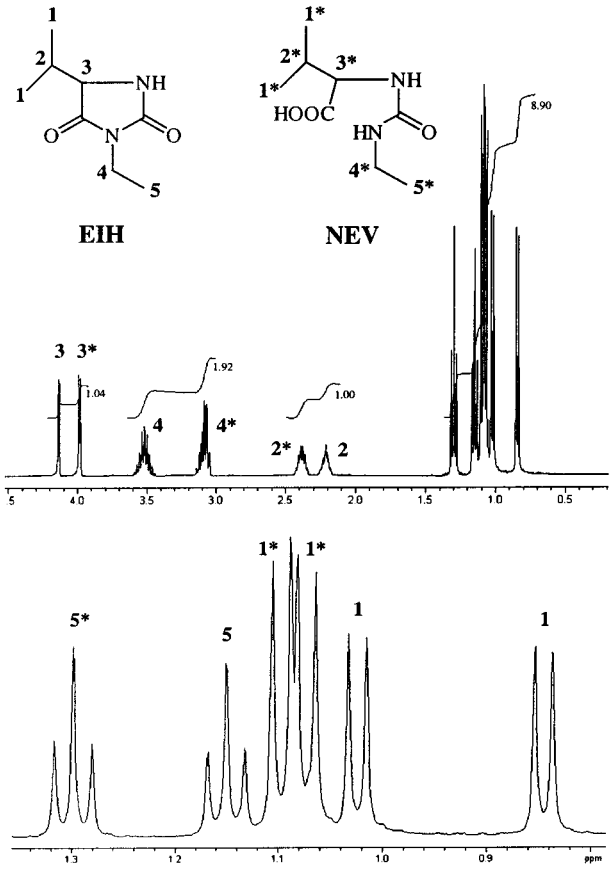

Figure 5. ^{1}H NMR spectra (D_2O) of the mixture 3-ethyl-5-isopropylhydantoin (EIH) and *N*-ethylcarbamoyl-D/L-valine (NEVal). For signal assignment please refer to text. Integration values are all normalized to hydrogen 2 (relative value: 1.00 for both EIH and NEVal).

^{13}C NMR Signal Assignment for EIH and NEVal. On the basis of chemical shift and the DEPT spectra, ^{13}C NMR signals between 12 and 20 ppm could be assigned to *C*H_3 groups, while signals for *C*H-, N*C*H_2-, and N*C*H-carbons, were found at ∼30, 34, and 60 ppm, respectively. Signals for nonprotonated *C*O-carbons were between 150 and 190 ppm (Figure 6). On the basis of the 2D-HETCOR spectrum signals at δ 15.2 and 18.4, 30.3, and 62.8 ppm were assigned to **1**, **2**, and **3**, respectively. As a consequence (and also based on the 2D-HETCOR), the second set of signals at δ 17.3 and 17.8, 29.4, and 58.8 ppm were assigned to **1***, **2***, and **3***. The signal at δ 159.8 ppm (labeled **4**) was nearly identical (within 0.4 ppm) to the N*C*O-group in MIH and, therefore, assigned to the N*C*O-group of EIH. As a consequence (and based on the DEPT spectrum), δ 172.1 ppm was assigned to **4*** (*C*OOH) in NEVal. The signal at δ 177.3 ppm was nearly identical to the N*C*ON-signal in MIH (δ 177.8 ppm) and occurred at twice the relative intensity compared to **4** or **4***. Since carbons **4** (in EIH) and **4*** (in NEVal) are less similar in chemical environments than carbons **5** and **5***, δ 177.3 ppm was assigned to both (**5**/**5***), the N*C*ON-group in EIH and NEVal. Signals for EIH (**2**, **3**, and **4**) are always slightly higher than those for NEVal (**2***, **3***, and **4***). On the basis of the relative height and the DEPT spectra, δ 12.2 and 33.8 ppm could be assigned to **7** and **6** of the ethyl moiety from EIH. The second set of signals (δ 13.0 and 35.3 ppm) could be assigned to **7*** and **6*** of the ethyl group from NEVal. The assignments for **6**/**6*** and **7**/**7*** would be also in accordance with the 2D-HETCOR.

[2] The signals can be assigned to either EIH or NEVal since signal assignment is based only on relative integration (for ^{1}H NMR) or relative height (for ^{13}C NMR).

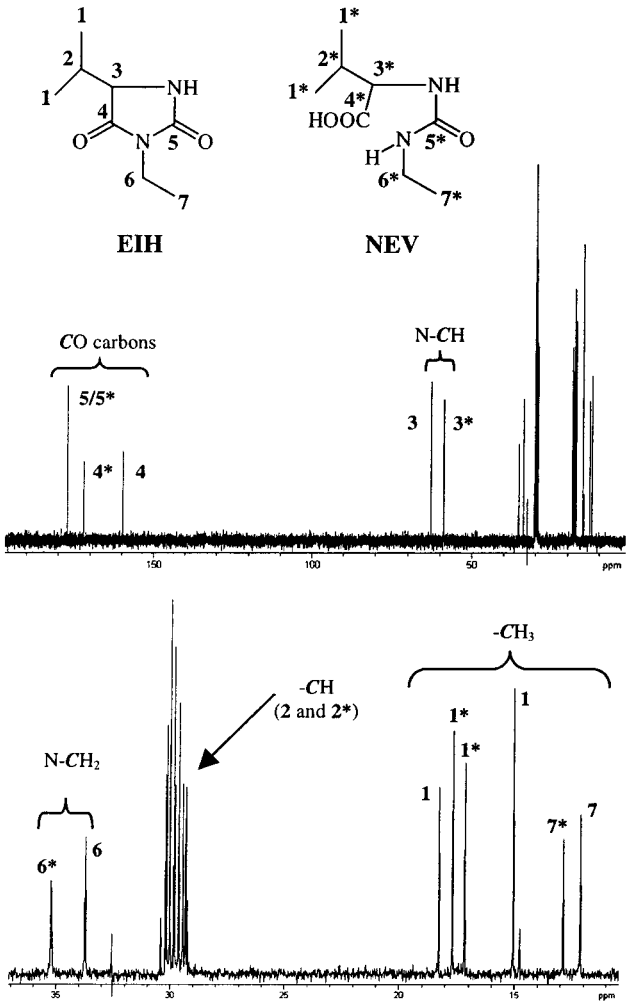

Figure 6. ^{13}C NMR spectra of the mixture 3-ethyl-5-isopropylhydantoin (EIH) and *N*-ethylcarbamoyl-D/L-valine (NEVal). For signal assignment please refer to text.

However, since the ^{13}C signals for the ethyl moiety were only assigned based on their relative height (and the corresponding ^{1}H signals on their relative integration), **6**/**6*** and **7**/**7*** may be interchangeable. ^{13}C NMR: δ 12.2, 13.0 (CH_2CH_3);[2] 15.2 (EIH-CH_3); 17.3 (NEVal-CH_3); 17.8 (NEVal-CH_3); 18.4 (EIH-CH_3); 29.4 (EIH-*C*H); 30.3 (NEVal-*C*H); 33.8, 35.3 (CH_2);[2] 58.8 (NEVal-N*C*H); 62.8 (EIH−N*C*H); 159.8 (EIH−N*C*O); 172.1 (NEVal-*C*OOH); and 177.3 (EIH and NEVal-N*C*ON). FAB-MS: *m*/*z* 171 [EIH + 1]; 303 [EIH + Cs^+]; 189 [NEVal + 1], and 321 [NEVal + Cs^+].

Preparation of Globin. A total of 2 × 10 mL of blood was drawn from the vein of exposed and nonexposed persons using a disposable syringe containing EDTA as anticoagulant. Red blood cells were separated from the plasma by centrifugation of the blood samples. Erythrocytes were washed with 0.9% saline and then lysed. The cell fragments were removed by centrifugation. After blood samples were lysed, globin was isolated immediately to avoid artifactual formation of adducts (*35*). To prepare between 150 and 250 mg of globin, a 50 mM hydrochloric acid solution in 2-propanol was added to 2 mL of lysate. Then, ethyl acetate was added slowly to the supernatant, and the solution was stored at 4 °C overnight. The precipitated globin was separated by centrifugation, washed several times with ethyl acetate and *n*-hexane, and dried in a vacuum desiccator (*36*, *37*). Globin was stored at −28 °C until analysis was carried out.

In Situ Experiments. The theoretical formation of *N*-methylcarbamoylated Hb at the N-terminal valine after exposure to MIC was tested in in-situ experiments. For this purpose, a blood sample was drawn from one volunteer. The individual was healthy, male, nonsmoker, nonexposed to DMF or MIC, and did not take any drugs prior to blood sampling. Red blood cells were separated from the plasma and cells were washed and lysed. Then, the sample was divided in two parts each containing 2 mL of lysed blood. A total of 20 μL of MIC was added to sample 1, while sample 2 served as a control. After both samples were shaken rapidly, they were incubated at 37 °C for 2 h. Then globin was isolated and stored at −28 °C until analysis was carried out. A double experiment was carried out.

Population Samples. MIH levels from 35 persons exposed to DMF in the polyacrylic fiber industry were studied. Workers were employed in different parts of the facility, e.g., working directly at the spinning machines, preparing DMF/water solutions, washing fibers, or crimping fibers (post-fiber treatment). In the company there is a potential co-exposure to carbon disulfide (CS_2). However, CS_2 is not known to form a carbamoylating intermediate in its metabolism. All study subjects were male. Eighteen persons were smokers, one person took snuff, and 16 persons were nonsmokers. Exposure assessment was carried out for the workers by measuring NMF in their urine samples using a standardized method of the Deutsche Forschungsgemeinschaft DFG (*38*). The levels were determined to be between 1.3 and 46.5 mg/L (mean = 17.0 mg/L, median = 9.8 mg/L). The results were compared to those derived from samples of 42 healthy persons who were not known to have been exposed to MIC or DMF. Twenty-two persons were male, 20 female. Smoking status was assessed by questionnaire (12 smokers, 30 nonsmokers). The persons did not use any drugs with carbamoylating properties (e.g., disulfiram) and did not participate in chemoprevention studies or clinical trials for drug development. NMF in all of their urine samples was determined to be lower limit of detection (<0.1 mg/L).

Method Calibration and Validation. Calibration standards were prepared in the range of 5 and 500 nmol of MIH/g of globin (0.78 and 78.0 μg of MIH/g of globin). For this purpose, 3.12 mg of MIH were dissolved in 100 mL of ethanol. From this stock solution, six different calibration standards between 156 and 15.6 mg/L in ethanol were prepared. One milliliter of standard 1 was added to 200 mg of globin, 1 mL of standard 2 to 200 mg of globin, etc., and the globin samples were dried in a vacuum desiccator overnight. The globin was derived from nonsmoking persons not exposed to DMF or MIC. Blank samples containing concentrated HAc/HCl (v/v, 3 mL) were also prepared to check for possible MIH contamination during sample cleanup. In every analytical series a complete set of calibration standards and one blank sample were included. Using this kind of calibration, losses during sample preparation were compensated. To prepare a solution of the internal standard (IS), 3.4 mg of the EIH/NEVal-mixture were dissolved in 100 mL ethanol. After sample preparation, the quotient of the area counts under the curve between MIH and EIH was plotted as a function of the concentration of MIH. The calibration curve was used for the calculation of MIH concentrations (nmol/g of globin) in each blood sample. The method was validated. The linearity of the calibration curve was determined up to a concentration of 1000 nmol/g of globin. Precision in the series (n = 8) and from day to day (n = 8) was determined at two concentrations (25 and 100 nmol/g of globin), while (relative) recovery (n = 5) was determined at 50 and 200 nmol/g of globin.

Sample Preparation and GC/MS Analysis. For the analysis of *N*-methylcarbamoylated valine of globin, 100 μL of the internal standard were added to 200 mg globin in a 20-mL-screwed-top vial. Then the globin was dissolved in 3 mL of a mixture of HAc/HCl (1:1, v/v) and incubated for 1 h at 110 °C. After the sample cooled, it was neutralized stepwise using 10 M NaOH solution. Saturated saline solution (1 mL) was added, and the sample was shaken rapidly for 10 min. The pH was adjusted to 3−5 using concentrated HCl, and then the sample was extracted with 5 mL of ethyl acetate. The sample was shaken for 5 min and centrifuged at 3000*g* for 10 min. The extraction step was repeated. The organic phases were combined in a new vial and evaporated to ∼1.5 mL. The solution was

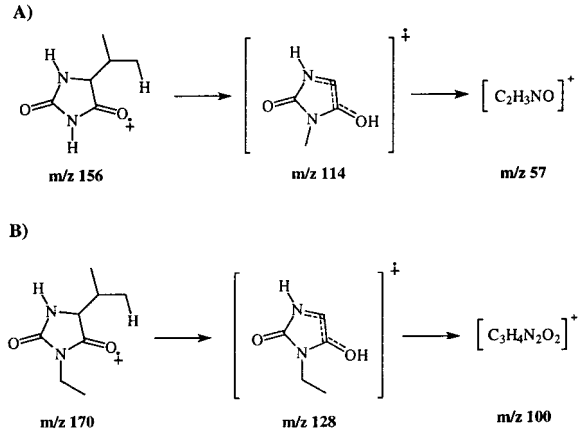

Figure 7. (A) Fragmentation of 3-methyl-5-isopropylhydantoin (*m*/*z* 156) yields *m*/*z* 114 (McLafferty rearrangement) and *m*/*z* 57. The ratio was found to be 1:160:35, respectively. The high abundance of *m*/*z* 114 in EI-MS is due to stabilization by electron delocalization (mesomeric effect). (B) Fragmentation of 3-ethyl-5-isopropylhydantoin is nearly identical. *m*/*z* 128 is formed by McLafferty rearrangement and *m*/*z* 100 by subsequent loss of ethene and H-transfer. The ratio for *m*/*z* 170:128:100 was determined to be 1:280:135, respectively.

transferred to a 1.7-mL-crimp-top vial and evaporated to dryness under a stream of nitrogen. The sample was dried in a vacuum desiccator over NaOH overnight and resolved in 1 mL of ethyl acetate again on the following day. After shaking for at least 15 min and centrifugation for 10 min at 3500*g* the supernatant was transferred to a new 1.7-mL-screwed-top vial and concentrated to 500 μL under a stream of nitrogen. One microliter was analyzed by GC/MS. Quantitative determination of Hb adducts derived from workers employed in the polyacrylic fiber industry and control subjects was carried out by EI-MS and multiple ion detection of three characteristic ions for MIH and EIH. The dwell time was maintained at 80 ms/ion trace. Recorded ion traces for MIH were *m*/*z* 156, 114, and 57, while *m*/*z* 170, 128, and 100 were recorded for EIH (Figure 7). The observed retention times were, t_R (MIH), 24.6 min, t_R (EIH), 26.7 min. Recorded ion trace for MIH during PCI-MS was *m*/*z* 157 [M + 1] after proton transfer from CH_5^+. The retention time was determined to be $t_R = 28.6$ min. PCI-MS was used to verify the outcome by GC/EI-MS. However, the method was not used for the quantification of adducts.

Results and Discussion

Synthesis of Standards. A simple and rapid synthesis for MIH is presented. For this purpose, D/L-valine was carbamoylated with MIC to NMVal (step 1). Instead of isolation, NMVal was subsequently treated with concentrated acids to give MIH (step 2). Step 1 was carried out in water over a period of 2 h at pH 9 since basic pH levels were reported to be advantageous for the reaction of D/L-valine and isocyanates (*39*, *40*). The pH adjustment was carried out using a freshly prepared 1 M Na_2O_2 solution. The kinetics of the formation of MIH was studied in dependence on the reaction time for step 1 (15, 30, 60, and 120 min). A strong increase in yield of MIH was investigated with increasing time. Yields of MIH were determined to be 23.8, 59.2, 76.1, and 80.1%, respectively. On the other side, step 2 was always completed after 15 min under reflux in concentrated acids. As a consequence, the rate-determining step is the formation of NMVal. Overall, the experiment points to a remarkable stability of MIC in aqueous solutions, which may be due to the use of Na_2O_2 as an alkalizing agent. However, as pH 9 is only ~2 levels above physiological pH, it is within the bounds of probability that MIC is transported in its "active" form throughout the body. On the other side, MIC was not directly determined in body fluids yet. Therefore, distribution in vivo may also occur via a semistabile, reversible mechanism, e.g., methylcarbamoylated thiols or methylcarbamoyl phosphate.

In contrast to MIH, different results were obtained for the synthesis of EIH. A considerable amount of ring-opened NEVal was traced after EIC reacted with D/L-valine for 2 h and reflux in concentrated acids for 15 min. A ratio of ~1:1 for EIH/NEVal was found according to the ^{1}H NMR spectrum. In analogy to the formation of MIH the kinetics for the formation of EIH was studied in dependence on the reaction times for step 1 and step 2. First, the reaction time for step 1 (D/L-valine with EIC) was increased to 6 and 12 h, while reaction time for step 2 (reflux in concentrated acids) was kept constant. Then, reaction time for step 1 was kept constant (2 h), while reflux in concentrated acid was extended to 1 and 2 h. Neither of the experiments changed the outcome as was determined by ^{1}H NMR. Always a ratio ~1:1 was found for EIH/NEVal. The results by ^{1}H NMR show that EIC can react with the amino group of D/L-valine to form NEVal. NEVal, however, is only converted to EIH in a minor extend. Decreased orbital overlapping caused by increasing repulsive interactions (sterical hindrance) during hydantoin formation may be of major role for the observed outcome.

Separation of EIH and NEVal using various organic solvents and solvent mixtures for recrystallation was not successful probably because of their structural similarity. No attempts were made to separate the compounds using column chromatography because the mixture is only used as internal standard. The ratio of ~1:1 was not assumed to undergo any changes during the cleanup procedure, which may influence the quantitative result. One can argue that a ratio of ~1:1 and difficulties to separate the substances may also point to the possibility that for some reason the ring-closed (or ring-opened) D- and L-isomers were obtained. However, based on stereochemistry alone, reaction of D/L-valine with a nonchiral molecule should not produce two products of different chirality.

Reliability of the Method. Differences in yields for the formation of hydantoins derived from the N-terminal valine are well-known from the literature. Calibration using the final hydantoins or the single amino acids (in our case MIH and NMVal) can lead to different results compared to radiolabeled Hb adduct standards. On the other side, there is only a negligible difference between the results derived from calibration using radiolabeled Hb standards and those derived from calibration with peptide standards (*41*, *42*). Ring closure to the hydantoin and the presence of at least one peptide bond (which is cleaved during cleanup) is therefore of crucial relevance during Hb adduct analysis. As a consequence, peptide standards or radiolabeled Hb standards should be used for calibration in Hb adduct analyses, which are based on the formation of hydantoins. With such standards, the N-terminal function of Hb is simulated during cleanup, and different conversion yields to the corresponding hydantoin are taken into account. To study the differences between dipeptide and hydantoin calibration, the dipeptide standards *N*-methylcarbamoyl-D/L-valine-D/L-leucine anilide and *N*-ethylcarbamoyl-D/L-valine-D/L-leucine anilide were also prepared, and the two calibration

Table 1. Reliability Criteria for the Determination of Hb Adducts after Exposure to DMF[a]

concentration	precision ($n = 8$) in series	precision ($n = 8$) day to day	relative recovery ($n = 5$)
25	7.2	12.4	
50			89.6
100	6.8	7.1	
200			92.7

[a] All concentrations are given in nanomoles of MIH per gram of globin, while precision and relative recovery is given as a percentage. n = number of investigated samples.

procedures were directly compared.[3] Surprisingly, no differences were observed. One explanation may be that ring-closure to MIH in the presence of concentrated acids and heat is nearly 100% once NMVal is formed. Since dipeptide standards are not commercially available, must be synthesized via a multistep procedure and are not advantageous compared to the use of MIH and EIH, there is no need to use them in the particular case of exposure to DMF or MIC. Therefore, future biological monitoring can be carried out using MIH for calibration, which makes the procedure easier from a practical point of view.

Under the conditions described here for sample preparation and the operational conditions for GC/EI-MS, a detection limit of 0.4 nmol of MIH/g of globin was calculated from a signal-to-noise ratio of 3:1 investigating *m*/*z* 114. The limit of detection for two ions (*m*/*z* 114, 57), however, is only 5 nmol/g because of the high abundance of *m*/*z* 114. The calibration curve was determined to be linear up to 1000 nmol/g of globin. Precision and recovery for the whole method were found to be in an acceptable range (Table 1).

In Situ Experiments. In the globin samples derived from the in situ experiments MIH could be clearly identified in MIC treated blood. Using GC/EI-MS and GC/PCI-MS MIH was identified based on its retention time and peak ratios compared to a standard solution of MIH in ethyl acetate (10 mg/L). The amount of MIH formed in the in situ experiments was sufficient to obtain a total ion count spectrum of MIH by MS. However, the results showed that PCI was less sensitive than EI. Possibly, negative chemical ionization (NCI-MS) may lead to better results. Unfortunately, we were not able to carry out GC/NCI-MS. However, the results derived from EI-MS and PCI-MS directly confirm those obtained by Ramachandran et al. (*33*), who demonstrated that MIC after inhalation serve as a *N*-methylcarbamoylating substance in vitro and in vivo in rats and rabbits. In our in situ samples not treated with MIC, MIH was identified in low amounts near the limit of detection using GC/EI-MS operating in the single ion monitoring mode for *m*/*z* 114. However, no peak could be detected in the chromatogram using GC/PCI-MS, which may be due to its lower sensitivity compared to EI-MS.

Population Samples. Thirty-five globin samples from persons exposed to DMF in the polyacrylic fiber industry were analyzed. A GC/EI-MS chromatogram derived from a globin sample of an exposed individual is shown in Figure 8. MIH could be identified in all blood samples based on its retention time and peak ratios. A mean of 120 nmol/g of globin (Table 2) was found in exposed

[3] H.U.K., Sieber, W., Burkhart-Reichl, A. and J.A. (2000) unpublished results.

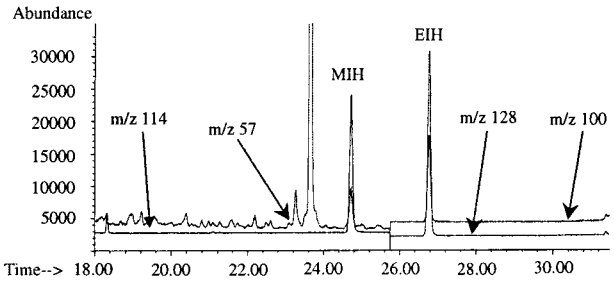

Figure 8. Chromatogram derived from a blood sample of an exposed worker in the polyacrylic fiber industry containing 73.8 nmol of 3-methyl-5-isopropylhydantoin/gram globin (*m*/*z* 156 not shown). The ratio of *m*/*z* 156:114:57 was determined to be 1:171:38.

Table 2. Descriptive Statistical Analysis for Two Population Samples Investigated in the Present Study[a]

investigated subjects	range	mean	median	5%	95%
exposed workers ($n = 35$)	26.1−412.0	120.3	109.7	29.8	214.3
	21.3−465.0[b]	123.6[b]	121.1[b]	25.3[b]	221.1[b]
general population ($n = 42$)	<LOD-16.6	1.8	1.4	0.4	2.8

[a] All concentrations are given in nanomoles of MIH per gram of globin. n = number of investigated persons, LOD = limit of detection, 5%, 95% = 5 and 95 percentiles. [b] Concentrations obtained by using dipeptide standards for calibration instead of MIH.

workers. In five spot checks, the presence of MIH was also verified by GC/PCI-MS. No statistical differences in MIH levels could be observed between smokers and nonsmokers (nonparametric Wilcoxon test).

In comparison to a previous publication (*43*), where we reported a mean value of 450.8 nmol of MIH/g of globin in 10 individuals exposed to DMF, Hb adduct levels were found to be lower in the present study. The difference may be mainly due to different exposure situations at the workplace. The exposure of the workers to DMF was verified by measurements of NMF in urine samples. NMF analysis in the previous publication (*43*) revealed concentrations between 8.8 and 84.6 mg/L (mean = 43.9 mg/L). Although NMF is only an index of daily exposure to DMF, the results indicated high exposures to DMF at the workplace in the previous study.[4] However, in the study presented here, NMF levels were determined to be between 1.3 and 46.5 mg/L (mean = 17.0 mg/L). Therefore, the exposure situation was lower, which is in line with lower Hb adduct concentrations.

Assuming that MIC is responsible for both, formation of MIH and AMCC, MIH concentrations should be lower in rodents since Mráz et al. (*13*) reported decreased formation of AMCC in rodents compared to humans. This suggestion is supported by the observation that metabolism of DMF to AMCC and MIH is closely tied together. Built-up of MIH levels paralleled the excretion course of AMCC after a single ip dose of 1000 mg of DMF/kg of body wt in rats (*44*). In addition, the levels of MIH and cumulative excretion of AMCC between the first and fourth day also correlated. Unfortunately, the results in rats by Mraz et al. (*44*) cannot be directly compared to those presented here in humans since dosage and route of uptake greatly differ from the situation at the workplace.

[4] The biological exposure index for NMF in urine is 15 mg/L.

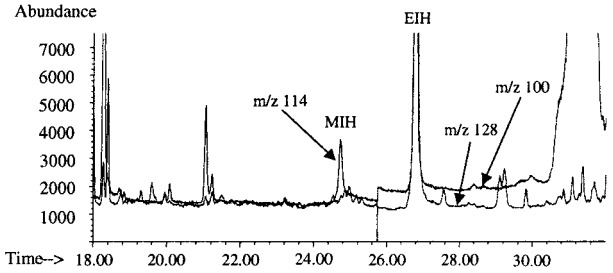

Figure 9. Chromatogram derived from a blood sample of a person not exposed to DMF or MIC. Nevertheless a small amount of MIH could be quantified (1.7 nmol/g globin). Only *m/z* 114 can be observed at such low concentrations.

To compare our results to those derived from persons nonexposed to DMF or MIC, blood samples of 42 individuals from the general population were analyzed. In contrast to exposed workers, MIH concentrations of the controls were up to 100 times lower and were determined to be in the range of 0.4 and 16.6 nmol of MIH/g of globin (Table 2). A chromatogram derived from a control individual is presented in Figure 9. No statistical differences in MIH concentrations between males and females were determined. Although a slight trend to higher MIH levels could be observed in smokers, the difference between smokers and nonsmokers was also not significant.

In contrast to reagent blanks, blood samples of the general population contained small amounts of MIH. Therefore, MIH is not totally selective for the exposure to DMF or MIC at the workplace. Since the general population is not exposed to DMF or MIC, the question remains as to why MIH was found in their blood samples. Possible explanations are speculative at the moment and should be investigated in future studies. The occurrence of methyl isothiocyanate (MITC) in the diet may be important. MITC was detected in wine samples (*45*, *46*). Moreover, thioglucosinolates, which can release MITC during enzymatic degradation, are present in cabbage and other cruciferous vegetables (*47*). MITC can yield methylamine and CO_2 through the intermediacy of MIC (*48*). A completely different explanation, however, would be carbamoylation of Hb subsequently followed by methylation. Carbamoylated Hb (CHb) is formed in humans mainly via the reaction of Hb with cyanate, a product of in vivo urea dissociation. Studies within the 90s (*49*, *50*, *51*) revealed high levels of CHb in healthy, nonexposed persons (between 200 and 450 nmol/g of Hb). Methylation of CHb, e.g., by *S*-adenosylmethionine (*52*), would yield *N*-methylcarbamoylated Hb.

Overall, the results evidence the formation of Hb adducts at the N-terminal valine of globin after exposure to DMF. Hb adducts may serve as a new biomarker on the molecular level to assess exposure to DMF. Therefore the method may be used in future biological monitoring. The study also provides an indirect evidence for the formation of MIC as a reactive intermediate during metabolism of DMF in humans since (1) the observed Hb adducts were proven to be identical to those derived from in situ reaction of human blood samples with MIC and (2) adducts were also identical to those which were reported after the Bhopal incidence in 1984. Therefore, the pathological outcome (toxic hepatitis, malformations, etc.) after exposure to DMF may be mediated by MIC. As Hb adducts are accepted biomarkers of mutagenic relevance (*53*), the study also shed some new light on the suspected carcinogenicity of DMF.

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References

(1) Lauwerys, R. R., Kivits, A., Lhoir, M., Rigolet, P., Houbeau, D., Buchet, J. P., and Roels, H. A. (1980) Biological surveillance of workers exposed to dimethylformamide and the influence of skin protection on its percutaneous absorption. *Int. Arch. Occup. Environ. Health* **45**, 189−203.

(2) Kennedy, G. L., Jr. (2001) Biological effects of acetamide, formamide, and their monomethyl and dimethyl derivatives: an update. *Crit. Rev. Toxicol.* **31**, 139−222.

(3) Fail, P. A., George, J. D., Grizzle, T. B., and Heindel, J. J. (1998) Formamide and dimethylformamide: reproductive assessment by continuous breeding in mice. *Reprod. Toxicol.* **12**, 317−332.

(4) Walrath, J., Fayerweather, W. E., Gilby, B. G., and Pell, S. (1989) A case-control study of cancer among Du Pont employees with potential for exposure to dimethylformamide. *J. Occup. Med.* **31**, 432−438.

(5) Ducatman, A. M., Conwill, D. E., and Crawl, C. (1986) Germ-cell tumors of the testicle among aircraft repairman. *J. Urol.* **136**, 834−836.

(6) Levin, S. M., Baker, D. B., Landrigan, P. J., Monaghan, S. V., Frumin, E., Braithwaite, M., and Towne, W. (1987) Testicular cancer in leather tanners exposed to dimethylformamide. *Lancet* **333**, 1153.

(7) Chen, J. L., and Kennedy, G. L. (1988) Dimethylformamide and testicular cancer. *Lancet* **334**, 55.

(8) Calvert, G. M., Fajen, J. M., Hills, B. W., and Halperin, W. E. (1990) Testicular cancer, dimethylformamide, and leather tanneries. *Lancet* **336**, 1253−1254.

(9) Gollins, W. J. (1991) Dimethylformamide and testicular cancer. *Lancet* **337**, 306−307.

(10) Malley, L. A., Slone, T. W., Van Pelt, C., Elliott, G. S., Ross, P. E., Stadler, J. C., and Kennedy, G. L. (1994) Chronic toxicity/oncogenicity of dimethylformamide in rats and mice following inhalation exposure. *Fund. Appl. Toxicol.* **23**, 268−279.

(11) International Agency for Research on Cancer (1989) *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. Some Organic Solvents, Resin Monomers and Related Compounds, Pigments and Occupational Exposures in Paint Manufacture and Painting*, Vol. 47, Lyon.

(12) Ducatman, A. M. (1989) Dimethylformamide, metal dyes, and testicular cancer. *Lancet* **335**, 911.

(13) Mráz, J., Cross, H., Gescher, A., Threadgill, M. D., and Flek, J. (1989) Differences between rodents and humans in the metabolic toxification of *N,N*-dimethylformamide. *Toxicol. Appl. Pharmacol.* **98**, 507−516.

(14) Mráz, J., Gescher, A., Cross, H., Shaw, A. J., and Flek, J. (1991) New findings in the metabolism of *N,N*-dimethylformamide-consequences for evaluation of occupational risk. *Sci. Total. Environ.* **101**, 131−134.

(15) Kestell, P., Threadgill, M. D., Gescher, A., Gledhill, A. P., Shaw, A. J., and Farmer, P. B. (1987) An investigation of the relationship between the hepatotoxicity and the metabolism of *N*-alkylformamides. *J. Pharmacol. Exp. Ther.* **240**, 265−270.

(16) Mráz, J., and Nohová, H. (1992) Absorption, metabolism and elimination of *N,N*-dimethylformamide in humans. *Int. Arch. Occup. Environ. Health* **64**, 85−92.

(17) Gescher, A. (1993) Metabolism of *N,N*-dimethylformamide: key to the understanding of its toxicity. *Chem. Res. Toxicol.* **6**, 245−251.

(18) Midorikawa, K., Murata, M., Oikawa, S., Tada-Oikawa, S., and Kawanishi, S. (2000) DNA damage by dimethylformamide: Role of hydrogen peroxide generated during degradation. *Chem. Res. Toxicol.* **13**, 309−315.

(19) Cordeiro, R. F., and Savarese, T. M. (1986) Role of glutathione depletion in the mechanism of action of *N*-methylformamide and *N*,*N*-dimethylformamide in a cultured human colon carcinoma cell line. *Cancer Res.* **46**, 1297−1305.
(20) Shaw, A. J., Gescher, A., and Mráz, J. (1988) Cytotoxicity and metabolism of the hepatotoxin *N*-methylformamide and related formamides in mouse hepatocytes. *Toxicol. Appl. Pharmacol.* **95**, 162−170.
(21) Mráz, J., Jheeta, P., Gescher, A., Hyland, R., Thummel, K., and Threadgill, M. D. (1993) Investigation of the mechanistic basis of *N*,*N*-dimethylformamide toxicity. Metabolism of *N*,*N*-dimethylformamide and its deuterated isotopomers by cytochrome P450 2E1. *Chem. Res. Toxicol.* **6**, 197−207.
(22) Ehrenberg, L., Granath, F., and Törnqvist, M. (1996) Macromolecule adducts as biomarkers of exposure to environmental mutagens in human populations. *Environ. Health Perspect.* **104** (Suppl. 3), 423−428.
(23) Törnqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through Hb adducts by a modified Edman degradation method. *Anal. Biochem.* **154**, 255−266.
(24) Walker, V. E., Wu, K. Y., Upton, P. B., Ranasinghe, A., Scheller, N., Cho, M. H., Vergnes, J. S., Skopek, T. R., and Swenberg, J. A. (2000) Biomarkers of exposure and effect as indicators of potential carcinogenic risk arising from in vivo metabolism of ethylene to ethylene oxide. *Carcinogenesis* **21**, 1661−1669.
(25) Walker, V. E., MacNeela, J. P., Swenberg, J. A., Turner, M. J., Jr., and Fennell, T. R., (1992) Molecular dosimetry of ethylene oxide: formation and persistence of *N*-(2-hydroxyethyl)valine in hemoglobin following repeated exposures of rats and mice. *Cancer Res.* **52**, 4320−4327.
(26) Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Törnqvist, M. (2000) Acrylamide: a cooking carcinogen? *Chem. Res. Toxicol.* **13**, 517−522.
(27) Perez, H. L., Segerbäck, D., and Osterman-Golkar, S. (1999) Adducts of acrylonitrile with hemoglobin in nonsmokers and in participants in a smoking cessation program. *Chem. Res. Toxicol.* **12**, 869−873.
(28) Tavares, R., Borba, H., Monteiro, M., Proenca, M. J., Lynce, N., Rueff, J., Bailey, E., Sweetman, G. M., Lawrence, R. M., and Farmer, P. B. (1996) Monitoring of exposure to acrylonitrile by determination of *N*-(2-cyanoethyl)valine at the *N*-terminal position of Hb. *Carcinogenesis* **17**, 2655−2660.
(29) Sabbioni, G., Hartley, R., Henschler, D., Hollrigl-Rosta, A., Köber, R., and Schneider, S. (2000) Isocyanate-specific hemoglobin adduct in rats exposed to 4,4′-methylenediphenyl diisocyanate. *Chem. Res. Toxicol.* **13**, 82−89.
(30) Sriramachari, S., Rao, G. J., Sharma, V. K., Jadhav, R. K., Saraf, A. K., and Chandra, H. (1991) GC−NPD and GC-MS analysis of preserved tissue of Bhopal gas disaster: evidence of methyl carbamoylation in post-mortem blood. *Med. Sci. Law* **31**, 289−293.
(31) Hazardous Substance Data Bank, HSDB (1998) Methyl isocyanate and ethyl isocyanate. *National Library of Medicine*, (http://toxnet.nlm.nih.gov/).
(32) Kaushik, M. P., Sikder, A. K., and Jaiswal, D. K. (1987) A convenient laboratory synthesis of methyl isocyanate. *Current Sci.* **56**, 1008.
(33) Ramachandran, P. K., Gandhe, B. R., Venkateswaran, K. S., Kaushik, M. P., Vijayaraghavan, R., Agarwal, G. S., Gopalan, N., Suryanarayana, M. V., Shinde, S. K., and Sriramachari, S. (1988) Gas chromatographic studies of the carbamoylation of Hb by methyl isocyanate in rats and rabbits. *J. Chromatogr.* **426**, 239−247.
(34) Venkateswaran, K. S., Raghuveeran, C. D., Gopalan, N., Agarwal, G. S., Kaushik, M. P., and Vijayaraghavan, R. (1992) Monitoring of haemoglobin-methyl isocyanate adduct by high-performance liquid chromatography with diode array detector. *Biochem. Int.* **28**, 745−750.
(35) Törnqvist, M. (1990) Formation of reactive species that lead to hemoglobin adducts during storage of blood samples. *Carcinogenesis* **11**, 51−54.
(36) Mowrer, J., Törnqvist, M., Jensen, S., and Ehrenberg, L. (1986) Modified Edman degradation applied to hemoglobin for monitoring occupational exposure to alkylating agents. *Toxicol. Environ. Chem.* **11**, 215−231.
(37) Bader, M., Lewalter, J., and Angerer, J. (1995) Analysis of *N*-alkylated amino acids in human Hb: evidence for elevated *N*-methylvaline levels in smokers. *Int. Arch. Occup. Environ. Health* **67**, 237−242.
(38) DFG, Deutsche Forschungsgemeinschaft (1997) in *Analyses of Hazardous Substances in Biological Materials* (Angerer, J., and Schaller, K. H., Ed.) Vol. 5, pp 97−108, VCH Weinheim.
(39) Rivett, D. E., and Wilshire, J. F. K. (1965) Reactions of *N*,*N*-diphenylcarbamoyl chloride. *Aust. J. Chem.* **18**, 1667−1675.
(40) Möller, M., Henschler, D., and Sabbioni, G. (1998) Synthesis and spectroscopic characterization of 4-chlorophenyl isocyanate (=1-chloro-4-isocyanatobenzene) adducts with amino acids as potential dosimeters for the biomonitoring of isocyanate exposure. *Helv. Chim. Acta* **81**, 1254−1263.
(41) Törnqvist, M., Magnusson, A. L., Farmer, P. B., Tang, Y. S., Jeffrey, A. M., Wazneh, L., Beulink, G. D., van der Waal, H., and van Sittert, N. J. (1992) Ring test for low levels of *N*-(2-hydroxyethyl)valine in human Hb. *Anal. Biochem.* **203**, 357−360.
(42) Pauwels, W., Farmer, P. B., Osterman-Golkar, S., Severi, M., Cordero, R., Bailey, E., and Veulemans, H. (1997) Ring test for the determination of *N*-terminal valine adducts of styrene 7,8-oxide with Hb by the modified Edman degradation technique. *J. Chromatogr. B* **702**, 77−83.
(43) Angerer, J., Göen, T., Krämer, A., and Käfferlein, H. U. (1998) *N*-methylcarbamoyl adducts at the *N*-terminal valine of globin in workers exposed to *N*,*N*-dimethylformamide. *Arch. Toxicol.* **72**, 309−313.
(44) Mráz, J., Gálova, E., Nohová, H., Boušková, and Hornychová, M. (1998) *Formation of N-methylcarbamoyl adduct with N-terminal valine of globin following the exposure to N,N-dimethylformamide (DMF) and N-methylformamide (MF) in rats*, 4th International Symposium on Biological Monitoring in Occupational and Environmental Health, 23−25 Sept. 1998, p 125, Seoul, Korea.
(45) Saito, I., Yamada, S., Oshima, H., Ikai, Y., Oka, H., and Hayakawa, J. (1994) Analysis of methyl isothiocyanate in wine by gas chromatography with dual detection. *J. AOAC Int.* **77**, 1296−1299.
(46) Rostron, C. (1992) Methyl isothiocyanate in wine. *Food Chem. Toxicol.* **30**, 821−823.
(47) Fenwick, G. R., Heaney, R. K., and Mullin, W. J. (1983) Glucosinolates and their breakdown products in food and food plants. *Crit. Rev. Food Sci. Nutr.* **18**, 123−201.
(48) Valentine, W. M., Amarnath, V., Amarnath, K., and Graham, D. G. (1995) Characterization of protein adducts produced by *N*-methyldithiocarbamate and *N*-methyldithiocarbamate esters. *Chem. Res. Toxciol.* **8**, 254−261.
(49) Balion, C. M., Draisey, T. F., and Thibert, R. J. (1998) Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. *Kidney Int.* **53**, 488−495.
(50) Han, J. S., Kim, Y. S., Chin, H. J., Jeon, U. S., Ahn, C., Kim, S., Lee, J. S., Jang, I. J., and Shin, S. G. (1997) Temporal changes and reversibility of carbamylated hemoglobin in renal failure. *Am. J. Kidney Dis.* **30**, 36−40.
(51) Stim, J., Shaykh, M., Anwar, F., Ansari, A., Arruda, J. A., and Dunea, G. (1995) Factors determining hemoglobin carbamylation in renal failure. *Kidney Int.* **48**, 1605−1610.
(52) Törnqvist, M., and Kautiainen, A. (1993) Adducted proteins for identification of endogenous electrophiles. *Environ. Health Perspect.* **99**: 39−44.
(53) IPCS, International Program on Chemical Safety (1993) *Biomarkers and risk assessment*, Environmental Health Criteria 155, WHO, Geneva.

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