Comparison of the Hemoglobin Adducts Formed by Administration of N-Methylolacrylamide and Acrylamide to Rats

Timothy R. Fennell,*1 Rodney W. Snyder,*2 Wojciech L. Krol,*3 and Susan C. J. Sumner*4

*CIIT Centers for Health Research, P.O. Box 12137, Research Triangle Park, North Carolina 27709

Received August 16, 2002; accepted October 29, 2002

Acrylamide (AM) and N-methylolacrylamide (NMA) are used in the formulation of grouting materials. AM undergoes metabolism to a reactive epoxide, glycidamide (GA). Both AM and GA react with hemoglobin to form adducts that can be related to exposure to AM. The objective of this study was to evaluate the extent to which NMA could form the same adducts as AM. N-(2-carbamoyethyl)valine (AAVal derived from AM) and N-(2-carbamoyl-2-hydroxyethyl)valine (GAVal derived from GA) were measured following a single oral dose of AM (50 mg/kg) or NMA (71 mg/kg) in male F344 rats. AAVal and GAVal were measured by a modified Edman degradation to produce phenylthiohydantoin derivatives and liquid chromatography/tandem mass spectrometry. In AM-treated rats, AAVal was 21 ± 1.7 pmol/mg globin (mean ± SD, n = 4), and GAVal was 7.9 ± 0.8 pmol/mg. In NMA-treated rats, AAVal was 41 ± 4.9 pmol/mg, and GAVal was 1.4 ± 0.1 pmol/mg. Whether AAVal was derived from reaction of NMA with globin followed by loss of the hydroxymethyl group, or the hydroxyethyl group to form AM, is not known. However, the higher ratio of AAVal:GAVal in NMA-treated rats (29 vs. 2.6 in AM-treated rats) suggests that reaction of NMA with globin is the predominant route to AAVal in NMA-treated rats. The detection of GAVal in NMA-treated rats indicates oxidation of NMA, either directly or following conversion to AM. The lower levels of GAVal on NMA administration compared with AM treatment. The lower levels of GAVal on NMA administration indicates oxidation of NMA, either directly or following conjugation with glutathione, or by oxidation to glycidamide (GA), a reactive epoxide (Fig. 1) (Calleman et al., 1990; Sumner et al., 1992). The genotoxic effects of AM are thought to be mediated by its metabolism to GA, which is mutagenic (Adler et al., 2000; Barfknecht et al., 1988; Butterworth et al., 1992; Generoso et al., 1996; Hashimoto and Tanii, 1985) and can react with DNA to form adducts (Segerbäck et al., 1995).

DNA adducts from GA have been reported following administration of AM to rodents (Segerbäck et al., 1995). AM is also reactive, and the direct reactivity of AM with cellular components may also play a role in its toxicity. Hemoglobin adducts from direct reaction with AM and from reaction with GA have been described in rodents administered AM, in exposed people, and in cigarette smokers (Bailey et al., 1986; Bergmark, 1997; Bergmark et al., 1991, 1993; Calleman et al., 1990, 1994). Recently the detection of acrylamide in fried foods has raised considerable concern about human exposure to low levels of AM in the diet (Tareke et al., 2002).

N-Methylolacrylamide (NMA) is produced by the reaction of formaldehyde with AM, and is used for the production of grouting agents (Fig. 1). NMA is carcinogenic in mice, producing tumors of the hardener gland, liver, and lung in both sexes, and benign granulosa-cell neoplasms of the ovary in females (Bucher et al., 1990). NMA was not carcinogenic in rats at the doses tested. NMA is neurotoxic in both rats and mice (Bucher et al., 1990) and has been estimated to have approximately 30% of the neurotoxic potency of an equivalent dose of acrylamide (Edwards, 1975). Like AM, NMA reacts rapidly with glutathione (Hashimoto and Aldridge, 1970) and is metabolized by conjugation with glutathione, resulting in N-acetyl-S-(3-hydroxymethylamino-3-oxopropyl)cysteine as the major urinary metabolite in rats and mice (Mathews, 2001).

The accidental release of AM and NMA from a tunnel construction project in Sweden resulted in the exposure of
workers, ground water contamination, the death of fish, and poisoning of cattle (Hagmar et al., 2001). In the exposed workers, N-(2-carbamoyl-2-hydroxyethyl)valine, formed by reaction of AM with the N-terminal valine residue of hemoglobin, was measured as an indicator of internal dose. A clear association was described between levels of the hemoglobin adduct and symptoms of peripheral nervous-system toxicity (Hagmar et al., 2001). The extent of formation of globin adducts from GA was not reported in this study. Whether the N-(2-carbamoyl-2-hydroxyethyl)valine arose from reaction of hemoglobin with AM or from NMA could not be distinguished.

In this study, we investigated the hypothesis that AM and NMA can give rise to the same adducts in globin, but are metabolized quantitatively in a different manner. This hypothesis was tested by measuring the formation of hemoglobin adducts from AM and GA in rats administered AM or NMA by gavage. The specific adducts measured were N-(2-carbamoyl-2-hydroxyethyl)valine, formed by direct reaction of AM with the N-terminal valine residue, and N-(2-carbamoyl-2-hydroxyethyl)valine, formed by reaction of GA with the N-terminal valine residue. To enable a parallel measurement of the extent of metabolism by 11C nuclear magnetic resonance (11C-NMR) spectroscopy reported elsewhere, the AM used was 13C-labeled (Sumner et al., 1992). AAVal and GAVal formed in hemoglobin were measured to compare the internal dose of AM vs. GA.

**MATERIALS AND METHODS**

**Chemicals.** [1,2,3-13C]AM (lot number PR-11085) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA) with a 98% chemical purity and a 99% enrichment. 13C-NMR spectra of the [1,2,3-13C]AM, acquired by the vendor prior to study initiation and acquired at CIIT after delivery, were consistent with the chemical structure: multiplets at 127–131 ppm and a doublet (Jcc = 51 Hz) at 171 ppm, consistent with the 13C-labeled carbons for the vinyl group (C=CH) and carbonyl group (C=O), respectively.

Methylolacrylamide (NMA, N-hydroxymethylacrylamide, CAS No 924–42–5) was obtained from TCI America (Portland, OR) as a powder. The vendor specified a minimum purity of 99%. 1H- and 13C-NMR analysis appeared consistent with the specified purity and did not indicate the presence of any free formaldehyde or acrylamide.

N-(2-carbamoyl-2-hydroxyethyl)valine-leu-anilide (AAVal-leu-anilide) was obtained from Bachem (King of Prussia, PA). Phenylisothiocyanate was obtained from Aldrich (Milwaukee, WI). Valine and valine-13C were obtained from Sigma (St. Louis, MO) and Isotec, Inc. (Miamisburg, OH), respectively.

**Synthesis of glycidamide.** Glycidamide was synthesized by H2O2 oxidation of acrylonitrile (Payne and Williams, 1961), and stored at −20°C. The 1H nuclear magnetic resonance (1H-NMR) spectrum for GA in CDCl3 (7.24 ppm) contained three 1-proton multiplets centered at 3.45 ppm (CH2), 2.95 ppm (CH2), and 2.79 ppm (CH3), and a broad 2-proton singlet at 6.2 ppm (NH2). The 13C-NMR spectrum (in CDCl3, 77 ppm) contained signals at 48 ppm (CH3), 50 ppm (CH2), and 173 (CONH2) that are consistent with the structure of GA.

**Synthesis of N-(2-carbamoyl-2-hydroxyethyl)valine.** AAVal, AAVal-13C5 was synthesized and purified, as described previously, by Bergmark et al. (1993). AM (10.01 g) and valine (1.76 g) were reacted in 30 ml water and 2.6 ml triethylamine for 6 days at room temperature. The 1H-NMR spectrum of AAVal in D2O (4.9 ppm) had signals at 3.6 ppm (doublet, CH2), 3.4 ppm (multiplet, CH2-NH), 2.8 ppm (triplet, CH2-NH), 2.3 (multiplet, CH2), and 1.1 ppm (two doublets, valine CH3). Integration of the signals provided a ratio appropriate for the number of assigned hydrogens. The 13C-NMR spectrum showed 2 carbonyl signals at 176 and 179 ppm, a signal at 72 ppm (CH3), a signal at 47 ppm (CH2-N), 2 signals near 33 ppm (CH3 and CH2-CO), and 2 signals near 21 ppm (2 CH3).

**Synthesis of N-(2-carbamoyl-2-hydroxyethyl)valine-13C5 (AAVal-13C5).** Labeled material for use as an internal standard was prepared on a smaller scale, essentially as described above for the unlabeled standard. 13C-Valine (62.5 mg) was reacted with AM (88.19 mg) in 1 ml of water and 87.5 µl of triethylamine for 6 days at room temperature. The NMR spectra of AAVal prepared from 13C-Val contained 13C-1H and 13C-13C coupling patterns for signals derived from the valine portion of the molecule, while signals derived from the AM portion of the molecule did not contain these patterns. The 1H-NMR spectrum of 13C-AAVal had signals at 3.4 ppm (multiplet, CH2-NH and CH2), 2.8 ppm (triplet, CH2-NH), 2.3 (multiplet, CH2), and 1.1 ppm (two doublets, valine CH3). A doublet of multiplets (at 2.1 and 2.5 ppm) were centered on 2.3 ppm, the shift for the CH3 proton. A doublet of multiplets (at 0.9 and 1.35 ppm) were centered at 1.1 ppm, consistent with the shift for the 2 CH3 protons. Integration of the signals provided a ratio appropriate for the number of assigned hydrogens. Signals derived from the valine-labeled carbons were located in the 13C-NMR spectrum near 176 ppm (CO, doublet), 72 ppm (CH3, 2 doublets), 33 ppm (CH2-CH3, 2 doublets), and 21 ppm (CH2-CH3, 2 doublets).

**Synthesis of N-(2-carbamoyl-2-hydroxyethyl)valine.** N-(2-carbamoyl-2-hydroxyethyl)valine (GAVal) was synthesized and purified as described previously by Bergmark et al. (1993). GA (1.25 g) and valine (0.583 g) were reacted in 5 ml of water and 87 µl of triethylamine for 24 h at 45°C. In addition to the expected signals for Val CH3 (3.65 ppm, 2 doublets), CH2 (2.35 ppm, multiplet), and CH3 (1.1 ppm, 2 doublets), multiplets for the GA-derived protons were observed at 4.65 ppm (CHOH) and at 3.3–3.6 ppm (CH2) in the 1H-NMR spectrum of GAVal in D2O. Integration of the spectrum provided the appropriate integration ratios for the assigned signals. Signals derived from the valine-labeled carbons were located in the 13C-NMR spectrum near 165 ppm (CO, doublet), 72 ppm (CH3, 2 doublets), 33 ppm (CH2-CH3, 2 doublets), and 21 ppm (CH2-CH3, 2 doublets).

**Synthesis of N-(2-carbamoyl-2-hydroxyethyl)valine-13C5 (GAVal-13C5).** Synthesis of N-(2-carbamoyl-2-hydroxyethyl)valine-13C5 (GAVal-13C5) for use as an internal standard material was conducted as described above, but on a smaller scale. 13C-Valine (57.3 mg) was reacted with GA (0.121 g) in 0.976 ml water and 85.4 µl of triethylamine for 6 days at room temperature. Signals
in the 13C-NMR spectrum of GAVal-13C5, were at shifts consistent with those obtained for unlabeled GAVal, and contained splitting patterns and relative signal intensity consistent with incorporation of the labeled carbons of 13C-

**Synthesis of AAVal phenylthiohydantoin.** To prepare the AAVal phenylthiohydantoin derivative (AAVal PTH), AAVal (12.2 mg) was incubated with phenylisothiocyanate (30 μl) in 1.5 ml of 0.5 M potassium bicarbonate:1-propanol (2:1), pH 8.6, for 2 h at 45°C. The reaction product was isolated by extracting with n-heptane (2×2 ml), drying under N2, redissolving in toluene, drying under N2, redissolving in methanol, and purifying by HPLC using a Beckman Ultrasphere ODS column (0.45×25 cm) eluted with 35% methanol/ water. The 1H-NMR spectrum of AAVal PTH (CDCl3) contained signals for the phenyl ring protons at 7.2–7.6 ppm. Additional signals were observed at 4.3 ppm (doublet, CH2, 3.85 and 4.35 ppm (multiplets, CH2-N, 2.6 and 3.0 ppm (multiplets, CH2-CO), 2.5 ppm (multiplet, CH2), and 0.95 and 1.25 ppm (2 doublets, valine CH2)). Nondetectable CH3 signals suggested hindered rotation of the AM side chain in AAVal PTH compared with AAVal. The 13C-NMR spectra of AAVal PTH contained signals for the Val portion of the molecule at 68 ppm (CH3) 30 ppm (CH2), and 16 and 18 ppm (2 CH2). Signals for AM-derived carbons were at 34 ppm (CH2-N), 42 ppm (CH2-CO), and 129–130 ppm for the phenyl ring carbons.

**Synthesis of AAVal PTH-13C5.** AAVal-13C5 (9.1 mg) was incubated with phenylisothiocyanate (20 μl) in 1.5 ml of 0.5 M potassium bicarbonate:1-propanol (2:1), pH 8.6, for 2 h at 45°C, and the product was isolated as described above. The 1H-NMR spectrum of 13C5-Val, AAVal PTH contained signals (multiplets near 4.3, 3.8, 3.0, and 2.6 ppm) for the AM portion of the molecule consistent with those detected for the unlabeled AAVal PTH. Patterns consistent with 13C-1H coupling were observed for signals from the Val-13C5 portion of the molecule. The valine 13CH3 gave rise to triplets at 4.0 and 4.5 ppm (centered at 4.3 ppm). The valine 13CH2 gave rise to multiplets at 2.3 and 2.7 ppm (centered at 2.5 ppm). The valine methyl groups gave rise to multiplets at 0.7 and 1.2 (centered at 0.95), and 1.0 and 1.45 ppm (centered at 1.2 ppm). Signals in the 13C NMR spectrum of 13C5-Val were consistent with the AAVal PTH, containing the 13C-13C splitting patterns and relative intensities consistent with the presence of 13C5-Val.

**Synthesis of GAVal PTH.** GAVal (12.0 mg) was incubated with phenylisothiocyanate (30 ml) in 1.5 ml of 0.5 M potassium bicarbonate:1-propanol (2:1), pH 8.6, for 2 h at 45°C. The reaction product was isolated by extraction with n-heptane (2×2 ml), drying under N2, redissolving in toluene, drying under N2, redissolving in methanol, and then isolating on HPLC using a Beckman Ultrasphere ODS column (0.45×25 cm) eluted with 35% methanol/ water at 30% acetonitrile for 10 min, 40% acetonitrile for 3.5 min, and 100% acetonitrile for 1.5 min. The 1H-NMR spectrum of GAVal PTH (in CDCl3) contained a sharp signal at 4.6 ppm (CHOH) and two broader signals at 4.0 and 4.5 ppm (CH2) derived from the GA-protons. Signals for the Val-derived protons were located near 0.9 and 1.3 ppm (CH3, 2 doublets) 2.5 ppm (CH2, multiple), and 4.3 ppm (CH3). Ring protons were present between 7.2 and 7.6 ppm. The 13C NMR spectrum of GAVal PTH-13C5 (in CDCl3) has intense multiplets for the Val-13C, portion of the molecule at 69 ppm (CH3), 30 ppm (CH2), and 15 and 18 ppm (2 CH2).

**Animals.** Male Fischer F344 rats were purchased from Charles River Laboratories (Raleigh, NC). At the time of dosing, the rats were 9–10 weeks old. They were supplied food (NIH 07 diet, Zeigler brothers, Gardner, PA) and reverse osmosis water ad libitum and maintained on a 12-h light-dark cycle (0700–1900 h for light phase) at a temperature of 64–79°F and relative humidity of 30–70%.

**Dosing and sample collection.** Male Fischer 344 rats (4 per group) were administered [1,2,3-13C]AM at a gavage at a nominal dose of 50 mg/kg body weight. NMA was administered by gavage to four male F344 rats at a nominal dose of 71 mg/kg body weight, targeted to provide an equimolar dose administered for AM and NMA. The AM and NMA dose solutions were prepared in distilled water and delivered at 1 ml/kg body weight. A control group of four additional F344 rats were administered distilled water. The dose administered was calculated by weighing the dosing syringe before and after dosing. The actual doses administered were 59.5 ± 8.0 mg AM/kg (0.80 ± 0.11 mmol/kg) and 73.1 ± 3.9 mg NMA/kg (0.72 ± 0.03 mmol/kg). The control rats and rats treated with [1,2,3-13C]AM were placed in glass metabolism cages after administration of the labeled material, and urine was collected over dry ice for 0–24 h after dosing. The urine samples were stored at −80°C. The rats administered NMA were placed in polycarbonate cages after dosing. At 24 h after administration, rats were euthanized by exposure to CO2, and blood was collected by cardiac puncture in a heparinized syringe. The blood was separated into red blood cells and plasma, and the red blood cell fraction was washed three times with 0.9% (w/v) saline and stored at −20°C for adduct analysis.

**Analysis of AAVal and GAVal in hemoglobin.** AAVal and GAVal, formed by reaction of AM and GA respectively with the N-terminal valine residue in hemoglobin, were measured by an LC-MS/MS method. Globin was isolated from washed red cells (Mowrer et al., 1986). Globin samples (approximately 20 mg) were derivatized with phenylisothiocyanate (5 μl) in formamide (1.5 ml) with 1 N NaOH (5 μl) to form adduct phenylthiohydantoin derivatives in a manner analogous to the modified Edman degradation (Bergmark, 1997; Perez et al., 1999; Törmäyst et al., 1986). AAVal PTH-13C5 (26.6 pmol) and GAVal PTH-13C5 (36.0 pmol) were added as internal standards, and the samples were extracted using a Waters Oasis HLB 3 ml (60 mg) extraction cartridge (Milford, MA). The derivatized adducts were eluted with methanol, dried, and reconstituted in 100 ml of MeOH:H2O (50:50, containing 0.1% formic acid). Analysis was conducted using a PE Series 200 HPLC system interfaced to a PE Sciex API 3000 LC-MS with a Turboionspray interface. Chromatography was conducted on a Phenomenex Luna PhenylHexyl Column (30 × 2 × 3 mm) eluted with 0.1% acetic acid in water and methanol at a flow rate of 350 μl/min, with a gradient of 45–55% methanol in 2.1 min. The elution of adducts was monitored by multiple-reaction monitoring (MRM) in the negative-ion mode for the following ions:

- AAVal-PTH: m/z 304 → 233 (M-H → M-H-CH2-CH2-CONH)
- GAVal-PTH: m/z 320 → 233 (M-H → M-H-CH2-CHOH-CONH)
- AAVal-PTH-13C5: m/z 309 → 238 (M-H → M-H-13CH2-CH2-CONH)

Quantitation of AAVal was conducted using the ratio of analyte to internal standard, with a calibration curve generated using AAVal-leu-anilide. Quantitation of GAVal was conducted using the ratio of analyte to internal standard.

For samples from rats administered a single dose of [1,2,3-13C]AM, the 13CAAVal and 13CGAVal adducts formed can be distinguished in the negative-ion mode since the 13C-containing adduct side chain is lost from the adduct PTH in the collision cell. An additional set of ions is monitored to quantitate the adducts formed:

- 13C-AAVal-PTH: m/z 307 → 233 (M-H → M-H-13CH2-CH2-CONH)

**Statistical analysis.** Statistical analysis was conducted using Instat 2.01 (Graphpad Software, San Diego, CA). One-way analysis of variance
ANOVA with the Tukey–Kramer post test was used to compare control, AM-treated, and NMA-treated groups.

RESULTS

Hemoglobin Adducts of AM and GA

For mass spectral analysis of AAVal and GAVal, the AAVal- and GAVal-PTH derivatives were analyzed in the negative ion mode with a turboionspray interface. In the negative ion mode, the major ion formed was the parent ion (M-H\(^{-}\)), and the major daughter ions resulted from loss of the AM or GA side chain (Figs. 2 and 3). This provided the capability to distinguish between the adducts derived from AM, from [1,2,3-\(^{13}\)C]AM, and from the internal standard labeled with valine-\(^{13}\)C\(_{5}\), since the loss of the AM and GA side chains results in three distinct reactions that can be monitored to detect each form of the adduct. For example, in the negative ion mode, AAVal PTH is monitored by m/z 304 → 233 (M-H\(^{-}\) → M-H\(^{-}\) - CH\(_{2}\)CH\(_{2}\)-CONH\(_{2}\)), while the internal standard AAVal-PTH-\(^{13}\)C\(_{3}\) is monitored by m/z 309 → 238 (M-H\(^{-}\) → M-H\(^{-}\) - CH\(_{2}\)CH\(_{2}\)-CONH\(_{2}\)), and \(^{13}\)C\(_{5}\)-AAVal-PTH derived from administration of [1,2,3-\(^{13}\)C]AM is monitored by m/z 307 → 233 (M-H\(^{-}\)-\(^{13}\)CH\(_{2}\)-\(^{13}\)CH\(_{2}\)-\(^{13}\)CONH\(_{2}\)).

Conditions for the HPLC analysis of AAVal PTH and GAVal PTH were developed to enable rapid analysis of samples. Thus chromatography was carried out using a short column (Phenomenex Luna PhenylHexyl Column, 50 × 2 × 3 mm), enabling rapid separation and elution of the adducts in 3 min, and recycling of the column for the next injection within 10 min. To enable rapid sample processing and avoid extensive solvent extractions involved in the original method for the modified Edman degradation (Törnqvist et al., 1986), a solid-
phase extraction method was developed to separate the adduct derivatives from the solvent, remaining protein, derivatizing reagent, and its products. Following extraction, the eluted adduct derivatives were dried and reconstituted in mobile phase for LC-MS analysis.

For standardization of the quantitative analysis, AAVal-leu-anilide was added to samples containing unmodified globin. This peptide provides a model for the AAVal in globin, and will cyclize and cleave in a similar manner (Lawrence et al., 1996; Osterman-Golkar et al., 1994; Törnvist et al., 1986). The samples were derivatized with PITC, and after addition of the internal standard AAVal-PTH\(^{13}\)C\(_5\), the PTH derivatives were extracted and analyzed by LC-MS/MS. A standard curve for AAVal PTH generated with AAVal-leu-anilide indicated a linear response with a background present in untreated hemoglobin, consistent with the observations of others (Bergmark, 1997; Perez et al., 1999; Tareke et al., 2000). This background limits evaluation of the sensitivity that may be obtained for the analysis method. However, for the analysis of samples of AAVal PTH without added globin, the limit of detection is estimated to be approximately 0.5 fmol/injection. Injection of 5-µl aliquots from a 100-µl sample derived from 20 mg of globin gives an estimated limit of detection of 0.5 fmol/mg globin. Greater sensitivity could be achieved by increasing the volume of the aliquot injected and by increasing the amount of globin analyzed. The standard curve generated was used for the quantitative analysis to convert the ratio of analyte to internal standard to amount of adduct present in each sample. Comparison of the amount of AAVal-leu-anilide added with the amount of internal standard added indicated that approximately 70% of the added analyte was recovered. A similar calibration could not be performed for GAVal with a peptide standard,

**FIG. 3.** Daughter ion spectra of GAValPTH (m/z 320, top) and GAVal PTH\(^{13}\)C\(_5\) (m/z 325, bottom) in the negative ion mode.
because this is not commercially available. Therefore, quantitation of GAVal was based on the peak area ratio of analyte:internal standard, and the amount of GAVal-PTH standard added.

Adducts in AM- and NMA-Treated Rats

Samples from rats administered [1,2,3-\textsuperscript{13}C]AM or unlabeled NMA were analyzed for the adducts formed by both the \textsuperscript{13}C-enriched and natural abundance forms of AA and GA. A chromatogram for AAVal in globin from a rat administered [1,2,3-\textsuperscript{13}C]AM by gavage is shown in Figure 4. Three separate chromatograms are shown for two forms of the analyte and the internal standard. A similar set of chromatograms is shown in Figure 5 for GAVal in the same animal. For quantitation, the two peaks for the isomers of GAVal-PTH were integrated together. Typical chromatograms for AAVal in NMA-treated rats are shown in Figure 6 and for GAVal in Figure 7.

The results of the analysis of GAVal and AAVal are presented in Table 1. In the 50-mg/kg \textsuperscript{13}C AM-treated rats, little change was observed in the amount of unlabeled AAVal and GAVal compared to the control group. However, \textsuperscript{13}C AAVal was 21 ± 1.7 pmol/mg globin (mean ± SD, n = 4), and \textsuperscript{13}C GAVal was 7.9 ± 0.8 pmol/mg. In the NMA-treated rats, AAVal was 41 ± 4.9 pmol/mg globin, and GAVal was 1.4 ± 0.1 pmol/mg. Thus AAVal and GAVal can be detected following administration of NMA. The ratio of AAVal to GAVal in the NMA-treated rats was considerably higher (29) than that following administration of an equimolar dose of AM to rats (2.6 in AM-treated rats at 50 mg/kg body weight).
The AAVal detected in globin from NMA-treated rats may have arisen by one of two mechanisms (Fig. 8). One possibility is that NMA undergoes loss of the hydroxymethyl group to form AM, which can then react with globin to form AAVal. The second possibility is that NMA reacts directly with globin and then loses the hydroxymethyl group to form AAVal. Both reactions, involving loss of formaldehyde, could occur on a chemical basis without the involvement of metabolism. These mechanisms cannot be directly distinguished from the AAVal data alone. The same issue exists for GAVal (Fig. 8). This adduct may be formed following dissociation of NMA to give AM, oxidation to GA, and reaction with globin. Alternatively, GAVal could be formed following oxidation of NMA to an epoxide and reaction with globin to form an adduct, which then releases the hydroxymethyl group. If loss of the hydroxymethyl group to form AM with the oxidation to GA is the route for AAVal and GAVal formation, one would expect that the ratio of AAVal to GAVal would be similar in the AM- and NMA-treatment groups. The much higher ratio of AAVal:GAVal in the NMA-treated rats (29 vs. 2.6 in AM-treated rats) suggests that reaction of NMA with globin is the predominant route to these adducts in NMA-treated rats rather than conversion to AM. The detection of GAVal in NMA-treated rats indicates oxidation of NMA, either directly or following conversion to AA. The lower levels of GAVal on NMA administration suggest a much lower level of epoxide formed in these animals compared with AM treatment.

A further question that was investigated was whether valine...
adducts containing a hydroxymethyl group could be detected in rats administered NMA. For this purpose, LC-MS/MS of the valine PTH extracts was performed as described above for AAVal and GAVal. However, reaction monitoring for a hydroxymethylated AAVal was carried out by monitoring $m/z$ 334 and for hydroxymethylated GAVal by monitoring 350 (data not shown). An increase in peak area of approximately 10-fold was observed in monitoring 334 in the NMA-treated rats compared with the control rats and those administered AM. Little change was seen in monitoring 350. While these observations suggest that adducts can be formed by direct reaction of NMA with hemoglobin, they are by no means conclusive. The preparation of authentic adduct standards and PTH-derivative standards would enable determination of retention time and fragmentation characteristics. This, in turn, would enable the choice of appropriate conditions for adduct quantitation.

**DISCUSSION**

Paulsson et al. (2002) recently reported a study in which AM and NMA were administered to rats and mice for measurement of AAVal and GAVal by modified Edman degradation, with pentafluorophenylisothiocyanate to form adduct phenylthiohydantoins. GAVal was further derivatized with acidic acetone to form an N-(2,2-dimethyl-4-oxazolidinonylmethyl)valine derivative. AM was administered to male Sprague-Dawley rats by ip injection in saline at a dose of 100 mg AA/kg body weight or...
142 mg NMA/kg body weight (an equimolar dose). In addition, male CBA mice were administered 25, 50, or 100 mg AM/kg, or 35, 71, or 142 mg NMA/kg body weight by ip injection. In this study, we confirmed the observation that NMA administration results in the detection of the same adducts formed by AM administration. However, there are considerable quantitative differences between our observations and those reported previously. A comparison of the two data sets is presented in Table 2, where the adduct levels have been normalized for the administered dose. The data obtained from AAVal following AM administration are similar in the two studies. The amount of GAVal obtained per unit dose was higher in this study and may be consistent with the saturation of oxidation and nonlinearity observed in previous studies (Calleman et al., 1992). Whereas Paulsson et al. (2002) described a higher amount of AAVal formation with AM treatment (ratio of AM:NMA treatment of 2.7), we found a much higher formation with NMA treatment (ratio of AM:NMA treatment of 0.53). While Paulsson et al. (2002) described a similar ratio of GAVal:AAVal in rats between AM (0.26) and NMA treatment (0.23), we observed considerable differences between AM and NMA, with ratios of GAVal:AAVal of 0.38 for AM treatment and 0.03 for NMA treatment. Possible reasons for these differences could be the route of exposure (ip injection vs. gavage administration) or nature of the NMA preparation (supplied as a solution or obtained as a solid).

An important issue in predicting and relating possible adverse effects of AM and NMA to hemoglobin adducts is
whether the methylol group is lost before or after formation of the adduct. Rephrasing, the question is whether NMA is converted to AM \textit{in vivo} prior to adduct formation (possible pathways leading from NMA to AAVal and GAVal are illustrated in Figure 8). While Paulsson \textit{et al.} (2002) were unable to answer this question, the data from this study may provide an answer. If NMA is converted to AM \textit{in vivo} prior to reacting with hemoglobin, one would expect similar ratios of GAVal:AAVal to be observed with NMA and AM administration. However, we observed very different ratios of these two adducts on administration of AM and NMA. Thus it would appear that NMA reacts with hemoglobin prior to the loss of formaldehyde. Whether this loss occurs in the animal or during the process of isolation and analysis cannot be addressed at this point. In a study of the metabolism and disposition of \textsuperscript{14}C-labeled NMA in male rats, Mathews (2001) found that 79\% of an oral dose of 150 mg/kg NMA was excreted in the urine. Unchanged NMA was excreted in the urine (30\% of the dose), and N-acetyl-S-(3-hydroxymethylamino-3-oxopropyl)cysteine was the major urinary metabolite (about 30\% of the dose). Other metabolites were detected in urine in this study but not identified (about 10\% of the dose). This indicates that at least 60\% of the dose administered is derived from NMA, rather than AM. Characterization of the additional metabolites could indicate whether all of the NMA is metabolized without conversion to AM.

The considerably higher levels of AAVal in NMA-treated rats suggest that the internal dose of NMA is higher than that of AM. To calculate the rate of elimination ($k_{el}$) of AM and GA, Bergmark \textit{et al.} (1991) used the relationship:

$$k_{el} = \frac{[RX]_0 k_{cys}}{[RY]/[Y]}$$

between hemoglobin adduct levels expressed as [RY]/[Y], the initial concentration of injected compound [RX]$_0$, and the rate constant for reaction of AM and GA with cysteine in hemoglobin. Calculation of [RX]$_0$ involved the assumption that AM and GA were rapidly and evenly distributed to tissues (Miller \textit{et al.}, 1982) and that 1 kg body weight equals 1 liter. In this situation, a knowledge of the rate constants of reaction of AM and NMA with hemoglobin to form N-terminal valine adducts would prove useful in determining the comparative internal dose, and to estimate the relative rates of elimination of AM and NMA.

An ambiguity remains in using AAVal and GAVal in assessing exposure to mixtures of AM and NMA. If AAVal data alone were used to estimate exposure to AM but the exposure was in reality to NMA, the exposure using the data generated in this study would be overestimated on a molar basis by a factor of two. The amount of GAVal formed on exposure to

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AAVal</th>
<th>\textsuperscript{13}C-AAVal</th>
<th>GAVal</th>
<th>\textsuperscript{13}C-GAVal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>156 ± 9</td>
<td>19 ± 2</td>
<td>117 ± 16</td>
<td>ND</td>
</tr>
<tr>
<td>\textsuperscript{13}C-Acrylamide</td>
<td>168 ± 22</td>
<td>20847 ± 1674*</td>
<td>114 ± 9</td>
<td>7876 ± 757*</td>
</tr>
<tr>
<td>NMA</td>
<td>0556 ± 4945* **</td>
<td>22 ± 6</td>
<td>1351 ± 122* **</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textbf{Note.} Groups of 4 rats were administered 0.80 ± 0.11 mmol AM or 0.72 ± 0.03 mmol NMA/kg body weight by gavage. Blood was collected at 24 h after dosing for analysis of adducts. Values represent mean ± SD, n = 4. ND = not detected.

*Significantly different from control, $p < 0.001$.

**Significantly different from \textsuperscript{13}C-AAVal from acrylamide treatment, $p < 0.001$.
AM would be considerably higher per mole of AAVal when compared with NMA.

Further investigations should focus on whether NMA undergoes oxidative metabolism to produce hydroxymethylglycidamide and whether the hemoglobin adducts of NMA and hydroxymethylglycidamide can be detected. The approach used in this study with LC-MS/MS is much more amenable to the analysis of polar adducts than is the more widely used GC-MS approach. Analysis for the presence of NMA and hydroxymethylglycidamide adducts may provide a means to distinguish NMA and AM exposure. However, the stability of these adducts in vivo, and during isolation and preparation for analysis will need to be evaluated.

The approach used in this study with LC-MS/MS of valine hemoglobin adducts has considerable advantages over the more widely used GC-MS approach. This new method requires less labor-intensive sample preparation: sample analysis is more rapid as a result of shorter chromatography runs, and the method much more amenable to the analysis of polar adducts. This method has been applied to the quantitation of adducts from other reactive chemicals, the results of which will be communicated elsewhere.

ACKNOWLEDGMENTS

This study was supported by SNF SA (primary scientific contact, Marvin A. Friedman). The authors would like to acknowledge the support of the CIIT Animal Care Staff. Dr. Barbara Kuyper is acknowledged for editorial review.

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