Identification of N^2-(1-Carboxyethyl)guanine (CEG) as a Guanine Advanced Glycosylation End Product†

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ABSTRACT: Reducing sugars such as glucose react nonenzymatically with protein amino groups to initiate a posttranslational modification process known as advanced glycosylation. Nucleotide bases also participate in advanced glycosylation reactions, producing DNA-linked advanced glycosylation endproducts (AGEs) that cause mutations and DNA transposition. Although several protein-derived AGEs have been isolated and structurally characterized, AGE-modified nucleotides have not yet been reported. We systematically examined the reactivities of the model nucleotide bases 9-methylguanine (9-mG), 9-methyladenine (9-mA), and 1-methylcytosine (1-mC) toward glucose and several glucose-derived reactants. In "fast" reactions performed at refluxing temperature and physiological pH, 1 equiv of nucleotide base was reacted with 10 equiv of D-glucose, D-glucose 6-phosphate (G-6-P), D-glucose 6-phosphate/lysine (G-6-P/Lys), the Schiff base 1-*n*-propylamino-*N*-D-glucoside (SB), or the Amadori product 1-*n*-propylamino-*N*-D-fructose (AP). In every reaction involving 9-mG, N^2-(1-carboxyethyl)-9-methylguanine (CEmG) was a major product which was produced. N^2-(1-carboxyethyl)-9-methylguanine also formed from 9-mG and AP in long-term incubations performed at 37 °C. Direct treatment of 9-mG with methylglyoxal (MG), a Maillard reaction propagator that forms from the decomposition of AP, also produced CEmG in high yield. N^2-(1-Carboxyethyl)-9-methylguanine appears to result from the nucleophilic addition of the primary amino group of guanine to the ketone group of MG followed by an intramolecular rearrangement. Methylglyoxal is a known prokaryotic mutagen and was shown additionally to be mutagenic in a eukaryotic shuttle vector assay system. These data implicate MG as a glucose-derived mutagen and suggest that CEmG is a major product that forms as a result of DNA-advanced glycosylation.

DNA mutations play a central role in cellular dysfunction and oncogenesis and have been proposed to contribute to many of the pathophysiological sequelae of normal aging (Finch, 1990; Mullaart *et al.*, 1990). The DNA within resting, postmitotic cells is long-lived and potentially is susceptible to a variety of extrinsic and intrinsic sources of chemical damage. These include spontaneous deamination and depurination events and oxidative reactions caused by background radiation and free radical formation. Nucleotide modifications induce DNA mutations by mispairing or by serving as targets for error-prone DNA repair enzymes that misincorporate bases at a significant frequency (Finch, 1990; Mullaart *et al.*, 1990; Warner & Price, 1989).

One form of intrinsic macromolecular damage that has received increased attention recently is a modification reaction initiated by the spontaneous reaction of reducing sugars with the primary amino groups of proteins (Bucala & Cerami, 1992). This process, the Maillard reaction, proceeds from reversible Schiff base and Amadori products to a class of irreversibly bound, structurally heterogeneous products that have been termed advanced glycosylation endproducts or AGEs. AGEs possess cross-linking and fluorescent properties and accumulate on long-lived, extracellular proteins. AGEs alter the structural and functional properties of proteins and account in part for the increase in collagen cross-linking and connective tissue fluorescence that has been observed in aged proteins *in vivo*. AGEs produce arterial and connective tissue rigidity (Schnider & Kohn, 1982; Monnier *et al.*, 1984), chemically inactivate the endothelium-derived relaxing factor nitric oxide (Bucala *et al.*, 1992), and serve as signals for macrophage endocytosis via distinct AGE "scavenger" receptors (Vlassara *et al.*, 1985). The accumulation of protein-linked AGEs thus has been implicated in many of the pathophysiological alterations associated with normal aging.

Recent studies suggest that DNA nucleotides also undergo advanced glycosylation *in vitro* and *in vivo* (Bucala *et al.*, 1984). Although the aromatic properties of purine and pyridinium rings render nucleotide primary amino groups less reactive toward sugars than the aliphatic primary amino groups of amino acids, the long half-life of DNA in postmitotic cells favors the gradual, time-dependent accumulation of DNA-linked AGEs *in vivo*. *In vitro* studies have demonstrated that DNA reacts with glucose and glucose-derived reactive intermediates to produce covalent

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[1] Abbreviations: AGE, advanced glycosylation endproduct; AP (Amadori product), 1-*n*-propylamino-*N*-D-fructose; CEG, N^2-(1-carboxyethyl)guanine; CEdGMP, N^2-(1-carboxyethyl)-2-deoxyguanosine 5′-monophosphate; CEmG, N^2-(1-carboxyethyl)-9-methylguanine; 1-DG, 1-deoxyglucosone; 3-DG, 3-deoxyglucosone; dGMP, 2-deoxyguanosine 5′-monophosphate; G-6-P, D-glucose-6-phosphate; G-6-P/Lys, D-glucose 6-phosphate/D,L-lysine; HMFF, 5-(hydroxymethyl)furfuranyl; 9-mA, 9-methyladenine; 1-mC, 1-methylcytosine, 9-mG, 9-methylguanine; MG, methylglyoxal; OmG, 8-oxo-7-*H*-9-methylguanine; SB (Schiff base), 1-*n*-propylamino-*N*-D-glucoside.

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addition products that cause mutations in bacteria (Bucala *et al.*, 1984; Lee & Cerami, 1987a). In *Escherichia coli*, the mutagenic effect of AGE-modified DNA results in part from the activation of host-derived, transposable elements such as INS-1 and $\gamma\delta$ (Bucala *et al.*, 1985; Lee & Cerami, 1991). Glucose-derived reactive intermediates also have been shown to cause mutations in plasmids which have been transfected into mammalian cells (Bucala *et al.*, 1993). In this case, mutations are due to the transposition of host elements derived from the *Alu* family of repetitive DNA sequences. These data suggest that AGE-mediated DNA damage may play an important role in the chromosomal alterations and DNA transpositions that frequently are associated with aged cells and oncogenic transformation (Bucala *et al.*, 1993; Cairns, 1981; Solomon *et al.*, 1990).

Although AGEs have been isolated and characterized from the reaction of model protein amines with glucose, AGE-modified nucleotide bases have not been previously reported. Such structural information might lead to the identification of the mutagenic glucose-derived "reactive intermediate" and provide insight into the DNA modifications responsible for transpositional activity. In the present study, we endeavored to characterize at the chemical level adducts that form from the reaction of DNA bases with glucose and several of its derivatives. We report the identification of N^2-(1-carboxyethyl)-9-methylguanine (CEmG) as a predominant product formed during the reaction of the model base 9-methylguanine (9-mG) and D-glucose, D-glucose 6-phosphate (G-6-P), D-glucose 6-phosphate/D,L-lysine (G-6-P/Lys), the Schiff base 1-*n*-propylamino-*N*-D-glucoside (SB), and the Amadori product 1-*n*-propylamino-*N*-D-fructose (AP). Also isolated was 8-oxo-7-*H*-9-methylguanine (OmG), the 9-methyl analog of a previously identified guanine oxidation product (Kasai *et al.*, 1984, 1986). N^2-(1-carboxyethyl)-9-methylguanine formed readily under physiological conditions by the direct reaction of 9-mG with methylglyoxal (MG), a known Maillard reaction intermediate. The naturally occurring nucleotide dGMP also was converted to the amino acid derivative CEdGMP by treatment with MG at neutral pH. These data indicate that MG represents one common reactive intermediate produced during the reaction of guanine with glucose and other glucose-derived reactants. Methylglyoxal is a known prokaryotic mutagen, and we present evidence that the CEG product is mutagenic in a model eukaryotic cell system.

MATERIALS AND METHODS

General. ^{1}H NMR spectra were recorded on a General Electric QE-300 (300 MHz) spectrometer. FAB-MS were obtained at the State University of New York (Stony Brook, NY) on a Kratos MS890/DS90 mass spectrometer system using xenon atoms as ion source at 7 kV or at the University of California at Riverside using a ZAB VG analytical spectrometer with xenon atoms as ion source at 8 kV. Ion spray mass spectrometry was performed at Rockfeller University using a custom-built instrument and an impact energy of 80 V. Infrared spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer, and UV−visible spectra were recorded on a Hewlett Packard 8450 spectrometer. Analytical thin layer chromatography (TLC) was performed on Whatman 2.5 × 7.5 cm, 250 μm layer fluorescent silica plates. Preparative TLC was performed on Analtec (Newark, DE) 20 × 20 cm 1000 μm GF silica plates. Scientific Adsorbents (Atlanta, GE) silica 60 (32−65 μm) was used for flash chromatography. Reverse-phase preparative TLC was performed on Analtec 250 μm C18 plates and reverse-phase HPLC utilized a Hewlett Packard HP1090 instrument. An Analtech/Applied Science 250 mm macrosphere C18 column was used for all samples except those containing 1-methylcytosine (1-mC) and its derivatives, which required a Bio-Rad Aminex ion exclusion HPX-72S 300 × 7.8 mm column. An aqueous buffer containing 0.5 M $(NH_4)_2SO_4$ was used as the mobile phase in all separations. The flow rate was 1.5 mL/min.

Reagents. The phosphate buffers (300 mM in Na) were adjusted to pH 7.4 and stabilized with EDTA (1 mM). Adenine, methylglyoxal (pyruvaldehyde) (MG), methyl iodide, *n*-propylamine, tetrabutylammonium hydroxide, 2,5-furandimethanol (FDM), and *o*-phenylenediamine were purchased from Aldrich (Milwaukee, WI). D-Glucose and 9-mG were purchased from Fluka (Buchs, Switzerland). Cytosine and 5-(hydroxymethyl)-2-furfuranyl (HMFF) were purchased from Lancaster (Windham, NH). D-Glucose-6 phosphate monosodium and L-lysine were obtained from Sigma (St. Louis, MO). 1-*N*-Propylamino-D-fructose oxalate was prepared from 1-*n*-propylamino-D-glucoside following the method of Micheel and Hagemann (1959). Spectral data of the oxalate were identical to data published previously (Chen & Cerami, 1993). 3-Deoxyglucosone (3-DG) was prepared essentially according to Madson and Feather (1981). The quinoxaline derivative of 3-DG was prepared by the method outlined below. 9-Methyladenine (9-mA) was prepared from adenine by the method of Hadayatullah (1982) (mp 301−302 °C, lit. 301−302 °C).

Synthesis of 1-n-Propylaminoglucoside. To 500 mL of methanol, 70 g (0.39 mol) of D-glucose and 50 g (0.85 mol) of *n*-propylamine were added. The mixture was heated at reflux under N_2 for several minutes until all of the solid D-glucose was dissolved. The resulting clear solution was cooled to room temperature, and the product was precipitated with anhydrous ethyl ether. The white solid was filtered and washed several times with ether. Drying *in vacuo* gave 72 g (83% yield). ^{1}H NMR (DMSO-d_6): δ 0.85 (t, J = 7.5 Hz, CH_3, 3 H), 1.4 (m, CH_2CH_2, 4 H), 2.4−4.8 (m, HOC*H*, O*H*, 11 H). Analysis for $C_9H_{11}NO_5$: C 48.86, H 8.66, N 6.33; found: C 48.89, H 8.79, N 6.24.

Synthesis of 2-(2,3,4-Trihydroxybutyl)quinoxaline. To 25 mL of phosphate buffer (pH 7, Na 0.3 M) containing 1 mM of EDTA, 50 mg (0.25 mmol) of 3-DG was added along with 27 mg (0.25 mmol) of *o*-phenylenediamine. The resulting solution was incubated at 37 °C for 22.5 h. Water was removed by rotary evaporation, and the organics were extracted from the solid residue into ethanol. The quinoxaline was recrystallized from ethanol to give 16 mg (25%) of a light beige solid (mp 127−128 °C). ^{1}H NMR (D_2O): δ 3.1 [dd, ^1J (H,H) = 14 Hz, ^2J (H,H) = 9.8 Hz, H1′, 1 H], 3.3 (dd, ^1J = 14 Hz, ^2J = 3.3 Hz, H1′, 1 H), 3.7 (m, H2′H4′, 3 H), 4.07 (m, H3′, 1 H), 7.7 (m, H5, H6, 2 H), 7.92 (m, H4, H7, 2 H), 8.7 (s, H1, 1 H).

Synthesis of 1-Methylcytosine (1-mC). To 15 mL of CH_2Cl_2, 555 mg (5 mmol) of cytosine was added along with 3.2 mL (10 mmol) of 40% tetrabutylammonium hydroxide. The mixture was stirred at room temperature for 10 min until all of the cytosine dissolved. To the resulting solution, 2.86 g (20 mmol) of methyl iodide was added and stirring was resumed overnight. After the addition of 50 mL of water,

the mixture was extracted with CH_2Cl_2. The aqueous layer was concentrated by rotary evaporation and the solid residue was recrystallized from ethanol to give 269 mg (43%) of 1-mC as a white solid, mp. 295−304 °C (dec.), lit. 296 (dec.) (Atkins & Hall, 1983). ^{1}H NMR (DMSO-d_6): δ 3.18 (s, CH_3, 3 H), 5.60 (d, J = 7.2 Hz, H5, 1 H), 6.9 (s, NH_2, 2 H), 7.54 (d, J = 7.2 Hz, H6, 1 H).

Isolation of N^2-(1-Carboxyethyl)-9-methylguanine (CEmG) from the Reaction of 9-mG with SB. To a 500-mL round bottom flask containing 250 mL of phosphate buffer (300 mmol Na, pH 7.4, 1 mM EDTA), 250 mg (1.5 mmol) of 9-mG and 3.32 g (15 mmol) of SB were added. The reactants were refluxed under N_2 for 16 h. The resulting solution was concentrated to dryness by rotary evaporation, and the residue was extracted with hot ethanol. Vigorous tirturation was applied to facilitate the extraction. Partial purification was achieved by flash column chromatography with silica gel using mixtures of ethyl acetate, methanol, and water with an increasing gradient of polarity. The fractions containing CEmG were pooled and further enriched by preparative TLC using silica gel and a mixture of ethyl acetate, methanol, and water (3:1:1) with 1% NH_4OH (CEmG: $R_f = 0.3$). Precipitation from ethanol afforded 23 mg (6%) of CEmG as a beige solid. ^{1}H NMR analysis indicated the presence of only minor impurities. ^{1}H NMR (DMSO-d_6): δ 1.31 (d, J = 6.6 Hz, -CH_3CHN^2, 3 H), 3.52 (s, 9-CH_3), 3.98 (q, -HCN^2, 1 H), 7.2 (s, N^2H, 1 H), 7.58 (s, H8, 1 H), 10.6 (br, N^1H, 1 H). FTIR (KBr): cm^{-1} 3530 (s, NH), 2970 (m, CH), 2930 (m, CH), 1680 (s, CO). Ion spray MS: m/z 238 (MH^+, 100), FAB-MS (Xe^+, 8 kV): m/z 238 (MH^+, 100), FAB-MS-HR for $C_9H_{11}N_5O_3(H^+)$: 238.09401; found: 238.09348.

Kinetics of N^2-(1-Carboxyethyl)-9-methylguanine. To a 100-mL round bottom flask containing 50 mL of phosphate buffer (300 mmol Na, pH 7.4, 1 mmol EDTA), 15 mg of 9-mG (0.091 mmol) was added along with 164 mg (0.91 mmol) of D-glucose. Parallel reactions contained 0.091 mmol of 9-mG alone or together with 277 mg (0.91 mmol) of G-6-P, 277 mg (0.91 mmol) of G-6-P/16.6 mg (0.091 mmol) of D,L-lysine, 197 mg (0.91 mmol) of SB, 283 mg (0.91 mmol) of AP or 33 mg (0.18 mmol) of MG (40% solution in H_2O, Aldrich). Each reaction mixture was heated overnight at reflux under N_2. Aliquots (typically 0.5 mL) then were removed at intervals and portions analyzed by reverse-phase HPLC for CEmG quantitation. D-Glucose, G-6-P, G-6-P/D,L-lysine, SB, and MG produced 69%, 46%, 51%, 61%, and 63% CEmG, respectively, while refluxing 9-mG alone gave no detectable CEmG. The reaction involving AP produced 23% CEmG after 24 h (see Figure 1).

37 °C Incubations: Detection of N^2-(1-Carboxyethyl)-9-methylguanine and Isolation of 8-Oxo-7-H-9-methylguanine. To 1 L of phosphate buffer (300 mmol Na, pH 7.4, containing 1 mM EDTA) 300 mg (1.8 mmol) of 9-mG was added. The solid was dissolved with vigorous stirring, and then 5.64 g (18 mmol) of 1-*n*-propylamino-D-fructose oxalate was added. The resulting solution was incubated at 37 °C for 28 weeks. A 20-mL aliquot was removed, concentrated to ca. 1/4 the original volume by rotary evaporation, and analyzed by HPLC. Eight hundred fifty millilters of the remaining solution was concentrated to dryness by rotary evaporation, and the resulting residue was extracted with hot ethanol. The ethanol soluble material was separated by flash chromatography on silica using mixtures of ethyl acetate, methanol, and water with an increasing gradient of polarity. Fractions containing CEmG were pooled and analyzed by ^{1}H NMR. Reverse-phase HPLC analysis indicated the presence of CEmG in 9% yield. Also isolated was 7 mg of 8-oxo-9-methylguanine (OmG) (3% yield) as a white solid ($R_f = 0.5$ on silica using ethyl acetate, methanol, and water 3:1:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.08 (s, 9-N-CH_3, 3 H), 6.46 (s, NH_2, 2 H), 10.51 (s, 1-NH, 1 H), 10.6 (s, 7-NH, 1 H). FAB-MS (Xe^+, 6−8 kV): m/z 182 (MH^+, 100%).

De Novo Synthesis of CEmG. To a 500-mL round bottom flask containing 200 mL of phosphate buffer (300 mmol Na, pH 7.4, containing 1 mM EDTA), 800 mg (4.85 mmol) of 9-mG and 1.92 mg (10.7 mmol) of a 40% aqueous solution of MG were added. The reactants were refluxed under N_2 for 24 h, and the resulting brown solution was concentrated to dryness by rotary evaporation. The residue was extracted thoroughly with hot ethanol, and the extraction was facilitated by vigorous tituration. Partial purification was achieved by flash silica column chromatography using mixtures of ethyl acetate, methanol, and water with an increasing gradient of polarity. A hot filtration with 100 mL of methanol removed insoluble impurities. The filtrate was concentrated to ca. 5 mL, and a solid containing mostly CEmG was precipitated out of solution with several drops of ethanol. The beige solid was filtered and dried *in vacuo* to give 352 mg (31%). Further purification of a 24 mg sample was achieved by reverse-phase (C18) thin layer chromatography ($R_f = 0.7$) with a 4:6 mixture of methanol and 0.5 M aqueous $(NH_4)_2SO_4$. The separation afforded 10 mg (13%) of a white solid mp 255 °C (dec).

Synthesis of N^2-(n-Butyl-1-carboxyethyl)-9-methylguanine. To a 50-mL round bottom flask, 10 mg (0.42 mmol) of CEmG was added along with 20 mL of butanol containing 1% concentrated H_2SO_4. The reaction mixture was heated under N_2 at 100% for 1 h. The reaction solution then was cooled to room temperature, and 50 mL of 5% aqueous sodium bicarbonate was added. The resulting mixture was concentrated to dryness by rotary evaporation and 50 mL of H_2O then added. The product was extracted (3 × 20 mL) with a 4:1 mixture of CH_2Cl_2/MeOH. The organic layers were combined and concentrated. Drying in vacuo afforded 6.5 mg of the butyl ester. ^{1}H NMR [CD_3OD/CF_3CO_2D (1:1)]: δ: 0.81 (t, J = 7.7 Hz, CH_2CH_3, 3 H), 1.30 (m, CH_2-CH_3, 2 H), 1.45 (d, J = 7.3 Hz, CH_3CHN^2, 3 H), 1.55 (m, $CH_2CH_2CH_3$, 2 H), 3.78 (s, 9-CH_3, 3 H), 4.11 (t, J = 6.96 Hz, CH_2-*n*-Pr, 2 H), 4.58 (q, J = 7.3 Hz, CH_3CHN^2, 1 H), 8.69 (s, H8, 1 H). DEI-MS (50 eV): 293 (M^+, 17.4), 192 (M^+, -CO_2-*n*-bu, 100). Exact mass for $C_{13}H_{14}N_5O_3$: 293.1488; found: 293.1496.

Isolation of Disodium N^2-(1-carboxyethyl)-2-deoxyguanosine 5′-Monophosphate (CEdGMP). To 400 mL of phosphate buffer (pH 7.4, Na 0.3 M), 4.75 g (13.7 mmol) of disodium 2-deoxyguanosine 5′-monophosphate (dGMP) and 3.7 g (20.5 mmol) of a 40% aqueous solution of MG were added. The reactants were refluxed for 20 h under N_2. The resulting brown solution was cooled to room temperature and then titrated with absolute ethanol until cloudiness persisted. The mixture was allowed to stand overnight and then suction filtered. This procedure of ethanol titration was repeated twice until essentially all of the buffer was removed from the ethanol/water solution. The final filtrate was

concentrated to dryness by rotary evaporation and triturated thoroughly with hot ethanol. The insoluble solid was filtered and dried *in vacuo* to afford 1.66 g of a beige solid. A 150 mg portion of this was purified by preparative TLC using a mixture of ethyl acetate, methanol, and water (2:1:1), R_f = 0.3. This afforded 45 mg (9% yield) of an inseparable mixture of diastereomers which were estimated by HPLC to comprise a 6.5:3.5 ratio. Approximately 100 μg of purified CEdGMP was analyzed per HPLC run. ^{1}H NMR (D_2O): δ 1.39 (d, 7.2 Hz, two overlapping doublets CH_3) 1.45−4.7 (m, aliphatic), 6.31 (m, NC*H*O), 8.02 (s, H8), 8.08 (s, H8′). FAB-MS (negative ion mode) *m/z* (relative intensity) 418 (M^-, 37.8), 213 (100, ribose hydrogen phosphate).

Eukaryotic Mutagenesis Assay. CEG mutagenesis was assessed in mammalian cells utilizing the *lacI*-containing shuttle vector pPy35 as described previously (Bucala *et al.*, 1993). Briefly, 5 μg of CsCl-purified pPy35 DNA was incubated with MG (0−10 μM) for 1 h at 37 °C in buffer containing 50 mM HEPES (pH 8.0) and 1 mM EDTA. For transfection, 1.7 × 10^6 X63Ag8.653 cells were resuspended in 1 mL of Opti-MEM (Gibco, Grand Island, NY) containing 25 μL of lipofectin (Gibco) and 5 μg of control or MG-modified DNA. After incubation for 3 h at 37 °C and in 5% CO_2, the cells were diluted by the addition of 5 mL of RPMI/10% FBS. Transfected cells were washed and resuspended in RPMI/10% FBS on the following day. Neomycin (G418, 600 μg/mL) was added after an additional 3−4 days of incubation. Episomal DNA was recovered by alkaline−SDS purification when lymphoid cell number reached 5 × 10^6 (2−3 weeks of culture). Recovered plasmids were transfected into the *E. coli* strain MC1061 F′Δ150kan and scored for *lacI* function by α-complementation on agar plates containing ampicillin and X-gal as described (Bucala *et al.*, 1993).

RESULTS

We developed a systematic approach to screen the reactivities of the individual nucleotide bases adenine, guanine, and cytosine toward glucose and several of its derivatives. Methyl-blocked bases were used instead of the naturally occurring N-glycosides to facilitate the characterization of reaction products. The ^{1}H NMR resonance of these methyl groups (predominant sharp singlets generally between 3.3 and 3.6 ppm) served as important diagnostic tools, indicating the number and approximate abundance of different products in crude reaction mixtures. In initial "fast reactions", individual nucleotide bases were heated at reflux for 24 h under nitrogen with 10 molar equiv of D-glucose, G-6-P, G-6-P/lys, SB, and AP. The typically crude reaction solutions then were analyzed by TLC, ^{1}H NMR, and reverse-phase or ion exclusion HPLC. NMR samples were prepared by concentrating crude reaction solutions to dryness and extracting the residues with DMSO-d_6. Ethanol extracted products also were analyzed by TLC and ^{1}H NMR.

9-Methyladenine did not react to any observable extent with glucose or any of the glucose-derived reactants. 1-Methylcytosine was somewhat reactive toward G-6-P and G-6-P/Lys and a trace amount of at least one product was detected by ^{1}H NMR after 2 days of reflux. 9-Methylguanine showed the highest reactivity in these studies. It reacted with D-glucose, G-6-P, G-6-P/lys, SB, and AP. In all reactions involving 9-mG, a single major adduct was identified by TLC and HPLC analyses. The ^{1}H NMR spectrum of this product in D_2O is simple and features the characteristic high field doublet and the lower field multiplet due to methyl and methine protons, respectively (spectrum not shown). Also present are the two singlets at 3.5 and 7.7 ppm due to the 9-methyl and aromatic protons on C-8 of guanine. In a ^{1}H NMR spectrum of a sample recorded in DMSO-d_6, the exchangeable N^1 and N^2 protons give rise to broad one proton resonances at 10.5 and 7.2 ppm, respectively. The ion-spray MS spectrum of this adduct contains a base peak at 238 *m/z* corresponding to the MH^+ ion and high resolution FAB-MS had a consistent, exact mass for $C_9H_{11}N_5O_3(H^+)$. Taken together, these data were consistent with a structure bearing either a 3-carbon lactylamido or carboxyethyl moiety attached to the N^2 position of 9-mG. The adduct then was converted successfully to its corresponding butyl ester with consistent ^{1}H NMR, FAB, FAB-high resolution spectra, thus establishing its structure as N^2-(1-carboxyethyl)-9-methylguanine (CEmG).

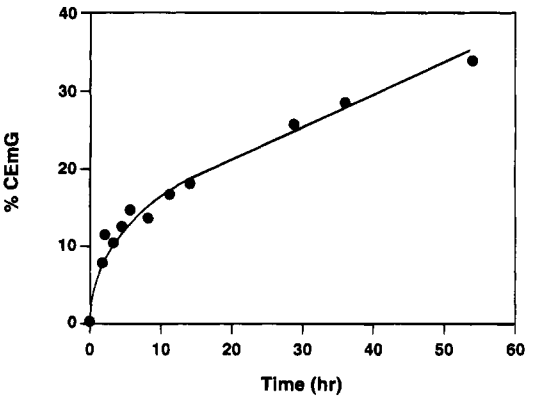

FIGURE 1: Time-dependent formation of CEmG from the reaction of 9-mG with 10 equiv of AP. CEmG was measured by HPLC as described in Materials and Methods.

The HPLC yields of CEmG obtained by reaction of 9-mG with 10 equiv each of D-glucose, G-6-P, G-6-P/lysine, SB, and AP were found to be 69%, 46%, 51%, 61%, and 23%, respectively. CEmG was not detected in any reactions that contained 9-mG alone. A representative kinetic analysis of the formation of CEmG from 9-mG and AP is shown in Figure 1 and indicates that CEmG forms in a slow, time-dependent fashion. N^2-(1-carboxyethyl)-9-methylguanine also was detected in long-term incubations (28 weeks) of 9-mG with AP which were performed at 37 °C and pH 7.4. CEmG was isolated from these reactions by HPLC chromatography utilizing C18 resin and an aqueous mobile phase of 0.5 M $(NH_4)_2SO_4$ and was observed to elute as a single peak after 10 min (Figure 2). The presence of CEmG in these long-term reactions and in samples obtained by partial purification was confirmed by analytical TLC, HPLC, and ^{1}H NMR. The overall HPLC yield of CEmG under these incubation conditions was 9%. Additional UV active (254 nm) products also were present in lower amounts. FAB-MS analyses did not yield useful information due to the very low abundance of individual products.

An additional derivative that formed in long-term incubations was 8-oxo-7-*H*-9-methylguanine (OmG). 8-Oxo-7-*H*-9-methylguanine and 9-mG co-elute from a reverse-phase HPLC column (Figure 2), but OmG has a slightly higher mobility on silica and was isolated in 3% yield by flash

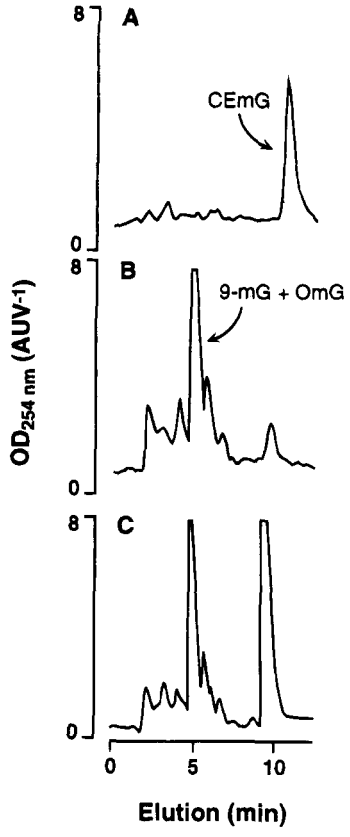

FIGURE 2: Reverse-phase HPLC profile of 9-mG reaction products. (A) Elution of purified CEmG. (B) Reaction products obtained by incubating 9-mG with AP at 37 °C and pH 7.4 for 28 weeks. The elution positions of 9-mG and OmG are indicated. The coelution of 9-mG and OmG was demonstrated in separate experiments by mixing pure samples of 9-mG and OmG prior to HPLC analysis (not shown). (C) Addition of purified CEmG to the reaction mixture chromatographed in panel B. HPLC was performed as described in Materials and Methods.

column chromatography. The ^{1}H NMR spectrum of OmG in DMSO-d_6 is consistent with the 8-oxo, 7-*H* tautomer rather than the 8-hydroxy form. X-ray crystallographic studies of the analogous 9-ethyl-8-hydroxyguanosine have shown that this purine also exists in the 6,8-diketo form (Doi *et al.*, 1991).

The HPLC profile of products formed in the long-term incubation at 37 °C and in the fast reactions involving 9-mG and AP were similar. Fast reactions proceeding for 1 or 2 days in the presence or absence of air also showed similar HPLC profiles. There were small differences in the relative intensities of the major peaks, and the long-term reaction also appeared to have more minor products. A fast reaction under N_2 involving 1 equiv of all three of the nucleotides and 10 equiv of AP produced CEmG as a major product, but no other novel products which could be identified by TLC, HPLC, and ^{1}H NMR.

In an attempt to identify the reactive intermediate producing CEmG, 9-mG was refluxed at pH 7.4 with 2 molar equiv of methylglyoxal (MG), a three-carbon product known to form from glucose during the Maillard reaction (Olsson *et al.*, 1981). As expected, CEmG was produced and was isolated in 31% yield. CEmG also was produced in 22% yield during long-term incubations (24 weeks at 37 °C) involving 9-mG and 2 molar equiv of MG. The isolation of adducts produced from the direct treatment of guanine (N-9 free) with MG under conditions of room temperature

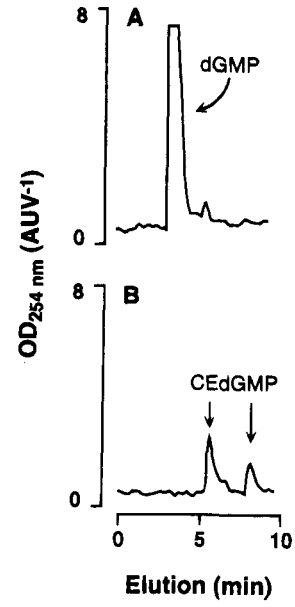

FIGURE 3: Reverse-phase HPLC profile of dGMP (A) and diastereomers of the product CEdGMP (B). HPLC and structural characterization was performed as described in Materials and Methods.

and pH 7 was reported first by Shapiro *et al.* (1969). Methylglyoxal reacts with deoxyguanosine to form an unstable cyclic structure which then can decompose under oxidizing conditions to form N^2-acetyl-dG (Shapiro *et al.*, 1969; Nukaya *et al.*, 1990). Although we detected other products in low abundance in crude reaction mixtures produced by the coincubation or reflux of 9-mG with MG, no further attempt was made at their isolation and identification. In view of the prolonged incubation conditions necessary to produce CEmG, it is conceivable that the carboxyethyl moiety also arises as a secondary product of the primary unstable, cyclic adduct.

The naturally occurring nucleotide dGMP reacts with 2 equiv of MG, affording products derived from the deoxyribose free base. Refluxing dGMP with 2 equiv of MG in phosphate buffer (pH 7.4) was found to produce the amino acid CEdGMP. This adduct was isolated by precipitation and chromatography in 9% yield and was characterized by ^{1}H NMR and FAB-MS. HPLC resolved CEdGMP into a 6.5:3.5 mixture of diastereomers (Figure 3). Refluxing dGMP alone under similar conditions followed by HPLC produced no detectable CEdGMP.

The formation of additional, lower abundance nucleotide products was studied by refluxing 10 equiv of each of the Maillard reaction intermediates MG, 3-deoxyglucosone (3-DG), diacetyl, lactic acid, and 5-(hydroxymethyl)furfuranyl (HMFF) with 9-mG, 9-mA, or 1-mC for 1 day. We followed the strategy that was utilized to identify CEmG and assumed that if these Maillard intermediates were capable of reacting with nucleotide bases under physiological conditions, even slightly, then larger quantities of modified nucleotides may form in the presence of a vast excess of a particular intermediate under refluxing conditions. Further understanding of the structural requirements necessary for reactivity with nucleotide bases also might be afforded by this analysis.

5-(Hydroxymethyl)furfuranyl was observed to react with 9-mG to produce a complex mixture of compounds. This

Table 1. Mutation Analysis of MG-Modified Shuttle Vector pPy35 after Recovery from Murine Lymphoid Cells[a]

methylglyoxal (μM)	transfections	total plasmids	total mutants	mutation frequency (%)
0	9	1.43×10^5	715	0.5%
0.1	7	2.02×10^5	2010	1.0%
1.0	6	1.42×10^5	2699	1.9%
10.0	9	3.01×10^5	10,235	3.4%

[a] Purified pPy35 DNA was incubated with the indicated concentration of MG for 1 h at 37 °C as described in Materials and Methods. "Transfections" refers to the total number of independent transfections analyzed for each methylglyoxal modification condition. "Total mutants" refers to the total number of mutants identified in all transfections. "Mutation frequency" was calculated by (total mutants total plasmids) × 100.

reaction occurred at a markedly slower rate than observed for the reaction of 9-mG with MG. It is likely that the carbonyl group of 5-(hydroxymethyl)furfuranyl reacts with 9-mG, since 2,5-furandimethanol did not react with 9-mG under similar conditions. Diacetyl, 3-DG, and dehydroascorbic acid did not react significantly with nucleotide bases under the conditions tested. Methylglyoxal was the only intermediate that reacted significantly with 1-mC, and none of the intermediates reacted with 9-mA.

Finally, we sought to address the biological activity of methylglyoxal *in vivo*. DNA modified by glucose and glucose-derived reactive intermediates has been shown to be mutagenized when studied in both prokaryotic and eukaryotic assay systems (Bucala *et al.*, 1985; 1993; Lee & Cerami, 1987, 1991). Methylglyoxal itself it mutagenic in *E. coli* (Olsson *et al.*, 1982); however, the mutagenicity of MG-modified DNA in eukaryotic cells has not been studied. Utilizing a previously described *lacI* shuttle vector system (Bucala *et al.*, 1993), plasmid pPy35 was modified by MG *in vitro* and transfected into the mouse lymphoid cell line X63.Ag8.653. After selection in culture for plasmid-containing cells, episomal DNA was purified and assayed for *lacI* mutations by α-complementation in *E. coli*. The mutation frequency increased from 0.5% for control, unmodified plasmid DNA to 3.4% for plasmid DNA modified with 10 μM MG (Table 1).

DISCUSSION

The amino groups of DNA react with glucose and glucose-derived reactive intermediates to produce AGEs that induce both DNA strand-breakage and mutations (Bucala *et al.*, 1984, 1985; Lee & Cerami, 1987a, 1991). In *E. coli*, the mutagenic effect of DNA-AGEs is due in part to the activation of host-derived transposable elements such as IS-1 and $\gamma\delta$ (Bucala *et al.*, 1985; Lee & Cerami, 1991). AGE-modified DNA also produces DNA transposition in mammalian cells. These transpositions result predominantly from the insertion of a small subset of *Alu*-containing, genomic DNA sequences that have been termed INS-1 elements (Bucala *et al.*, 1993). The present studies were undertaken in an attempt to characterize, at the chemical level, the precise nucleotide modifications that result from DNA advanced glycosylation and which may mediate mutational activity. Reaction products that form during the incubation of nucleotide bases with glucose and glucose-derived Maillard products thus were examined in a systematic fashion, first by using "fast" refluxing conditions and then under "long-term" conditions performed at physiological temperature. Although this approach allows for the facile identification of products at the chemical level, it is recognized that modified nucleotides which form in greatest abundance *in vitro* may not necessarily be representative of the products with the greatest biological activity *in vivo*. In general, AGE derivatives of DNA have been estimated to be minor spontaneous lesions, occurring at <1% of the frequency of thermal or oxygen-induced DNA products (Bucala *et al.*, 1994; Lindahl *et al.*, 1993). Nevertheless, the mutagenic potential of particular forms of DNA damage also depends greatly on the capacity of cells to repair specific nucleotide adducts.

The identification of CEmG in reactions of 9-mG with D-glucose, G-6-P, G-6-P/Lys, AP, or SB suggests that a common, reactive Maillard intermediate is produced during each of these reactions. Examination of the reaction products of nucleotide bases with various Maillard intermediates showed that only MG and HMFF had observable reactivity. These results suggest that an aldehyde moiety is a necessary component for the intermediate to react significantly with the relatively deactivated amino groups of nucleotides. The inability of the highly reactive aldehyde 3-DG to modify nucleotide bases is due most likely to its low stability (Madson & Feather, 1981). 3-Deoxyglucosone decomposes before it can react significantly with nucleotides.

The direct formation of CEG by MG treatment suggests that MG, a known Maillard reactant (Hodge, 1953), is the common, reactive intermediate that modifies nucleotide bases under *in vitro* conditions. The mechanism of formation of MG from AP has been proposed by Hodge (1953) and is shown in Figure 4. Amadori products decompose to 1-deoxyglucosone (1-DG) and a free amine. A retro-aldol fission of 1-DG generates MG and glyceraldehyde. A slower production of MG from glucose also has been observed in reactions not catalyzed by amines (Ledl & Schleicher, 1990). Therefore, in reactions involving nucleotide bases and glucose or G-6-P, initial Schiff base formation may not be essential for the production of MG. At any rate, nucleophilic addition to the ketone group of hydrated MG by the N2 of 9-mG gives the covalent hydrate intermediate which can lose H_2O to form an imine intermediate. This intermediate can then undergo a rearrangement to give CEmG. A nucleophilic addition to the aldehyde group of MG, followed by a hydride shift, would have given the lactic acid amide N^2-lactylamido-9-methylguanine. This adduct would have spectral properties very similar to those of CEmG. Conversion of the 9-mG adduct to the butyl ester, however, established its structure to be that of an N^2-(1-carboxyethyl) substituent. The 1-carboxyethyl adducts of guanine and guanosine have been isolated previously from various microbes, and it is possible that these adducts also form by the nonenzymatic reaction of DNA or nucleotides with MG (Ballio *et al.* 1960; Al-Khalid & Greenberg, 1961).

The oxidized base OmG also was identified to form in the long-term reactions that were performed under physiological conditions. The OmG tautomer 8-hydroxyguanine is known to be produced in DNA by exposure to ionizing radiation or after reaction with radical-generating agents (Kasai *et al.*, 1984). Hydroxylation of guanine at the C-8 position also has been reported to occur when 2-deoxyguanosine or DNA is incubated with preheated, solid glucose (200 °C, 20 min) (Kasai *et al.*, 1986). The adduct forms

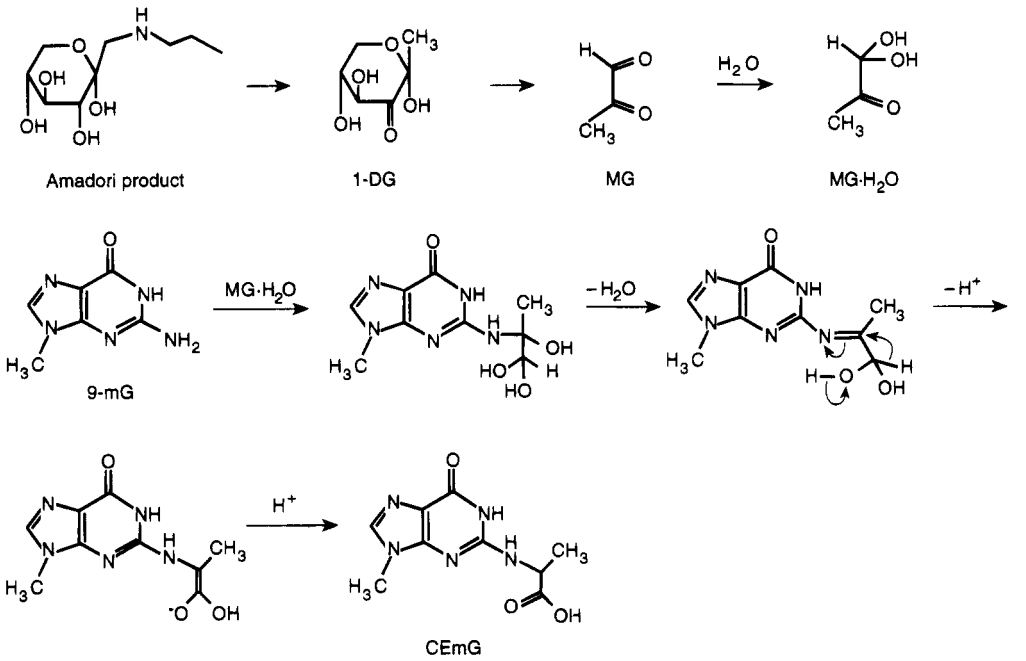

FIGURE 4: Proposed mechanism for the formation of CEmG from Amadori product and 9-methylguanine.

presumably by the attack of a hydroxyl radical on the 8-position of guanine. Ames and co-workers have reported a correlation between the urinary content of 8-hydroxyguanine and host age or basal metabolic rate (Fraga *et al.*, 1990) and have suggested that the prevalence this base may reflect cumulative cell exposure to reactive oxygen species. Reducing sugars have been shown to produce oxygen free radicals during metal-catalyzed autoxidation *in vitro* (Thornalley *et al.*, 1984). Although significant autoxidation is unlikely to occur *in vivo*, the present results suggest the possibility that glucose-dependent, Maillard processes contribute to the formation of OmG in tissues. Evidence in support of this possibility may be acquired by examining OmG/8-hydroxyguanine levels in diabetic tissues, where the content of Maillard products is increased markedly (Bucala & Cerami, 1992).

Previous studies have shown that when DNA is incubated with G-6-P together with [^{3}H]lysine, a reactive intermediate forms that covalently links the radioactive lysine to DNA (Lee & Cerami, 1993b). Insight into the chemical basis for this sugar-derived cross-linked was not obtained in this study. The cross-linking species that forms in these reactions either must be present in low abundance or else readily decomposes to species that could not be identified in the present experimental strategy.

Plasmid DNA modified by MG *in vitro* was found to be mutagenized after its introduction and replication in murine lymphoid cells. This result verifies the mutagenicity of MG that has been reported in *E. coli* (Ledl & Schleicher, 1990). Whether the mutations produced by incubation of shuttle vector DNA with MG were the result of DNA transposition as was observed in the case of G-6-P/Lys modified DNA (Bucala *et al.*, 1993) remains to be established. Nevertheless, the facile preparation of CEG-containing plasmid DNA bytreatment with MG *in vitro* will assist greatly in the further analysis of the mutagenic profile of this particular DNA-AGE.

Methylglyoxal has also been proposed to arise enzymatically in some organisms through the action of MG synthetase on the glycolytic intermediates dihydroxyacetone phosphate or glyceraldehyde 3-phosphate (Cooper, 1984). Methylglyoxal then may be degraded by glyoxalase I (lactoyl–glutathione methylglyoxal lyase), an enzyme that detoxifies MG by catalyzing the formation of *S*-lactoylglutathione. Glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase) then hydrolyzes the thiolester to lactate and free glutathione(Douglas & Shinkai, 1985). Shires *et al.* (1990) have reported that in cells which were depleted experimentally of glutathione, [^{14}C]glucose was incorporated into DNA concomitantly with an apparent rise in reactive, 3-carbon dicarbonyl compounds. These data implicate MG or other reactive glucose-derived products in the covalent modification of DNA *in vivo* and are consistent with the formation of CEG nucleotides described in the present study. Further investigations will be necessary to elucidate the importance of enzymatic versus nonenzymatic (Maillard) pathways for intracellular MG formation as well as the role of glutathione depletion in promoting MG-mediated DNA damage *in vivo*. We presently are endeavoring to quantitate the frequency of CEG occurrence *in vivo* by chemical and immunochemical methods.

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