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Detection of O^6-Carboxymethyl-2′-deoxyguanosine in DNA Following Reaction of Nitric Oxide with Glycine and in Human Blood DNA Using a Quantitative Immunoslot Blot Assay

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Previous research has shown that a range of nitrosated glycine derivatives react with DNA to form O^6-carboxymethylguanine and O^6-methylguanine DNA adducts [Harrison et al. (1999) *Chem. Res. Toxicol. 12,* 106−111). Nitrosated glycine derivatives may be formed in the gastrointestinal tract from the reaction of dietary glycine with nitrosating agents. The aim of this study was to further investigate the role of dietary glycine in the formation of O^6-guanine adducts at physiologically relevant concentrations. In vitro studies were performed by reacting 10 μM to 50 mM glycine with nitric oxide in the presence of oxygen. An HPLC assay was developed to measure the resulting nitrosated glycine derivative, diazoacetate anion. The amount of nitrosating agent present in the reaction mixture was determined by colorimetric measurement of nitrite, the hydrolysis product of N_2O_3. Diazoacetate anion formation depended linearly on glycine concentration. Solutions of nitrosated glycine reacted with 2′-deoxyguanosine and calf thymus DNA to give O^6-carboxymethyl-2′-deoxyguanosine and, at high concentrations of glycine and nitric oxide, O^6-methyl-2′-deoxyguanosine. At physiological concentrations of glycine and nitric oxide, diazoacetate anion was not detectable. Studies with synthetic diazoacetate anion showed that concentrations <14 μM did not give detectable O^6-carboxyethylguanine in DNA, even when a sensitive immunoslot blot assay was used. However, O^6-carboxymethylguanine was detected in human blood DNA samples obtained from three volunteers consuming a standardized high meat diet, using the immunoslot blot assay. O^6-Carboxymethylguanine levels ranged from 35 to 80 (detection limit = 15) O^6-carboxymethylguanine per 10^8 bases. These studies provide further evidence that nitrosated amino acids may be risk factors for gastrointestinal tract cancers.

Introduction

The endogenous transformation of amines to nitrosated derivatives is a well-established phenomenon in both experimental animals and humans. The in vivo transformation of secondary amines to *N*-nitroso compounds on coadministration with nitrite was first demonstrated by Sander and Bürkle in 1969 (*1*). Several groups showed that coadministration of various secondary and tertiary amines with nitrite gave rise to tumors (*2*, *3*). The potential of this pathway in humans was first shown by Ohshima and Bartsch using the formation of stable, noncarcinogenic *N*-nitrosoproline (*4*). The *N*-nitrosoproline test has been used in a number of studies and has consistently produced evidence that endogenous nitrosation occurs in humans (*5*). However, despite good evidence that endogenous nitrosation occurs in humans at sites (e.g., the stomach) where cancers develop (*6*) and a hypothesis that links the two phenomena (*7*), there has been considerable difficulty in establishing the identity of likely intermediates (*8*).

Building upon the large body of information that is known about both the kinetics of nitrosation of many different nitrogenous chemicals and their occurrence in the human diet, Shephard and Lutz (*9*) calculated the likely pattern of nitrosation at gastric pH and demonstrated that the main products would be derived from amino acids. While the *N*-nitrosoproline test made use of the naturally occurring amino acid L-proline, the remaining common amino acids have a primary amine function as the nitrogen α to the carboxylic acid and will not form stable *N*-nitroso derivatives in aqueous solution at physiological pH and temperature. To establish the relevance of the endogenous nitrosation of amino acids for human carcinogenesis, it is therefore necessary either to detect the formation of nitrosated intermediates or to observe characteristic reaction products with DNA or proteins or both.

In vitro studies with a range of nitrosated glycine derivatives led to the identification of two alkyl adducts at the O^6 position of guanine, O^6CMeG[1] and O^6MeG, with

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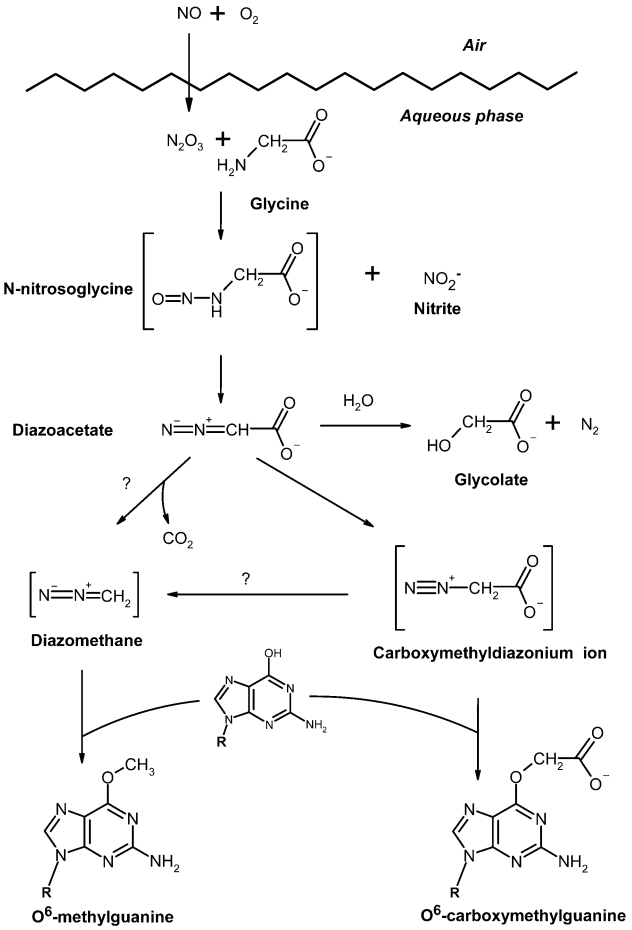
Scheme 1. Reaction Scheme Showing the Nitrosation of Glycine by NO-Derived N_2O_3 to Give Diazoacetate Anion and the Subsequent Formation of O^6-Methyl- and O^6CMeG Adducts[a]



[a] Intermediates shown in square brackets were not isolated or characterized directly. R denotes either a 2′-deoxyribose group or DNA. Pathways with a question mark are alternatives that cannot be distinguished at the moment.

more than 10 times higher levels of carboxymethylation than methylation (*10*). It would therefore be expected that if such nitrosated glycine derivatives were contributing to background levels of DNA alkylation in man, more O^6CMeG might be found than O^6MeG. Of additional interest is the finding that O^6CMeG is not repaired by O^6-alkylguanine transferase (*11*) and is thus likely to accumulate in the DNA of gastrointestinal tract tissues and possibly be a promutagenic lesion.

The aims of the work presented in this paper were to examine the possibility that nitrosated intermediates are formed from the reaction of glycine and a nitrosating agent derived from NO at physiologically relevant concentrations and to investigate the formation of resulting O^6-alkylguanines. The chemistry of the reactions under investigation is shown in Scheme 1. It is important to note that NO is not itself capable of nitrosation but is converted to a mixture of nitrosating agents by reaction with oxygen. The major nitrosating agent is dinitrogen trioxide (N_2O_3), which reacts with amines at neutral and alkaline pH (*12*). Nitrosation by N_2O_3 and its hydrolysis gives nitrite as a side product. Nitrite is unreactive as a nitrosating agent at neutral and alkaline pH values in the absence of suitably activated substrates (*12*).

Although the nitrosation product of glycine, the diazoacetate anion, has been known for almost 100 years (*13*), it is prepared by alkaline hydrolysis of ethyl diazoacetate and not by direct nitrosation. Here, we show that glycine itself is converted to diazoacetate anion under neutral and alkaline conditions and that the resulting reaction mixtures react further with 2′-deoxyguanosine and DNA to give O^6CMeG. Detection of low levels of O^6CMeG is facilitated by an immunoslot blot assay. We also show that this adduct is present in human DNA isolated from whole blood.

Experimental Procedures

Caution: *Reagents that generate carboxymethyldiazonium ions are alkylating agents and should be treated with extreme caution. Unused solutions of diazoacetate anion should be decomposed in 1 M acetic acid overnight.*

Chemicals. Potassium and lithium salts of diazoacetate anion were synthesized by hydrolysis of ethyl diazoacetate (Aldrich, U.K.) with equimolar concentrations of potassium or lithium hydroxide for 4 h according to the method of Kreevoy (*14*). The products were characterized by ^{13}C NMR and HPLC. Standards of O^6CMeG and O^6CMedG were synthesized as previously described (*15*). All other chemicals were of analytical or HPLC grade from commercial suppliers. Phosphate-buffered saline was prepared from commercially available tablets (Oxoid, U.K.) and had the following composition and pH: NaCl, 0.16 M; KCl, 0.0003 M; Na_2HPO_4, 0.008 M; KH_2PO_4, 0.001 M; pH 7.3. All DNA was from calf thymus (Sigma) unless indicated otherwise.

Chromatography. Nitrite and diazoacetate anion were analyzed by HPLC using a Phenomenex Luna C_{18} column (25 cm × 4.6 mm). Mobile phase A was 25 mM tetrabutylammonium hydrogen sulfate adjusted to pH 8 with NaOH, and mobile phase B was methanol. The flow rate was 0.75 mL/min. The HPLC column was eluted with 100% A for 20 min and a gradient up to 30% B over the next 10 min, followed by an equilibration to the starting conditions over 15 min. The analytes were detected by UV absorbance at 240 (nitrite) and 249 nm (diazoacetate anion). Quantitative analyses were carried out by reference to standard curves for each analyte.

O^6CMeG and O^6MeG were separated by HPLC using a Phenomenex Luna C_{18} column (15 cm × 4.6 mm, 5 μm). Mobile phase A was 0.1% (v/v) aqueous heptafluorobutyric acid, and mobile phase B was 0.1% (v/v) heptafluorbutyric acid in methanol. The flow rate was 0.75 mL/min. The HPLC column was eluted with 5% B for 5 min, 5−50% B over the next 15 min, and then 50% B for 5 min. This was followed by equilibration to the starting conditions over 10 min. The bases were detected by UV absorbance at 260 nm or by fluorescence at 378 nm (excitation at 286 nm).

Human DNA Samples. DNA was extracted from blood samples taken from human volunteers housed at the MRC Dunn Nutrition Centre (Cambridge, U.K.). Three male nonsmoking volunteers consumed a high meat diet (420 g per day based on pork and beef recipes) for 43 days using protocols similar to those previously published (*8*). The diets consisted of a 3 day rotation menu with fasting blood samples being taken on days 1, 4, 10, 22, 31, and 43, always after the sweet and sour pork menu. DNA was extracted from freshly collected whole blood using commercially available DNA extraction kits (Qiagen, U.K.) and stored as an aqueous solution at −80 °C until required for analysis.

Immunoslot Blot Assay for O^6CMeG. Human blood DNA or DNA samples treated with either diazoacetate anion or glycine−NO reaction mixtures were analyzed for O^6CMeG in triplicate using an immunoslot blot assay (*16*−*18*). The samples

[1] Abbreviations: NO, nitric oxide; O^6CMeG, O^6-carboxymethylguanine; O^6CMedG, O^6-carboxymethyldeoxyguanosine; O^6MeG, O^6-methylguanine; O^6MedG, O^6-methyldeoxyguanosine.

were analyzed by comparison with a standard curve constructed from DNA containing known levels of O^6CMeG.

The standard DNA was made as follows. Calf thymus DNA (5 mg/mL) dissolved in 10 mM K_2HPO_4, pH 8.0, was treated overnight at 37 °C with diazoacetate anion (5 mM). Following precipitation with propan-2-ol (1 vol) and washing with ethanol and 70% aqueous ethanol, the DNA was dissolved in water. Treated DNA (20 μg) was hydrolyzed in 0.1 M formic acid at 70 °C for 1 h. O^6CMeG was determined by HPLC with fluorescence detection as described above. Known amounts of the adducted DNA were mixed with nonadducted calf thymus DNA to give a series of DNA standards with 0−10 fmol O^6CMeG/μg DNA. These DNA samples were used to construct the standard curve for the immunoslot blot assay.

The immunoslot blot assay was carried out as follows. DNA samples were heat-denatured (100 °C for 5 min) and then cooled on ice, and aliquots containing 1 μg of the resulting single-stranded DNA were pipetted in triplicate into wells of a 72 well Minifold II blotting apparatus (Schleicher and Schuell) containing a nitrocellulose filter (Protran, 0.1 μm, Schleicher and Schuell, Dassel, Germany). The DNA samples were pulled through the filter under vacuum. The filter was removed and baked at 80 °C for 1.5 h, and nonspecific binding sites were blocked using nonfat milk powder (5% w/v) dissolved in phosphate-buffered saline containing 0.1% Tween-20 for a further 1 h. The filter was incubated with a working dilution (1 in 800) of polyclonal rabbit O^6CMeG primary antibody (*15*) for 2 h at room temperature prior to incubation at 4 °C overnight. The polyclonal antibody had previously been purified using the Mab Trap G II kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After the filter was washed in phosphate-buffered saline, it was incubated with a working dilution (1 in 40 000) of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma Chemical Co., Dorset, U.K.) for 2 h at room temperature. At the end of the incubation, the filter was bathed with chemiluminescent reagents (Super Signal West Dura Extended Duration Substrate, Pierce, IL) and exposed to chemiluminescent sensitive hyperfilm, and the DNA adduct levels in the samples were quantitated from the calibration curve using a Fluor-S MultiImager (Biorad, CA). The adduct values for each sample were then corrected for differences in DNA binding to the nitrocelluose filter, which was determined by incubating the filter with propidium iodide (50 μg in 50 mL of phosphate-buffered saline) for 3 h and subsequent reanalysis for fluorescence using the Fluor-S MultiImager.

Reactions of Glycine with NO under Various Conditions. NO (100% NO (Aldrich) or 10 000 ppm NO in N_2 (Argo International, Essex) was bubbled through 100 mL solutions of glycine in phosphate-buffered saline at pH 7.3. Aliquots of this solution (1 mL) were removed at regular intervals into tubes containing 2 M KOH (to prevent degradation of diazoacetate anion). These aliquots were analyzed for diazoacetate anion by HPLC and for nitrite using a colorimetric Griess assay (*19*). NO was also bubbled through solutions of glycine in 0.1 M NaOH. Concentrations of glycine used ranged from 10 μM to 50 mM.

Reaction with 2′-Deoxyguanosine. Aliquots of diazoacetate anion in phosphate-buffered saline (adjusted to pH 8.4) or 14 mL aliquots from the reaction of glycine with NO in phosphate-buffered saline were incubated with 2 mg of 2′-deoxyguanosine (2 mg/mL solution) to give a final volume of 15 mL and left overnight in a shaking water bath at 37 °C. At the end of the incubation, the samples were either analyzed immediately or frozen at −20 °C. Adducted nucleosides were isolated using a combination of O^6CMedG and O^6MedG immunoaffinity columns as described in detail elsewhere (*10*).

Reactions with DNA. A range of concentrations of diazoacetate anion in 0.5 mL of phosphate-buffered saline and glycine−NO reaction mixtures were incubated with 0.5 mg of DNA (final volume 1 mL). DNA was precipitated by addition of an equal volume of propan-2-ol at the end of the incubation and stored at −20 °C overnight. The solution was then centrifuged at 3000*g* for 30 min, and the DNA pellet was washed with 70% aqueous ethanol. The DNA was dried in air and dissolved in 0.5 mL of water. The amount of DNA was quantitated by UV spectrophotometry (GeneQuant spectrophotometer, Pharmacia Biotech, U.K.) by determining the absorbance at 260 nm. DNA purity was checked by determining the 260/280 nm ratio. These samples were then analyzed for O^6CMeG using the immunoslot blot assay.

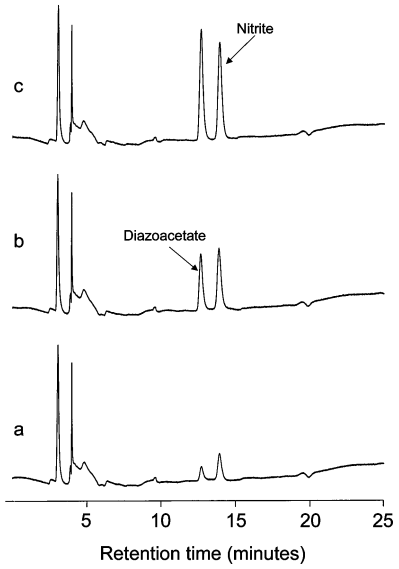


Figure 1. Typical HPLC chromatograms showing the formation of diazoacetate anion following reaction of 1 mM glycine in phosphate-buffered saline (adjusted to pH 10) after (a) 1 min and 30 s, (b) 3 min, and (c) 5 min exposure to NO.

Results

Characterization of Diazoacetate Anion. The product mixtures from the hydrolysis of ethyl diazoacetate with potassium or lithium hydroxide were analyzed by ^{13}C NMR at 250 MHz in D_2O. Characteristic resonances were seen for the diazoacetate anion: δ (ppm) 176.11 (**C**(O)O^-); 46.73 (**C**=N=N) and ethanol: δ (ppm) 57.75 (**C**H_2OH), 17.19 (**C**H_3). Attempts to isolate diazoacetate anion salts resulted in extensive decomposition as noted by previous workers (*14*), and the crude hydrolyzates were used without further purification.

Formation of Diazoacetate Anion from Glycine in Buffer at pH 10. Diazoacetate anion is known to be a very reactive, unstable intermediate, which undergoes rapid hydrolysis to glycolic acid in neutral and acidic conditions (*14*). The half-life of diazoacetate anion in phosphate-buffered saline was 15−30 s at pH 7.3 and 100 min in phosphate-buffered saline at pH 8.3. An HPLC method was developed that separated diazoacetate anion and nitrite. Glycine eluted with the solvent front, and glycolate (retention time ∼10 min) was observed as a small peak when concentrated solutions of diazoacetate anion had decomposed. The peaks were identified by coretention with commercially available standards and the synthesized diazoacetate anion. The limit of detection for diazoacetate anion was approximately 5 pmol. Figure 1 shows the formation of diazoacetate anion and nitrite over time from 1 mM glycine treated with NO in phosphate-buffered saline adjusted to pH 10. Incubation of glycine at pH 10 with up to 1 mM nitrite did not result in formation of detectable amounts of diazoacetate anion, thus confirming that conversion of NO to N_2O_3 (rather than nitrite) is required for nitrosation.

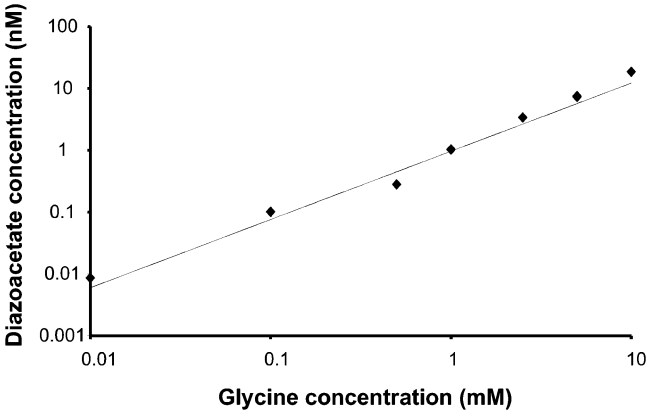


Figure 2. Formation of diazoacetate anion with increasing glycine concentration in 0.1 M NaOH and exposure to NO giving a final concentration of ~100 μM nitrite.

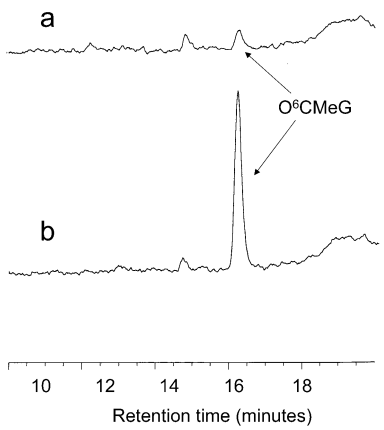


Figure 3. Chromatogram of two HPLC fluorescence analyses of O^6CMeG immunoaffinity column eluant from a reaction of 1 mM glycine with NO followed by incubation with 2′-deoxyguanosine overnight with (a) O^6CMeG immunoaffinity column eluant only and (b) O^6CMeG immunoaffinity column eluant with co-injected O^6CMeG standard.

These initial experiments showed that glycine can be nitrosated to form a nitrosated glycine intermediate, diazoacetate anion. These experiments were performed with treatments of NO that resulted in final nitrite concentrations of <500 μM, which are not representative of the conditions that might be found in the gastrointestinal tract in vivo. Thus, these studies were repeated, adjusting the conditions to encompass more physiologically relevant concentrations of glycine (<100 μM, *18*) and NO (giving a final nitrite concentration of <50 μM, *5*).

Nitrosation of Glycine at Physiologically Relevant Concentrations. To show that diazoacetate anion is formed from the reaction of NO with physiologically relevant levels of glycine under conditions where the hydrolysis of diazoacetate anion is minimized, the reactions were performed in 0.1 M NaOH. Diazoacetate anion formation was found to be linear with increasing glycine concentrations at NO treatments resulting in a final concentration of ~100 μM nitrite (Figure 2).

After we showed the formation of diazoacetate anion in 0.1 M NaOH, reactions of glycine with NO were performed in phosphate-buffered saline adjusted to pH 8.4. In these reactions, the final levels of diazoacetate anion detected were lower due to some concomitant degradation of diazoacetate anion. Diazoacetate anion levels of 1.6−1.7 nM were detected on nitrosation of 5 and 10 mM glycine solutions at pH 8.4. No detectable levels of diazoacetate anion were found for glycine concentrations <5 mM following treatment with NO to give a final concentration of 100 μM nitrite.

Formation of O^6CMedG from Nitrosated Glycine and 2′-Deoxyguanosine. Experiments were performed by bubbling pure NO for 10 min through glycine solutions (10 μM to 10 mM) in phosphate-buffered saline adjusted to pH 8.4. Aliquots of the reaction mixtures were incubated with 2′-deoxyguanosine overnight. The adducted nucleosides were isolated by passing the incubation solution through consecutive O^6CMedG and O^6MedG immunoaffinity columns followed by HPLC analysis as the O^6-alkylguanine bases (*10*). These experiments showed the formation of both O^6CMeG and O^6MeG at relatively high exposures to NO (giving a final nitrite concentration of >1 mM). The observed O^6CMeG:O^6MeG ratio was 16:1 (data not shown), and this corresponds closely with the ratio previously reported for reactions of diazoacetate anion with DNA (*10*).

Figure 3 shows the formation of O^6CMeG from 1 mM glycine and NO where the final nitrite concentration was 148 μM. The nitrosated glycine solution (14 mL) was immediately adjusted to pH 8.4 by the addition of 2 M KOH (100 μL) and incubated with 2 mg of 2′-deoxyguanosine overnight at 37 °C. O^6CMedG was isolated as described in the Experimental Procedures.

Immunoslot Blot for O^6CMeG in DNA. Harrison et al. (*10*) showed the formation of O^6CMeG and O^6MeG adducts following the reaction of 0.5−5 mM diazoacetate anion with DNA. In these experiments, the levels of O^6CMeG and O^6MeG adducts were determined by hydrolyzing the DNA to nucleosides and analyzing the O^6-alkyl guanine bases by HPLC/fluorescence following immunoaffinity purification as described above. Harrison et al. (*15*) also showed preliminary results of an immunoslot blot method for the detection of O^6CMeG adducts in intact DNA. This assay method has now been fully developed, and quantitation is obtained by comparison with standards determined by HPLC fluorescence. The detection limit of the immunoslot blot assay is ~0.4 fmol of O^6CMeG. Figure 4c shows a set of typical raw results from an immunoslot blot filter obtained using a 3 × 24 well manifold. Samples were applied to the filter in triplicate. Each set of three vertical bands corresponds to a single sample. Figure 4a shows the standard curve that was generated from the blot shown in Figure 4c. Figure 4b shows the levels of O^6CMeG generated in DNA under the following conditions: Concentrations of 0, 1, and 10 mM glycine in phosphate-buffered saline at pH 8.4 were treated with NO to give final nitrite levels of 563, 229, and 388 μM, respectively. Under these conditions, diazoacetate anion could not be detected in the reaction mixture using the HPLC conditions described above. Aliquots of these solutions (15 mL) were reacted with 5 mg of DNA overnight at 37 °C in duplicate. The DNA was precipitated and quantitated, and triplicate samples of 1 μg from each incubation were analyzed for O^6CMeG by immunoslot blot assay.

To determine the lowest concentrations of diazoacetate anion that would give detectable levels of O^6CMeG, synthetic diazoacetate anion (1.5 nM to 14.9 mM) in 0.5 mL of phosphate-buffered saline was mixed with 0.5 mL of DNA (1 mg/mL) at 37 °C overnight. At the end of the incubation, the DNA was precipitated, quantitated, and analyzed by immunoslot blot assay. O^6CMeG were de-

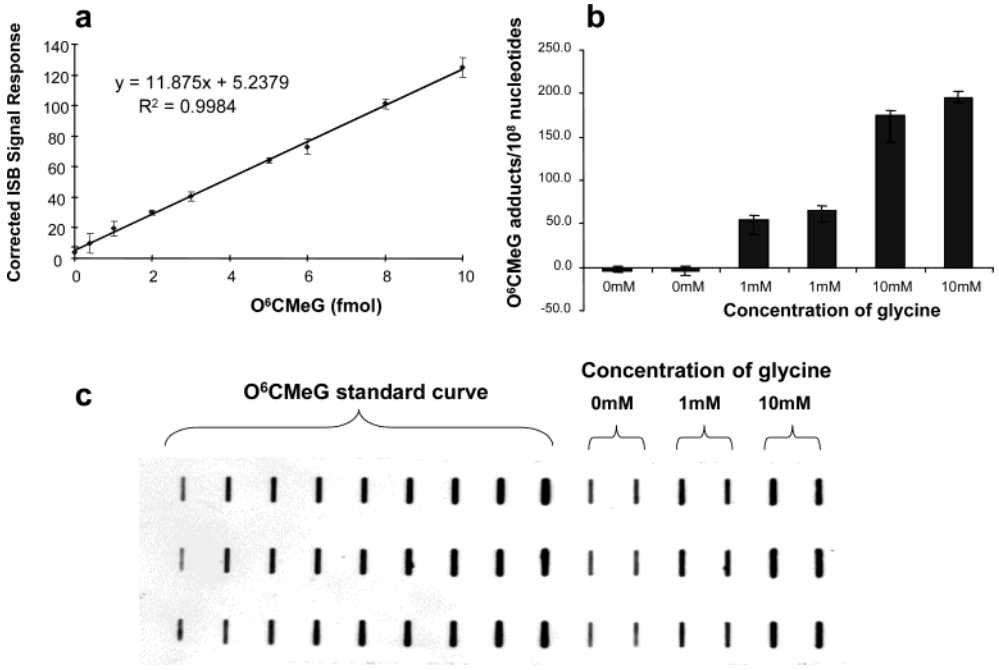


Figure 4. Typical standard curve for immunoslot blot assay of O^6CMeG in DNA (a) and representative results (b) derived from the immunoslot blot assay data shown in panel c.

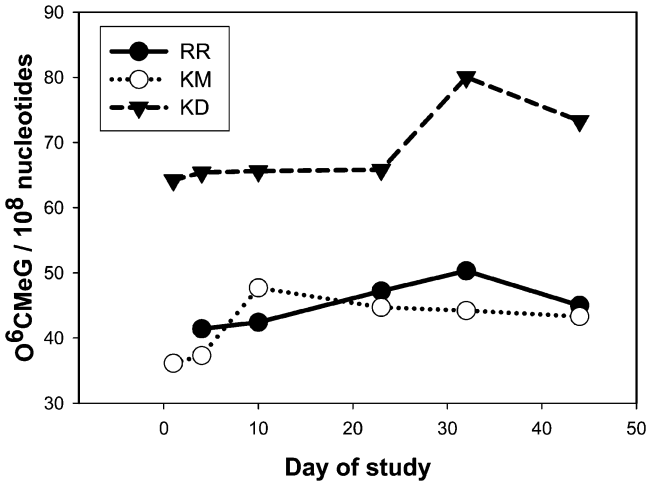


Figure 5. O^6CMeG in DNA isolated from whole blood of three volunteers (RR, KM, and KD) consuming standardized high meat diets for 43 days.

tected only at diazoacetate anion concentrations of >14 μM.

The immunoslot blot method was applied to a series of DNA samples extracted from whole blood obtained from three volunteers. For 43 days, the volunteers consumed an isocaloric standardized diet in which the proportions of protein (143−150 g), fiber (23−26 g), and fat (30% of total energy) remained constant. The results are summarized in Figure 5 and show that not only was O^6CMeG detectable in human DNA but that the levels of O^6CMeG remained relatively constant over the course of the study.

Discussion

To study the nitrosation of glycine in vitro, methods were developed to analyze the nitrosated glycine derivative, diazoacetate anion, and also to analyze adducts formed on intact DNA as a result of incubation with nitrosated glycine derivatives. Quantitation of diazoacetate anion was possible by HPLC−UV using a large counterion (tetrabutylammonium) and alkaline mobile phase conditions. Samples were analyzed as soon as possible after the incubation to reduce diazoacetate anion degradation. Diazoacetate anion had a retention time of ~12.5 min on this HPLC system. Thus, a maximum of ~25 min would have elapsed from removal of the sample from the incubation and the peak elution in the HPLC run.

Alkyl DNA adducts have previously been measured by fluorescence HPLC following immunoaffinity cleanup (*10*). The HPLC method quantitates the adducts as O^6-alkylguanine bases after acid hydrolysis of eluted O^6-alkyl-2′-deoxyguanosines. The limits of detection for the HPLC method are 3.7 O^6CMeG adducts/10^8 bases and 1.85 O^6MeG adducts/10^8 bases from 1 mg of DNA (*10*). In comparison, the immunoslot blot assay quantitates the levels of adducts from intact DNA and only 1 μg of DNA per well is needed for the assay, with a limit of detection of 15 O^6CMeG per 10^8 bases.

The chemistry and biochemistry of the formation of *N*-nitroso compounds and their subsequent decomposition to alkylating agents are complex (*12*). In a model of the aetiology of gastric cancer proposed by Correa (*7*), there are several mechanisms for the formation of *N*-nitroso compound intermediates, i.e., acid-catalyzed nitrosation, bacterially catalyzed nitrosation, and nitrosation from NO. Acid-catalyzed nitrosation occurs when dietary nitrate is reduced to nitrite in the oral cavity and converted to nitrous acid in the acidic stomach. Nitrite is also formed by nitrate-reducing bacteria present in the stomach at high pH. The discovery of the widespread occurrence of NO in the human body in both normal and inflammatory states provides another potential source of endogenous nitrosating agents (*20*). In the in vitro studies described in this paper, an estimate of the amount of nitrosating agent was achieved by the quantitation of nitrite formed from NO. Scheme 1 shows the postulated reaction of NO with glycine to form alkyl−DNA adducts.

HPLC analysis of the reaction solutions showed the formation of an intermediate derived from glycine, namely, diazoacetate anion (Figure 1). Experiments performed using millimole concentrations of glycine and >100 μmol levels of nitrosating agent (as measured by assaying nitrite ion), and at alkaline pH to stabilize diazoacetate anion, followed by reactions with 2′-deoxyguanosine showed the formation of O^6CMeG (Figure 2). A preliminary account of the nitrosation of glycine esters by another group also reported evidence for the formation of carboxymethylating agents (*21*).

Concentrations of glycine and exposures to NO were also chosen to encompass physiologically relevant levels of both reactants. Komorowska et al. (*22*) found glycine concentrations of 30−50 μM in basal and pentagastrin- or histamine-stimulated gastric juice. The concentrations of glycine used in our studies ranged from 10 μM to 10 mM. The incubations with gaseous NO were continued until the level of nitrite in the solution approached 100 μM. The level of nitrite in gastric juice in vivo varies considerably depending on the pH, which in turn can vary with different clinical conditions. Nitrite levels in gastric juice of 0.12 ± 0.02 and 53.59 ± 8.50 μM (mean ± standard deviation) in the pH range 1.13−2 and 7−8, respectively, were reported in a study by Xu and Reed (*5*). High concentrations of nitrite and high pH are typical in patients with achlorhydria, which is a predisposing condition for gastric cancer. Thus, the present study models the conditions present in patients with achlorhydria.

It was not possible to detect adducts from single treatments of DNA with nitrosated glycine at physiologically relevant levels of glycine and NO and at a pH of 8.4 that represents the higher end of gastric pH in patients with achlorhydria. Reactions with synthetic diazoacetate anion and DNA only resulted in detectable levels of O^6CMeG adducts when the concentration of diazoacetate anion was >14 μM.

We have previously used the immunoslot blot assay method to detect and quantitate DNA adducts in human DNA extracted from gastric biopsies (*23*) and blood (*24*). The biopsies were obtained from patients attending a dyspepsia clinic, and their *H. pylori* status was known, and this was not found to be a significant factor in the levels of adducts detected (*18*). The lack of O^6CMeG adducts in DNA treated with the lowest levels of glycine and NO used in our in vitro studies could be attributed to the fact that the samples were analyzed following single exposures to diazoacetate anion. In vivo, the gastric tissue DNA could be repeatedly exposed to diazoacetate anion over a period of time.

Results in this paper show that O^6CMeG is also present in human blood DNA. Monitoring of O^6CMeG DNA adduct formation in humans could be a biomarker of DNA damage arising from endogenous nitrosation. Further evidence that nitrosated amino acids have an important role in gastric cancer aetiology comes from the observation in human volunteers and in mice that increased red meat intake results in elevated levels of fecal total *N*-nitroso compounds (*8*, *25*−*27*). In addition, a number of epidemiological studies that have linked high protein intake with increased risk of gastrointestinal tract cancers (*28*).

In conclusion, these studies report the quantitative HPLC analysis of the nitrosated glycine intermediate, diazoacetate anion, and show that it is formed from glycine and NO at concentrations similar to those likely to occur in the gastrointestinal tract. In addition, glycine exposed to NO and then incubated with 2′-deoxyguanosine or DNA leads to formation of O^6CMeG and O^6-MeG. The immunoslot blot assay for O^6CMeG could be used in future molecular epidemiology studies on the role of nitrosated amino acids in the etiology of gastrointestinal tract cancers.

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