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ORIGINAL PAPER

The structural modification of DNA nucleosides by nonenzymatic glycation: an *in vitro* study based on the reactions of glyoxal and methylglyoxal with 2′-deoxyguanosine

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Abstract Methylglyoxal and glyoxal are generated from the oxidation of carbohydrates and lipids, and like D-glucose have been shown to nonenzymatically react with proteins to form advanced glycation end products (AGEs). AGEs can occur both *in vitro* and *in vivo*, and these compounds have been shown to exacerbate many of the long-term complications of diabetes. Earlier studies in our laboratory reported D-glucose, D-galactose, and D/L-glyceraldehyde formed AGEs with nucleosides. The objective of this study was to focus on purines and pyrimidines and to analyze these DNA nucleoside derived AGE adducts with glyoxal or methylglyoxal using a combination of analytical techniques. Studies using UV and fluorescence spectroscopy along with mass spectrometry provided for a thorough analysis of the nucleoside AGEs and demonstrated that methylglyoxal and glyoxal reacted with 2′-deoxyguanosine via the classic Amadori pathway, and did not react appreciably with 2′-deoxyadenosine, 2′-deoxythymidine, and 2′-deoxycytidine. Additional findings revealed that methylglyoxal was more reactive than glyoxal.

Keywords Advanced glycation end products · Maillard reaction products · Glyoxal · Methylglyoxal · 2′-Deoxyguanosine

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Introduction

The carbonyl groups of reducing sugars or sugarlike compounds can nonenzymatically react with the free amino groups of biomolecules to form a polymorphic group of compounds referred to as advanced glycation end products (AGEs) [1] or advanced glycation like end products (AGLEs) [2]. Nonenzymatic reactions can occur both *in vitro* and *in vivo* and affect the structure, function or conformation of many biomolecules. For example, irreversible cross-links of AGEs have been shown in some long-living matrix structural proteins such as type IV collagen, laminin, and fibronectin [3]. Similar modifications to proteins have also been reported in the aged population (nondiabetic), raising the possibility that nonenzymatic glycation may play a role in "diabetes induced early aging" [4]. Although the nonenzymatic glycation of biomolecules occurs naturally, in some metabolic disorders such as diabetes, the formation and accumulation of AGEs occurs faster, which contributes to many of the long-term complications of these diseases. This rapid formation and accumulation of AGEs is caused not only by D-glucose itself, but also by certain glucose-derived dicarbonyl intermediates such as glyoxal and methyglyoxal, which are both potent precursors of AGEs [5–10].

Although extensive studies have been conducted to investigate the glyoxal and methylglyoxal AGEs of proteins, only minimal attention has been devoted to investigating their nonenzymatic modification of purines and pyrimidines. Recent studies in our laboratory have demonstrated that like proteins, DNA is susceptible to nonenzymatic attack by sugar affecting the structure, stability, and conformation of DNA molecules [2].

The objective of this study was twofold. First, it was to evaluate the effect of reactant concentration, temperature, pH and ionic strength on the formation of glyoxal and methylglyoxal purine and pyrimidine AGE adducts and, second, it was to characterize the DNA nucleosides derived AGE adducts of these two compounds by using UV spectrophotometry, fluorescence spectroscopy, high-performance liquid chromatography (HPLC) and mass spectrometry.

In this report, we compare the reactivity of glyoxal and methylglyoxal with that of different purines and pyrimidines and describe factors influencing the formation of AGEs by these compounds.

Materials and methods

Chemicals and supplies

Analytical grade glyoxal, methylglyoxal, 2′-deoxyadenosine (dA), 2′-deoxyguanosine (dG), 2′-deoxycytidine (dC), 2′-deoxythymidine (dT), sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma Chemical (St. Louis, MO, USA). Disposable UV-transparent cuvettes (12.5 mm×12.5 mm×36 mm) were obtained from Fisher Scientific (New Lawn, NJ, USA). Unless otherwise indicated, all other reagents and solvents were of analytical grade and were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Preparation of buffers and reaction mixtures

Unless otherwise indicated, all reactions were conducted in 0.2 M phosphate buffer (pH 7.2) containing 0.02% sodium azide. Stock solutions of glyoxal and methyglyoxal were

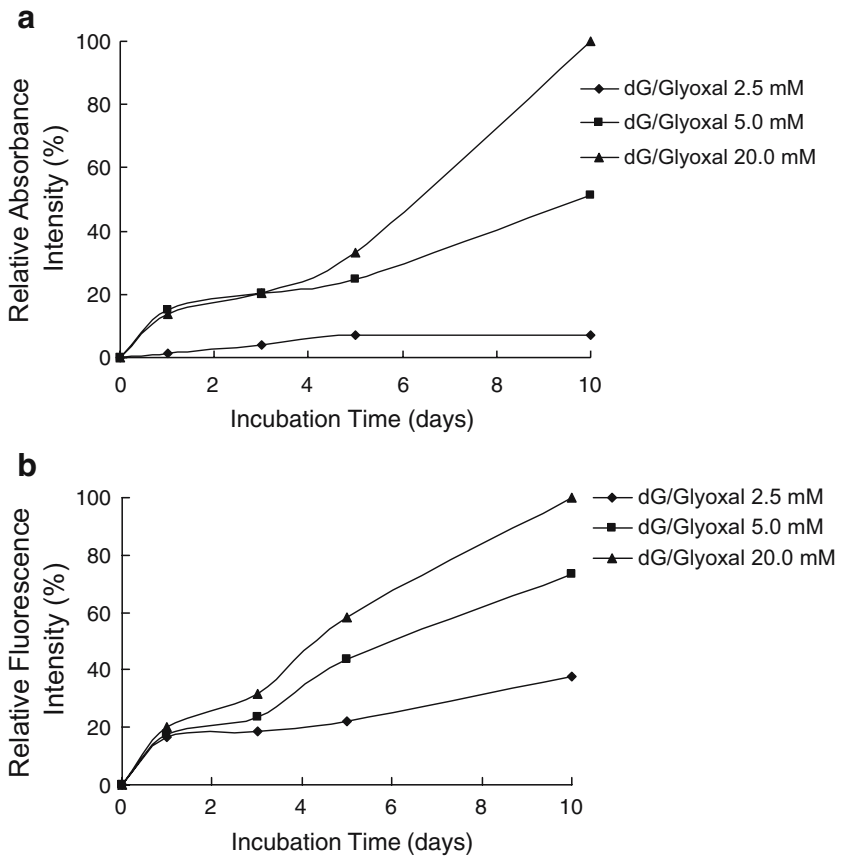

Fig. 1 UV and fluorescence spectral profiles of 2′-deoxyguanosine (dG; 5 mM) with glyoxal (2.5, 5.0, and 20 mM) at 37 °C for 10 days. UV spectral profiles (**a**) were measured at 260 nm. Fluorescence spectral profiles (**b**) were measured with an excitation wavelength of 370 nm and an emission wavelength of 420 nm. All points **a** and **b** represent the average of triplicate measurements, and every reading was found to be within 5% of its counterpart duplicate. All readings were compared with the highest reading, which was set at 100%

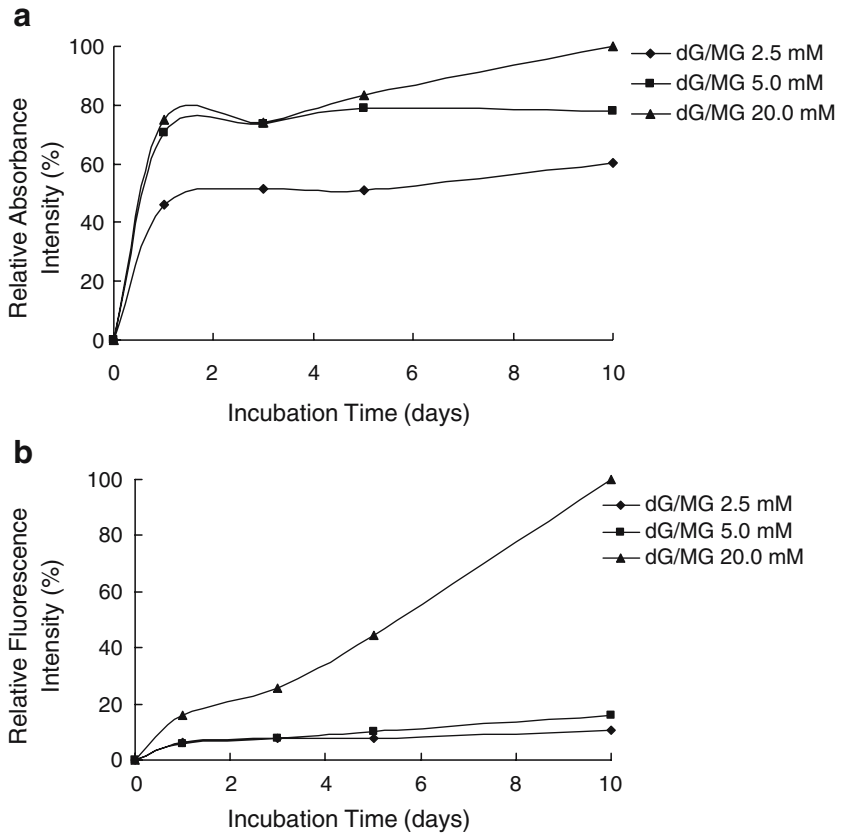

Fig. 2 UV and fluorescence spectral profiles of dG (5 mM) with methylglyoxal (2.5, 5.0, and 20 mM) at 37 °C for 10 days. UV spectral profiles (**a**) were measured at 260 nm. Fluorescence spectral profiles (**b**) were measured with an excitation wavelength of 370 nm and an emission wavelength of 420 nm. All points in **a** and **b** represent the average of triplicate measurements, and every reading was found to be within 5% of its counterpart duplicate. All readings were compared with the highest reading, which was set at 100%. *MG* methylglyoxal

prepared by dissolving 40 mM concentrations of each in the 0.2 M phosphate buffer. Stock solutions of the different deoxynucleosides were prepared by dissolving 10 mM concentrations of dG, dA, dT, or dC in the 0.2 M phosphate buffer. Final reaction mixtures containing methylglyoxal or glyoxal (2.5, 5, and 20 mM) and various nucleosides (5 mM) were prepared from the stock solutions. Controls included methylglyoxal or glyoxal at 2.5, 5, and 20 mM concentration or the different nucleosides each at 5 mM concentration. Blank tubes included freshly mixed methylglyoxal/glyoxal with different nucleosides. Unless otherwise indicated, all reaction mixtures and controls were incubated in the dark at 37 °C in a shaking water bath for up to 10 days and were frozen until their analysis.

Table 1 The effect of temperature on the formation of advanced glycation end products (AGEs), an evaluation by UV spectroscopy

Time (days)	37 °C		50 °C		75 °C	
	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG
1	0.106	0.361	0.114	0.444	0.114	0.577
3	0.144	0.365	0.238	0.479	0.238	0.672
5	0.215	0.402	0.339	0.709	0.339	0.761

All samples were measured in triplicate and were diluted 1:40 before testing. Each measured value was no larger than 5% of its counterpart triplicate reading and all tabulated values represent the mean of the triplicate readings. In each instance, the readings were blanked against the reading from the tubes which contained solutions of glycoxal and methylglyoxal (20 mM) to which freshly prepared 2′-deoxyguanosine was added (5 mM).
dG 2′-deoxyguanosine, *MG* methylglyoxal

Table 2 The effect of temperature on the formation of AGEs, an evaluation by fluorescence spectroscopy

Time (days)	37 °C		50 °C		75 °C	
	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG
1	0.452	1.915	0.731	6.722	3.098	2.122
3	0.662	3.043	0.922	9.367	3.668	4.767
5	1.146	5.291	1.277	8.897	4.664	4.297

All samples were measured in triplicate and were diluted 1:40 before testing. Each measured value was no larger than 5% of its counterpart triplicate reading and all tabulated values represent the mean of the triplicate readings. In each instance, the readings were blanked against the reading from the tubes which contained solutions of glycoxal and methylglyoxal (20 mM) to which freshly prepared 2′-deoxyguanosine was added (5 mM).

UV and fluorescence spectroscopy

The acquisition of UV spectra was performed at a wavelength of 260 nm with a DU 800 spectrophotometer (Beckmann). Fluorescence emission spectroscopy was performed at an excitation wavelength of 370 nm and an emission wavelength of 420 nm using an LS 55 luminescence spectrometer (PerkinElmer) equipped with a thermal cell for maintaining the samples at 25±1 °C. The above-mentioned excitation and emission wavelengths were found to be optimal for detecting the nucleoside AGEs.

High-performance liquid chromatography

HPLC was performed with a system (Hitachi High Technologies America, San Jose, CA, USA) consisting of a low-pressure gradient pump (L-7100), a four-channel degasser, a sequential autosampler (L-7200), and a high-sensitivity diode-array detector (190–800 nm). AGE species were separated on a Phenomenex HPLC amide column (5 μm×4.6 mm×50 cm). Mobile phase A consisted of 0.1 M ammonia acetate buffer in deionized water. Mobile phase B consisted of 100% acetonitrile. An isocratic condition consisting of 67% mobile phase A and 33% mobile phase B was applied for 15 min at a constant flow rate of 1.00 ml/min. Prior to HPLC analysis, all solvents were filtered with a 0.45-μm membrane (Millipore, Billerica, MA, USA), degassed for 15 min, and centrifuged. The monitoring wavelength was set at 260 nm. All HPLC runs were repeated three times to ensure reproducibility of the data and peak retention times, and unless otherwise indicated, no significant differences were observed between the different repeat runs.

Electrospray ionization mass spectrometry

An orthogonal time-of-flight (TOF) mass spectrometer (Applied Biosystems Mariner atmospheric pressure ionization TOF workstation, Framingham, MA, USA) equipped with standard electrospray ionization source was used. The mass-spectral data were collected at positive ion polarity. Nitrogen was used as the nebulizer, curtain, heater, and collision gas. The Sciex heater was set to 350 °C and the spray tip potential was set at 4,000 V. The instrument was outfitted with an integrated syringe pump and with a dual syringe rack for direct infusion onto the mass spectrometer. The mass spectrometry system was operated in full-scan mode (*m*/*z* 100–1000). Spectral acquisition was performed every 2 s and a total of ten spectra were accumulated. All ions measured were in the $[M+H]^+$ form.

Table 3 The effect of pH on the formation of AGEs, an evaluation by UV spectroscopy

Time (days)	pH 6.0		pH 7.2		pH 8.0	
	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG
1	0.040	0.213	0.106	0.361	0.092	0.042
3	0.068	0.230	0.144	0.365	0.184	0.427
5	0.092	0.356	0.215	0.402	0.472	0.687

All samples were measured in triplicate and were diluted 1:40 before testing. Each measured value was no larger than 5% of its counterpart triplicate reading and all tabulated values represent the mean of the triplicate readings. In each instance, the readings were blanked against the reading from the tubes which contained solutions of glycoxal and methylglyoxal (20 mM) to which freshly prepared 2′-deoxyguanosine was added (5 mM).

Table 4 The effect of pH on the formation of AGEs, an evaluation by fluorescence spectroscopy

Time (days)	pH 6.0		pH 7.2		pH 8.0	
	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG	dG/glyoxal	dG/MG
1	0.035	1.334	0.452	1.915	1.637	1.918
3	0.113	1.528	0.662	3.043	1.667	10.162
5	0.169	2.159	1.146	5.291	1.971	10.349

All samples were measured in triplicate and were diluted 1:40 before testing. Each measured value was no larger than 5% of its counterpart triplicate reading and all tabulated values represent the mean of the triplicate readings. In each instance, the readings were blanked against the reading from the tubes which contained solutions of glycoxal and methylglyoxal (20 mM) to which freshly prepared 2′-deoxyguanosine was added (5 mM).

Results

UV–vis and fluorescence spectroscopy

Preliminary experiments using a combination of analytical techniques revealed that of all the nucleosides (i.e., dA, dG, dC, and dT) evaluated in this study only dG was highly reactive with glyoxal and methylglyoxal. The results described herein will henceforth focus primarily on the reactions of dG with glyoxal and methylglyoxal.

The UV and fluorescence studies focused on two types of experiments. In the first series of experiments, emphasis was placed on incubating a constant concentration (5 mM) of each of the nucleosides with increasing concentrations of glyoxal or methylglyoxal (2.5, 5, and 20 mM). Figures 1 and 2 show the relative UV and fluorescence spectral profiles of dG with glyoxal and methylglyoxal at 37 °C for 10 days, respectively. All readings in each figure were compared with the highest reading, which was set at 100%. Tubes of blanks included solutions of glyoxal or methyl-

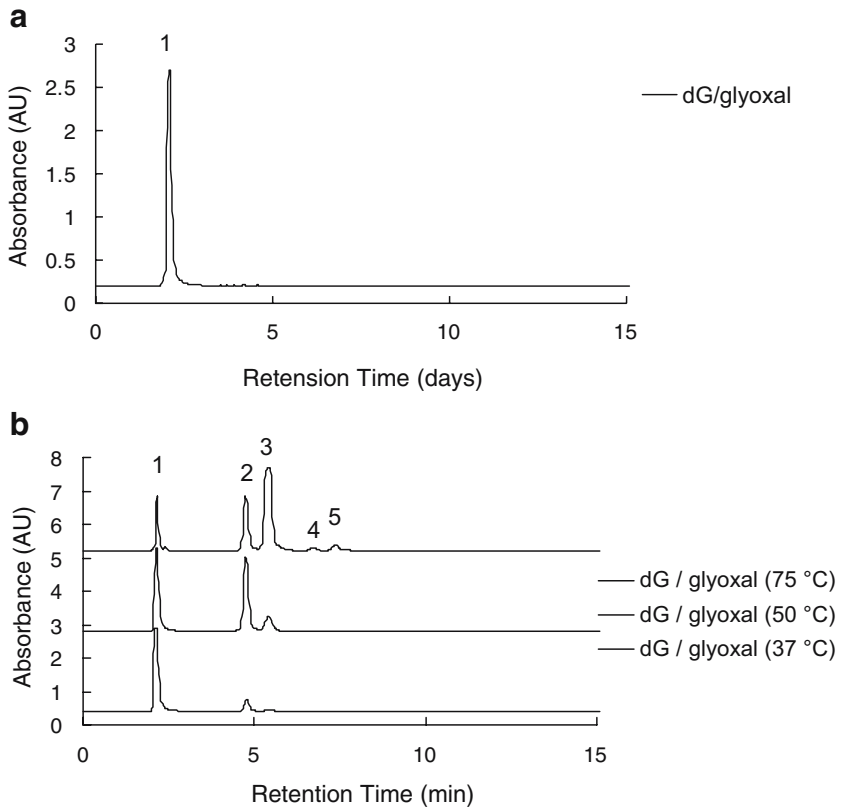

Fig. 3 High-performance liquid chromatography (HPLC) elution profiles of an initial mixture of dG with glyoxal (**a**) and mixtures of dG with glyoxal (**b**) incubated at 37, 50, and 75 °C for 3 days. With peak 1 representing the total amount of nonglycated nucleoside, the total yield of advanced glycation end products (AGEs) at 37, 50, and 75 °C was 16.3, 56.0, and 83.9%, respectively

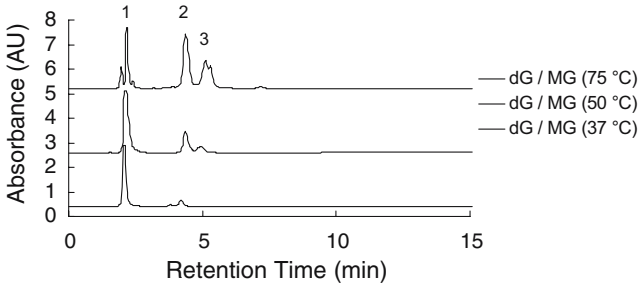

Fig. 4 HPLC elution profiles of mixtures of dG with methylglyoxal at 37, 50, and 75 °C for 3 days. With peak 1 representing the total amount of nonglycated nucleoside, the total yield of AGEs at 37, 50, and 75 °C was 15.0, 33.0, and 77.7%, respectively

glyoxal (20 mM) to which freshly prepared dG had just been added (5 mM).

The UV and fluorescence spectral profiles revealed that the formation of AGEs increased with time and with glyoxal and methylglyoxal concentrations. Using the UV and fluorescence readings as a measure of glycated products, we found that methylglyoxal was more reactive than glyoxal. Other findings showed that dG was a highly reactive nucleoside and that the reactivities of dA and dC were comparable (data not shown). As expected dT exhibited no reactivity, a finding that was attributed to the molecule not having a free amino group. Control solutions containing nucleosides alone, methylglyoxal alone, or glyoxal alone yielded no increases in UV and fluorescence readings, indicating that the changes seen earlier (Figs. 1, 2) were due to the formation of AGEs, and not to the instability of the nucleosides, methylglyoxal, or glyoxal (data not shown).

In the second series of experiments, focus was placed on evaluating the role of temperature and pH in the formation of AGEs. Tables 1 and 2 show the respective UV and fluorescence spectral data of incubation mixtures containing methylglyoxal and glyoxal (20 mM) with dG (5 mM) at 37, 50, and 75 °C for 5 days (Fig. S1). All readings were blanked against the reading from the tubes containing solutions of glyoxal or methylglyoxal (20 mM) to which freshly prepared dG had just been added (5 mM).

Both the UV and the fluorescence data confirmed that the formation of glycated products was a temperature-dependent and a time-dependent process and that increased temperature and time promoted AGE formation. As before, methylglyoxal was found to be more reactive than glycoxal. Another interesting observation was that in all the studies performed, the UV readings were less pronounced than their counterpart fluorescence readings, a finding which may have resulted from the inherent differences between the two methods. For example, as shown in Tables 1 and 2, the initial mixing of dG (5 mM) with glycoxal (20 mM) resulted in a solution with an average UV reading of 0.106 that changed to 0.215 after 5 days of incubation. For the same solution, the average fluorescence reading of the starting mixture was 0.452, changing to 1.146 after 5 days. This trend in the UV and fluorescence readings was consistent throughout the study (Tables 1, 2, 3, 4).

Tables 3 and 4 show the effect of pH on the formation of AGEs when methylglyoxal or glycoxal was reacted with dG at 37 °C for 5 days (Fig. S2). The data reveal that a shift in pH from an acid to an alkaline condition increased AGE formation, supporting the notion that the occurrence of AGEs proceeds via Schiff base intermediate products. Schiff base reactions are typically favored at pH conditions above neutrality [11]. The stimulatory effect of alkaline pH on glycation was previously demonstrated by incubating L-fucose, D-glucose and D-galactose with immunoglobulin G and albumin [12]. Tables 3 and 4 also demonstrate that a shift in pH from 6.0 to 8.0 allowed methylglyoxal to react more extensively with dG than did glycoxal.

HPLC analysis

Figure 3 compares the HPLC elution profiles of dG (5 mM) with glyoxal (20 mM) after initial mixing and after 3 days at 37, 50, and 75 °C, respectively. The HPLC profile of freshly mixed solutions of dG and glyoxal yielded one prominent peak at 2.61 min. An identical HPLC profile was observed for a freshly prepared solution of dG alone (data not shown). This suggested that peak 1 in Fig. 3a was mainly due to dG and not glyoxal. This conclusion was further supported by performing HPLC of a solution containing glyoxal alone, which resulted in an elution profile devoid of any UV absorption peaks (data not shown). As glyoxal cannot be simply detected by UV spectroscopy even at low wavelengths, one may convert glyoxal to glycolic acid in the presence of sodium hydroxide and then detect it in an ion chromatographic system [13]. Alternatively, the detection of glyoxal can be

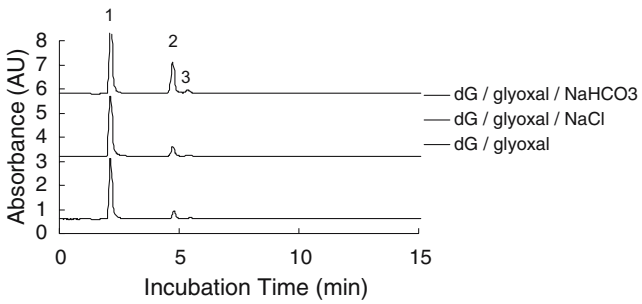

Fig. 5 HPLC elution profiles of incubation mixtures of dG with glyoxal in the presence and absence of salts after 3 days at 37 °C. With peak 1 representing the total amount of nonglycated nucleoside, the total yield of AGEs at 37°C was 14.9% in the absence of ionic salts, and 19.2 and 40.0% in the presence of NaCl and $NaHCO_3$, respectively

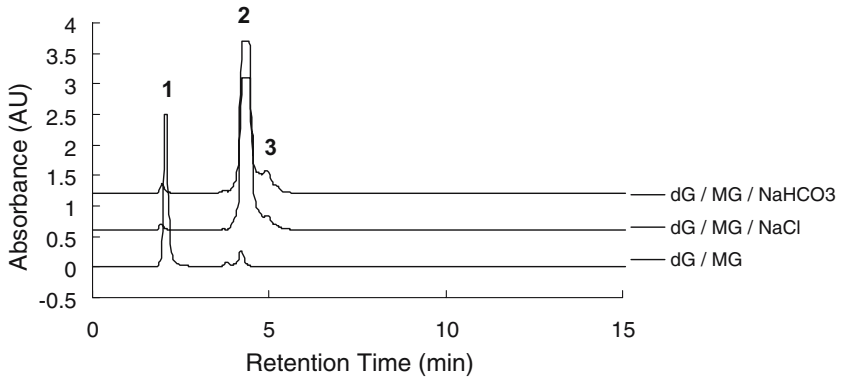

Fig. 6 HPLC elution profiles of incubation mixtures of dG with methylglyoxal in the presence and absence of salts after 3 days at 37 °C. With peak 1 representing the total amount of nonglycated nucleoside, the total yield of AGEs at 37 °C was 15.1% in the absence of ionic salts and 96.0 and 96.2% in the presence of NaCl and $NaHCO_3$, respectively

achieved by employing *meso*-stilbenediamine as a postderivatizing reagent [14]. Nevertheless, since our studies focused on the UV-absorbing AGEs of glyoxal and methylglyoxal, no efforts were devoted to implementing any of these techniques.

Increases in temperature promoted the formation of AGEs and caused new AGE products to form (Fig. 3b). At 37 °C, two peaks emerged, one at a retention time of 2.61 min (peak 1), and the other at a retention time of 4.75 min (peak 2). The intensity of peak 2 was significantly lower than that of peak 1, indicating that at 37 °C most of the dG was not glycated. As the temperature was increased from 37 to 50 °C, a new AGE product emerged. This product had a much lower intensity than that giving rise to peak 2 and was eluted at 5.47 min (peak 3). The chromatogram at 50 °C revealed that increases in temperature caused an increase in the intensity of peak 2. This suggested that the occurrence of AGEs was a temperature-dependent process, a finding substantiated earlier by UV and fluorescence studies (Tables 1, 2). Other findings revealed that an increase of the temperature to 75 °C resulted in two new additional AGEs being formed, with retention times of 6.72 min (peak 4) and 7.68 min (peak 5) minutes, respectively. The HPLC elution profile at 75 °C also demonstrated that the intensity of peak 3 was significantly higher than that of its counterpart peak at 50 °C, once again demonstrating the stimulatory effect of temperature on AGE formation.

Figure 4 shows the HPLC elution profiles of dG (5 mM) with methylglyoxal (20 mM) at 37, 50, and 75 °C. These profiles once more confirmed that AGE formation was a temperature-dependent and time-dependent phenomenon.

Figures 5 and 6 show the HPLC profiles of dG with both glyoxal and methylglyoxal in the absence and presence of the ionic salts sodium chloride and sodium bicarbonate. While both sodium chloride and sodium bicarbonate positively influenced AGE formation, the effect of sodium bicarbonate was more pronounced than that of sodium chloride. Additionally, the observation was made that in the presence of these salts the formation of the AGEs was more pronounced with methylglyoxal than with glyoxal, a conclusion reached by comparing the peak intensities of the AGE products.

Other findings demonstrated that incremental increases in pH caused different AGE products to form as the pH of

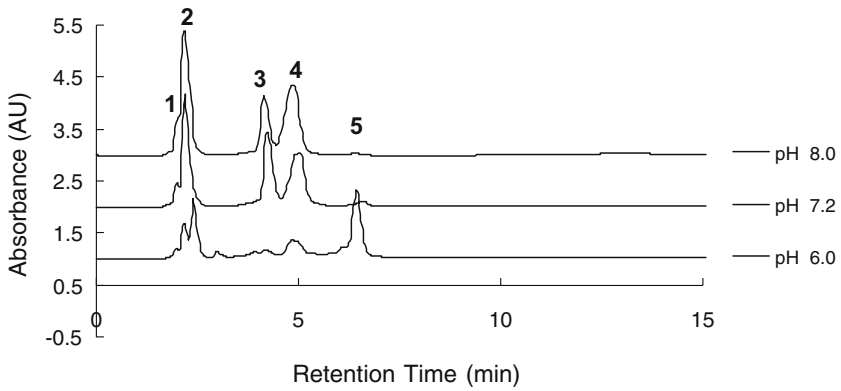

Fig. 7 HPLC elution profiles of incubation mixtures of dG with methylglyoxal after 5 days at 75 °C under various conditions of pH. With peak 1 representing the total amount of nonglycated nucleoside, the total yield of AGEs at pH 6.0, 7.2, and 8.0 was 47.8, 51.0, and 60.6, respectively

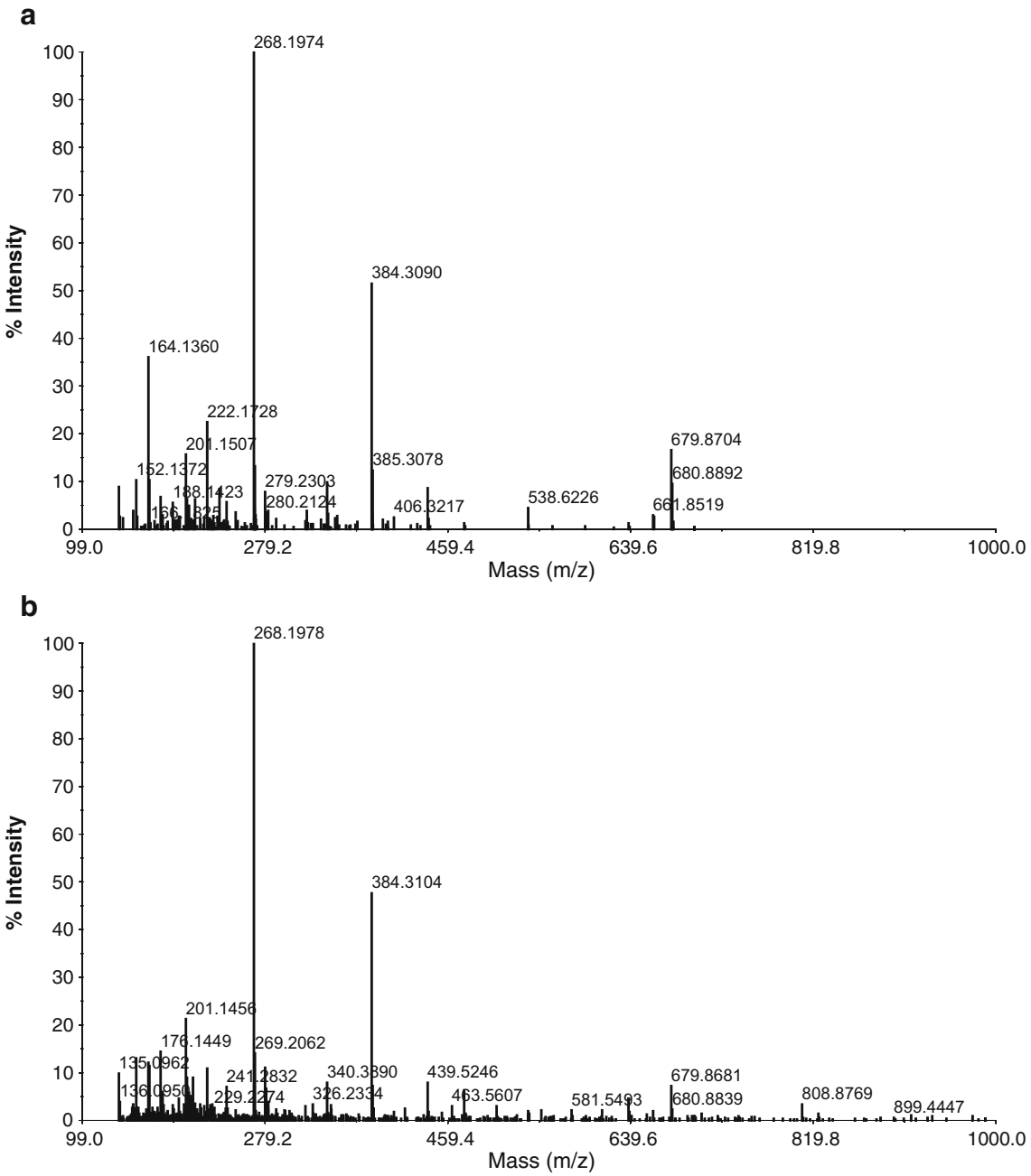

Fig. 8 Mass-spectral analysis of dG (5 mM) with methylglyoxal (20 mM) of HPLC peak 1 (**a**) and peak 2 (**b**) after a 3-day incubation at 75 °C

the incubation mixtures was increased from 6.0 to 7.2 and finally to 8.0 (Fig. 7).

Characterization of AGEs by mass spectrometry

In an attempt to elucidate the pathway for AGE formation, mass spectrometry was used to analyze each of the HPLC-resolved glycated products. Figure 8 shows the respective mass-spectral profiles of HPLC peaks 2 and 3 resulting from the elution of a mixture of dG (5 mM) with methylglyoxal (20 mM) incubated at 75 °C for 3 days (Fig. 4). The ion at *m*/*z* 340.39 in Fig. 8b is consistent with a $[\text{Schiff base}+\text{H}]^+$ molecule resulting from the condensation of dG (M_r 285.26) with methylglyoxal (M_r 70.06) in a dehydration reaction involving the loss of an H_2O molecule. The ion at *m*/*z* 679.87 in Fig. 8a and b is consistent with the formation of a $[\text{Schiff base}+\text{H}]^+$ dimer product. The ions at *m*/*z* 268.19 is speculated to result from the loss of a hydroxyl group from dG. The ion with *m*/*z* 384.31 is assumed to be a fragment formed by the degradation of methyglyoxal reacting with the Schiff base product, or its enaminol or Amadori intermediate. Figure 9 shows a

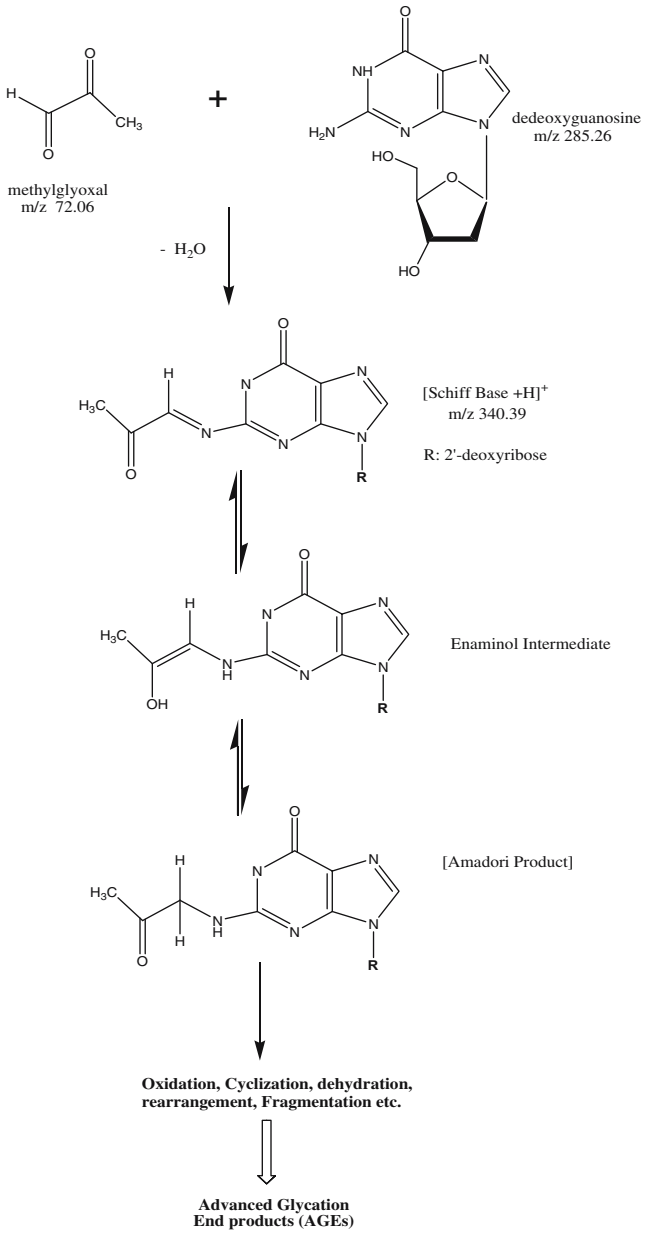

Fig. 9 Postulated mechanism pathway for the formation of AGEs from the reaction of dG with methylglyoxal

postulated mechanism pathway consistent with the mass-spectral data for the reaction of dG with methylglyoxal.

Discussion

The nonenzymatic glycation of proteins has been extensively studied, and the factors influencing the rate and extent of protein glycation by molecules such as D-glucose, D-galactose, and D/L-glyceraldehyde have been the topic of several reviews [15–17]. One reason for the interest in glycation reactions is due to the formation and accumulation of AGEs: molecules which have been shown to occur *in vivo* and to account for many of the chronic complications of diabetes. The occurrence of glycation reactions has been also implicated in the normal aging process and in the cross-linking of proteins, a phenomenon which has been shown to trigger cellular injury responses [18–20]. Additionally, protein glycation has been associated with β-amyloid deposits and the formation of neurofibrillary tangles in Alzheimer disease, and possibly other neurodegenerative diseases involving amyloidosis [21]. The occurrence of AGE-linked DNA has been demonstrated in the nuclei of epithelial cells, mesangial cells, and endothelial cells of the glomeruli in patients with diabetic nephropathy, suggesting that chronic hyperglycemia may induce a loss of genetic integrity in these patients and that the nuclei may serve as the major site of DNA glycation [22].

Our laboratory has recently focused on the glycation of nucleosides and has shown that many of the same factors influencing protein glycation can also influence the glycation of the purines and pyrimidines in DNA and RNA [2, 5, 11, 23]. Additionally, we demonstrated that dG was more reactive than dA, dC, and dT with D-galactose, D-glucose, or DL-glyceraldehyde serving as the model carbohydrates [2]. This increased susceptibility to sugar attack was affected by the concentration of reactants, ionic strength, temperature, and time of incubation [2, 23]. Our findings also revealed that AGEs of dG with glyceraldehyde exhibited a different HPLC profile relative to the AGEs of dG with glyoxal.

For a typical nonenzymatic glycation reaction to occur, the carbonyl group of a reducing sugar must first react with the free amino groups of a protein to form a Schiff base with the latter and the intermediate then undergoes an Amadori rearrangement to generate a stable ketoamine product. Our mass-spectroscopic data have shown that the reaction of dG with methylglyoxal proceeds via the classic Amadori pathway and yields glycation-like products similar to those generated between a nucleoside and a carbohydrate. Our data also revealed that alkaline conditions promoted AGE formation and caused the occurrence of new AGE products with time. AGE formation was also shown to proceed faster with increasing temperature and reactant concentrations, a finding verified by UV and fluorescence spectroscopy. Finally, our data showed that methylglyoxal was a more potent glycator of dG, a finding which was substantiated by comparing the HPLC elution profiles and the UV and fluorescence readings of mixtures containing dG with methylglyoxal and glyoxal.

The nonenzymatic reactions of nucleosides with sugar and sugarlike compounds such as glyoxal and methylglyoxal warrant further investigation, as these reactions may play an important role in the natural processes of aging and promote changes in the function and/or structure of DNA and RNA molecules.

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