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Nonenzymatic glycation of guanosine 5′-triphosphate by glyceraldehyde: An *in vitro* study of AGE formation

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Abstract

Guanosine 5′-triphosphate (GTP) plays a significant role in the bioenergetics, metabolism, and signaling of cells; consequently, any modifications to the structure of the molecule can have profound effects on a cell's survival and function. Previous studies in our laboratory demonstrated that like proteins, purines, and pyrimidines can nonenzymatically react with sugars to generate advanced glycation endproducts (AGEs) and that these AGEs can form *in vitro* under physiological conditions. The objective of this investigation was twofold. First, it was to evaluate the susceptibility of ATP, GTP, CTP, and TTP to nonenzymatic modification by D-glucose and DL-glyceraldehyde, and second to assess the effect of various factors such as temperature, pH and incubation time, and sugar concentration on the rate and extent of nucleotide triphosphate AGE formation. Of the four nucleotide triphosphates that were studied, only GTP was significantly reactive forming a heterogeneous group of compounds with DL-glyceraldehyde. D-Glucose exhibited no significant reactivity with any of the nucleotide triphosphates, a finding that was supported by UV and fluorescence spectroscopy. Capillary electrophoresis, high-performance liquid chromatography and mass spec-

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trometry allowed for a thorough analysis of the glycated GTP products and demonstrated that the modification of GTP by DL-glyceraldehyde occurred via the classical Amadori pathway.

Keywords: AGEs; Advanced glycation endproducts; Nonenzymatic glycation; GTP; Glyceraldehyde

1. Introduction

The free amino groups of bio-molecules can nonenzymatically react with the carbonyl groups of sugars to generate Amadori products that with time can rearrange to form a heterogeneous class of compounds referred as Maillard reaction products (MRP) or Advanced Glycation End Products (AGEs) [1]. Nonenzymatic glycation plays an important role in the pathophysiology of diabetes and studies have shown that the *in vivo* formation of AGEs may exacerbate such conditions as Alzheimer's disease, cataract formation, renal dysfunction, and atherosclerosis [2–6].

Recent studies in our laboratory demonstrated that sugars can nonenzymatically react with purine and pyrimidine bases in DNA and alter the structure and conformation of DNA molecules [7,8]. The studies also revealed that nucleosides were susceptible to nonenzymatic modification by sugars and that the rate and extent of glycation depended on the structure and chemistry of the reacting nucleoside and carbohydrate [9]. More importantly, the observation was made that many of the same factors influencing protein AGE formation also contributed to AGE formation by DNA nucleobases [7,9,10].

The objective of this study was to evaluate the nonenzymatic reactivity of ATP, GTP, CTP, and TTP with D-glucose and DL-glyceraldehyde and to assess the factors influencing their AGE formation. Why focus on the glycation of nucleotide triphosphates and more specifically, on the nonenzymatic modification of these molecules by D-glucose or DL-glyceraldehyde? Nucleotide triphosphates play a significant role in the bioenergetics of a cell. They serve as substrate for many key enzymes and as major reactants in several biochemical pathways. D-Glucose and DL-glyceraldehyde were selected as the model glycating sugars because each was reported to nonenzymatically react with proteins and because of the respective role D-glucose and DL-glyceraldehyde play in diabetes and the fructose pathway [10]. This report demonstrates that GTP can be glycated with DL-glyceraldehyde to form AGE products through the classical Amadori pathway.

2. Materials and methods

2.1. Chemicals and supplies

Ribose based purine and pyrimidine triphosphates (i.e., adenosine 5′-triphosphate, guanosine 5′-triphosphate, cytidine 5′-triphosphate, thymidine 5′-triphosphate), DL-glyceraldehyde, D-glucose, sodium tetraborate, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other reagents and solvents for HPLC and CE were of analytical grade and were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA).

2.2. Preparation of buffers and reaction mixtures

Unless indicated, all *in vitro* reactions were conducted in 0.2 M phosphate buffer pH 7.2 containing 0.02% sodium azide. Stock solutions of sugar (D-glucose or DL-glyceraldehyde) and different nucleotide triphosphates were prepared by separately adding 40 mM sugar or 10 mM of adenosine 5′-triphosphate, guanosine 5′-triphosphate, cytidine 5′-triphosphate, or thymidine 5′-triphosphate to the 0.2 M phosphate buffer and then vortexing each solution until all materials were dissolved. Reaction mixtures containing varying amounts of sugar (2.5, 5, and 20 mM) and different nucleotide triphosphates (5 mM) were then prepared from each of the stock solutions. Controls included either 2.5, 5 or 20 mM of each sugar alone or 5 mM of each of the nucleotide triphosphates only. 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 then frozen at −20 °C until their analysis.

2.3. UV and fluorescence spectroscopy

The acquisition of UV spectra was performed at a wavelength of 260 nm using a Beckman DU 800 spectrophotometer (Fullerton, CA, USA) that was equipped with a cuvette thermoelectric controller. Fluorescence emission spectroscopy was performed with a PerkinElmer LS 55 luminescence spectrometer (Shelton, CT, USA) that was equipped with a thermal cell that maintained the samples at 25 ± 1 °C. All samples were analyzed at an excitation and an emission wavelength of 370 and 420 nm, respectively.

2.4. High-performance liquid chromatography (HPLC)

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 auto-sampler (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 20 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. All HPLC runs were repeated three times to ensure reproducibility of the data and peak retention times.

2.5. Electrospray ionization mass spectrometry (ESI-MS)

An orthogonal time-of-flight (TOF) mass spectrometer (Applied Biosystems Mariner API-TOF Workstation, Framingham, MA, USA) equipped with standard electrospray ionization (ESI) source was used. The mass spectral data was 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 4000 V. The instrument was outfitted with an integrated syringe pump and with a dual syringe rack for direct infusion onto the mass spectrometer. The MS system was operated in full scan (m/z 100–1000). Spectral acquisition was performed every 2 s and a total of 10 spectra were accumulated. All ions measured were in the $[M + H]^+$ form.

2.6. Capillary electrophoresis (CE)

CE analyses were performed at ambient temperature on a Quanta 4000 CE System (Waters Company, Milford, MA, USA) equipped with a UV detector and a silica capillary that had an internal diameter of 50 μm, external diameter of 375 μm and a length of 60 cm (Polymicro Technologies, Phoenix, AZ, USA). Prior to use, the capillary was conditioned by flushing for 15 min with 1 N NaOH, 10 min with Milli-Q water and thereafter for 5 min with a tetraborate buffer, pH 9.2 (hereinafter "running buffer"). In between each run, the capillary was rinsed with 1 N NaOH for 1 min and then by the running buffer for 2 min. Acetone was used as a neutral marker with a consistent retention time of 5.78 min.

Samples were applied at the anionic end to the capillary with a hydrodynamic injector for 60 s. Electropherograms were monitored at 214 nm and data was analyzed by an integrator system (Spectra Physics Integrator, San Jose, CA, USA). All CE runs were repeated three times to ensure reproducibility of the data and the peak retention times.

3. Results

3.1. UV–visible and fluorescence spectroscopy

Preliminary studies using a combination of analytical techniques revealed that of all the nucleotide triphosphates (i.e., ATP, CTP, GTP, and TTP) and sugars that were evaluated only GTP and DL-glyceraldehyde (GA) were reactive. The results described herein will henceforth focus primarily on the reaction of GA with GTP.

Table 1 show the respective fluorescence and UV absorbance results that were observed when GA and GTP were reacted at 37 °C for 0, 1, 3, 5, and 10 days. The fluorescence experiments revealed that increases in fluorescence (370/420 nm) intensity directly related to increases in GA concentration and incubation time, a finding that we observed earlier when evaluating the reactivity of GA with various deoxynucleobases and proteins [7,10]. ATP and CTP incubated for 10 days at 37 °C exhibited no significant reactivity with GA or with D-glucose (data not shown). The absence of AGE products in incubation mixtures containing GA and TTP was not surprising as TTP lacks a free amino group which prevents the molecule from participating in glycation reactions.

Table 1
The effect of increasing GA concentration in the presence of constant amounts of GTP (5 mM) at 37 °C for 10 days

Time (day)	GA (2.5 mM)		GA (5 mM)		GA (20 mM)	
	Fluorescence	UV	Fluorescence	UV	Fluorescence	UV
0	6.349 ± 0.107	0.120 ± 0.001	6.599 ± 0.102	0.123 ± 0.009	6.799 ± 0.199	0.152 ± 0.001
1	9.233 ± 0.051	0.124 ± 0.003	9.320 ± 0.280	0.320 ± 0.002	10.235 ± 0.301	1.065 ± 0.004
3	9.488 ± 0.307	0.132 ± 0.002	9.839 ± 0.198	0.314 ± 0.001	11.398 ± 0.147	1.267 ± 0.003
5	9.392 ± 0.298	0.136 ± 0.002	10.261 ± 0.088	0.323 ± 0.002	12.880 ± 0.309	1.282 ± 0.004
10	9.938 ± 0.176	0.140 ± 0.003	10.474 ± 0.177	0.320 ± 0.001	14.764 ± 0.444	1.304 ± 0.004

All samples were measured in duplicate and were diluted 1:20 before testing. Fluorescence spectral profiles were measured at excitation and emission wave lengths of 370 and 420 nm, respectively. UV spectral profiles were measured at 260 nm. The spectroscopic data for every measurement was no larger than 10% of its counterpart duplicate reading.

The UV absorbance readings (Table 1) confirmed the fluorescence findings that the extent of AGE formation was dependent on both GA concentration and incubation time. Nevertheless, the fluorescence intensities at each of the GA concentrations were significantly more pronounced than the UV readings, a finding which we speculate to be due to the inherent differences between the two methods. The controls with GA alone or GTP alone exhibited no significant increase in fluorescence and UV absorbance readings confirming that the formation of AGEs was strictly due to the nonenzymatic reactions between GA and GTP.

Table 2 show the fluorescence and UV absorbance profiles of GA (20 mM) incubated with GTP (5 mM) at 50 and 75 °C for 5 days. Increases in temperature caused increases in the rate of AGE formation, a finding that was supported by both the fluorescence and UV data (Table 2). Table 3 demonstrates the fluorescence and UV profiles of GA (20 mM) with GTP (5 mM) at pH 6 and pH 8, respectively. As the pH shifted from acid to alkaline conditions, there was an increase in the fluorescence and UV readings. This observation indicated the formation of AGEs and supported the notion that the occurrence of AGEs proceeded via Schiff base intermediate products, especially since Schiff base reactions are favored at pH conditions above neutrality (see Table 3). Increases in the yield of fluorescence and UV absorbing products resulting from shifts in pH (i.e., from acid to alkaline conditions) were also reported to occur with proteins in the presence of D-glucose and D-galactose [11].

Table 2
The effect of temperature on the formation of AGEs

Time (day)	37 °C		50 °C		75 °C	
	Fluorescence	UV	Fluorescence	UV	Fluorescence	UV
0	6.799 ± 0.199	0.152 ± 0.001	6.799 ± 0.199	0.152 ± 0.001	6.799 ± 0.199	0.152 ± 0.001
1	10.235 ± 0.301	1.065 ± 0.004	16.860 ± 0.494	1.156 ± 0.000	67.292 ± 0.446	1.355 ± 0.005
3	11.398 ± 0.147	1.267 ± 0.003	36.425 ± 0.593	1.303 ± 0.004	78.398 ± 1.353	1.573 ± 0.005
5	12.B80 ± 0.309	1.282 ± 0.004	43.045 ± 0.296	1.330 ± 0.005	87.278 ± 0.710	1.770 ± 0.000

All samples were measured in duplicate and were diluted 1:20 before testing. Fluorescence spectral profiles were measured at excitation and emission wave lengths of 370 and 420 nm, respectively. UV spectral profiles were measured at 260 nm. The spectroscopic data for every measurement was no larger than 10% of its counterpart duplicate reading.

Table 3
The effect of pH on the formation of AGEs

Time (day)	pH (6.0)		pH (7.2)		pH (8.0)	
	Fluorescence	UV	Fluorescence	UV	Fluorescence	UV
0	6.463 ± 0.107	0.152 ± 0.001	6.799 ± 0.108	0.152 ± 0.001	6.849 ± 0.129	0.152 ± 0.001
1	6.809 ± 0.249	0.603 ± 0.004	10.235 ± 0.301	1.065 ± 0.004	10.715 ± 0.311	1.355 ± 0.005
3	8.966 ± 0.361	1.118 ± 0.003	11.398 ± 0.147	1.267 ± 0.003	22.460 ± 0.284	1.573 ± 0.005
5	11.198 ± 0.428	1.230 ± 0.001	12.880 ± 0.309	1.282 ± 0.004	32.392 ± 0.351	1.770 ± 0.000

All samples were measured in duplicate and were diluted 1:20 before testing. Fluorescence spectral profiles were measured at excitation and emission wave lengths of 370 and 420 nm, respectively. UV spectral profiles were measured at 260 nm. The spectroscopic data for every measurement was no larger than 10% of its counterpart duplicate reading.

Other experiments with GA and GTP demonstrated that ionic salts such as sodium chloride and sodium bicarbonate (i.e., up to 200 mM) had no appreciable effect on the rate of AGE formation (data not shown).

3.2. HPLC and mass spectrometry analysis

Fig. 1a–d show the HPLC elution profiles of GA (20 mM) with GTP (5 mM) after initial mixing and after 5 days of incubation at 37, 50, and 75 °C, respectively. The emergence of three prominent peaks (2, 3, and 4) with retention times of 2.13, 2.37, and 7.46 min in Fig. 4b revealed the increases in the heterogeneity of AGE species with time. Under the same elution conditions, control solutions containing either GA alone or GTP alone yielded only a single peak. This peak emerged 1.73 min after resolving each of the controls by HPLC (data not shown). Figs. 1c and d depict the HPLC elution profiles of GA–GTP mixtures at temperatures above 37 °C. The emergence of new peaks (peak 5 and 6) and the increases in the intensity of previous peaks (peak 2 and 3) demonstrates the important role which temperature plays in the formation of AGEs. Other HPLC studies revealed that increases in pH promoted AGE formation and that the progressive raise in the pH of incubation mixtures (i.e., containing GA and GTP) from pH 6 to pH 8 incited an increase in the heterogeneity of AGEs.

Fig. 2a and b show the mass spectral profiles of two HPLC peaks, (i) the peak that corresponded to the freshly prepared mixture of GA with GTP (peak 1 in Fig. 1a), and (ii) the peak that corresponded to mixtures of GA with GTP that were incubated at 37 °C for 5 days (peak 4 in Fig. 1b). The peak at $m/z = 546.7$ in Fig. 2a is consistent with $[GTP + Na]^+$ and the peak at $m/z = 637.7$ in Fig. 2b is consistent with the formation of a Schiff base whose structure is depicted as the $[\text{Amadori Product} + K + 4H]^{5+}$ in Fig. 3. The fragments with m/z at 538.64, 439.50, and 340.39 are speculated to result from the progressive dephosphorylation of the $[\text{Amadori Product} + K + 4H]^{5+}$ characterized by a loss of $m/z = 99.10$ $[H_3PO_4 + H]^+$ (Fig. 3). A further study of the mass spectrum in Fig. 2b suggest that the peak at $m/z = 241.27$ was consistent with a hydrated Schiff base derivative.

3.3. CE analysis

Fig. 4a–d show the CE profiles of GTP (5 mM) with GA (20 mM) following their immediate mixing and after 5-days of their incubation with each other at 37, 50, and 75 °C, respectively. CE analysis of the control solution containing GTP alone (data not shown) yielded a similar elution profile as the immediately mixed solutions of GTP with GA (Fig. 4a), a finding which suggests that the prominent peak in Fig. Fig. 4a appears to be due to the presence of GTP. The GA–GTP mixture at 37 °C was resolved in three major peaks with retention times at 12.40, 13.04, and 14.34 min (Fig. 4b), respectively. At 50 °C there was an increase in the heterogeneity of the AGE products and the CE elution profile showed four major peaks with retention times at 13.09, 14.50, 14.77, and 15.66 min, respectively (Fig. 4c). At 75 °C, five major peaks were observed (retention time = 13.48, 15.05, 16.48, 16.88, and 18.53 min) supporting the UV and fluorescence data that temperature had a positive effect on the formation of AGEs, a finding that was also substantiated by HPLC analysis (see Fig. 1b–d).

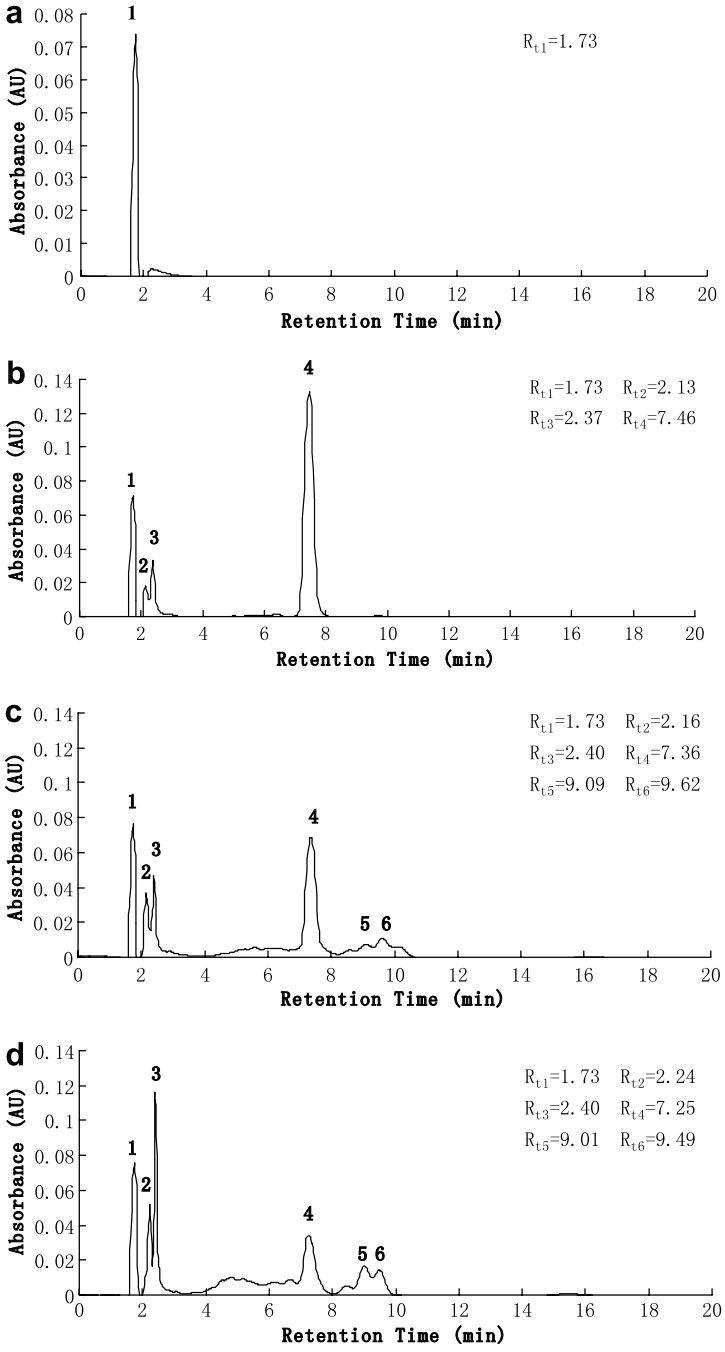

Fig. 1. HPLC elution profiles of GTP (5 mM) with GA (20 mM) after initial mixing (a), at 37 °C (b) at 50 °C (c), and at 75 °C (d) after 5 days of incubation, respectively. Repeat chromatographic studies by HPLC revealed no significant differences in the reproducibility of the data and in the elution profile and the retention times of the AGE peaks.

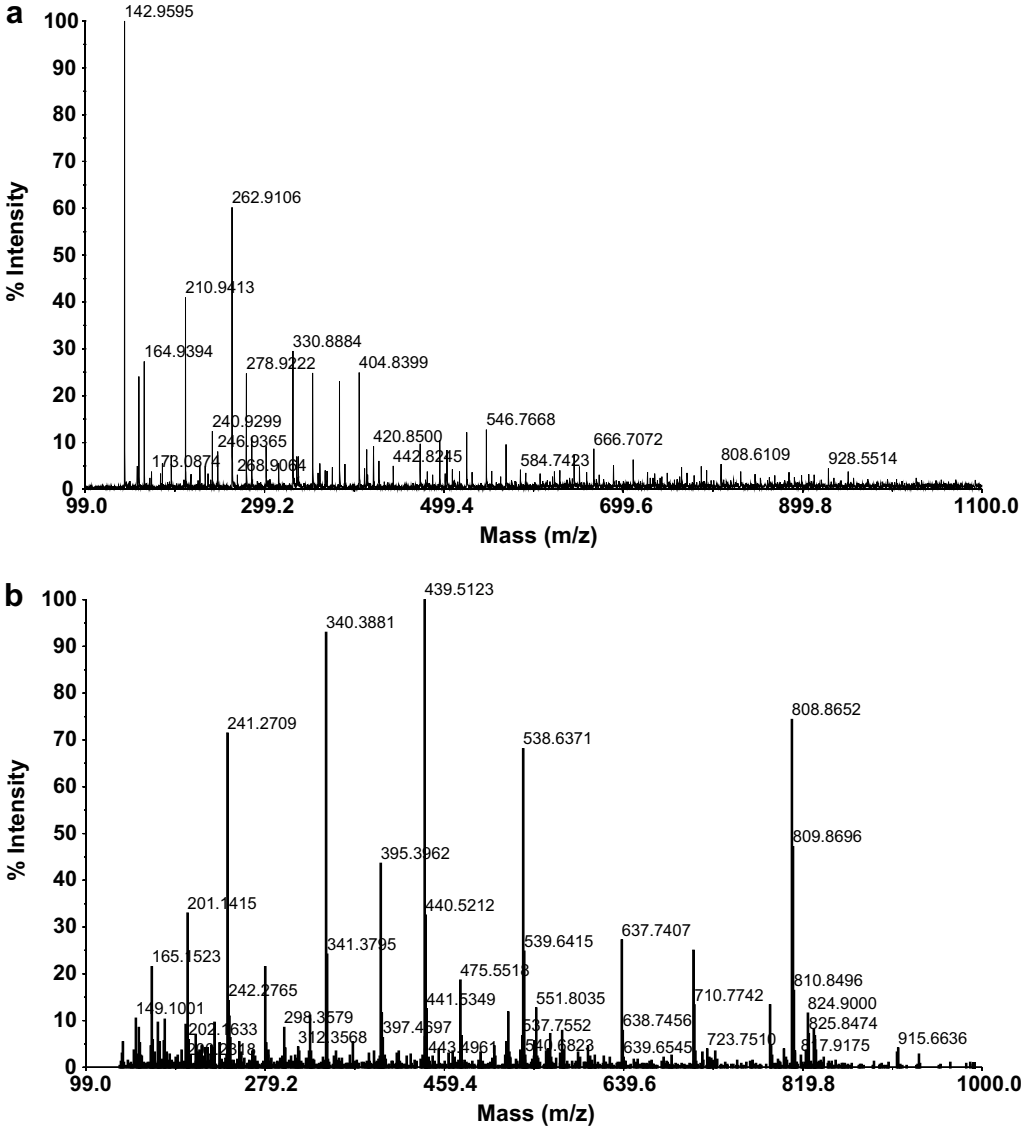

Fig. 2. Mass spectral analysis of a GA–GTP mixture following HPLC separation (a) freshly prepared mixture of GA with GTP, and (b) mixture of GA with GTP that was incubated at 37 °C for 5 days.

Additional CE studies revealed that similar to the temperature experiments, increases in pH promoted AGE formation and caused the formation of new AGE species in solutions that contained GA with GTP (data not shown). Other CE profiles demonstrated that the control tubes containing GA or GTP alone did not show any deterioration or decomposition over time and under varying temperature and pH conditions.

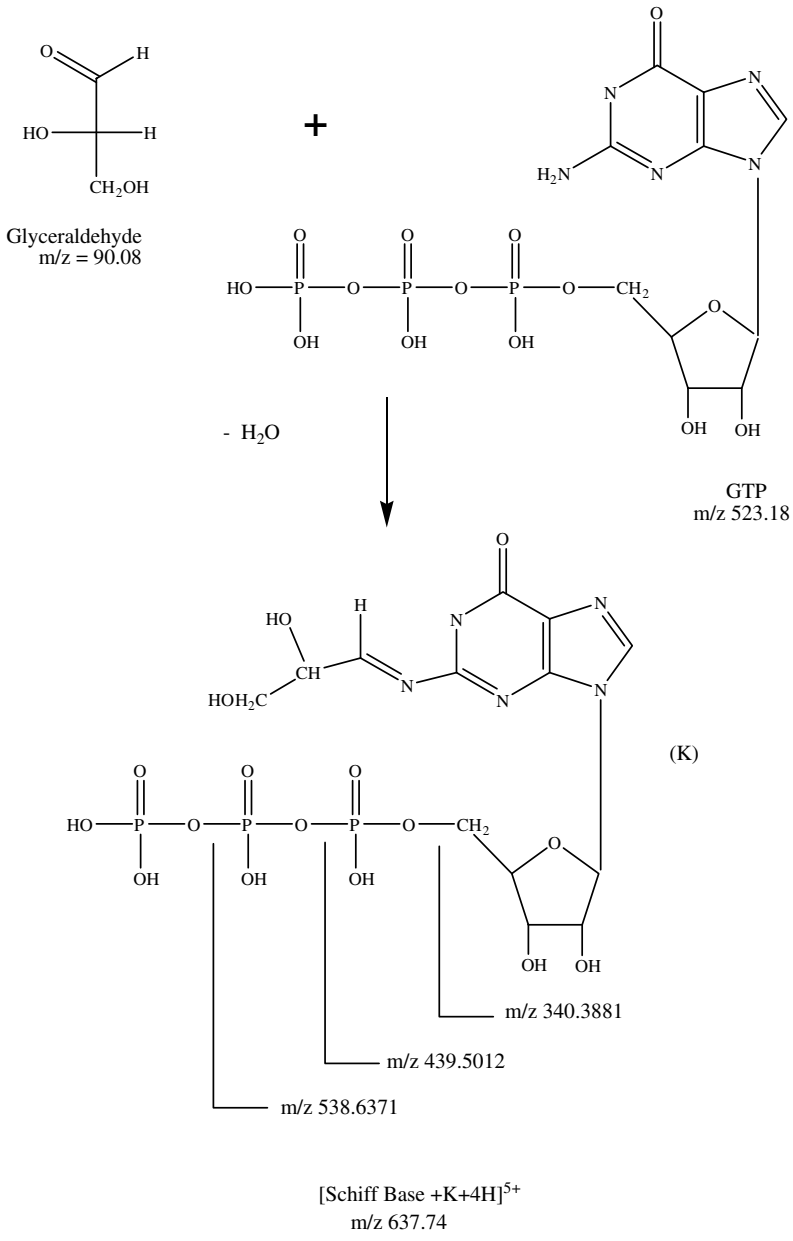

Fig. 3. Postulated mechanism pathway for Amadori product formation of a GA–GTP reaction mixture.

4. Discussion

The nonenzymatic reaction of reducing sugars with macromolecules such as proteins, and DNA has been the subject of several reviews and what makes this area interesting is that glycation reactions can occur *in vivo* accounting for many of the chronic complica-

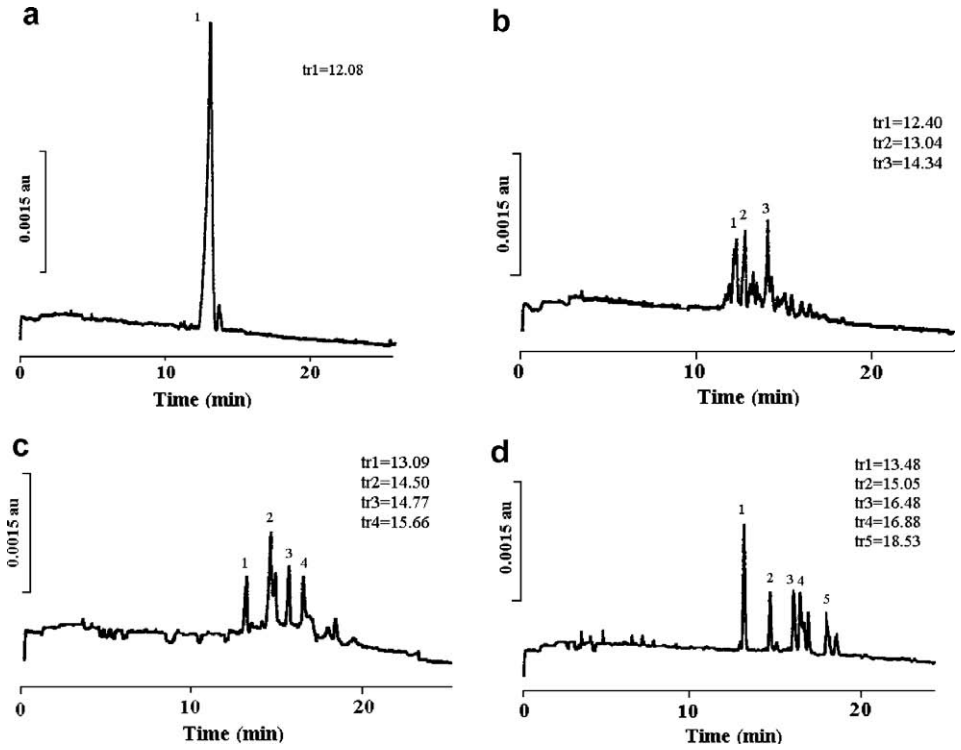

Fig. 4. Capillary electropherograms of GTP (5 mM) and GA (20 mM) after initial mixing (a), at 37 °C (b), at 50 °C (c), and at 75 °C (d) after 5 days of incubation, respectively. Repeat electrophoretic studies by CE revealed no significant differences in the reproducibility of the data and in the elution profile and the retention times of the AGE peaks.

tions in diabetes and other disorders of carbohydrate metabolism [12–15]. The formation of AGEs occurs via a number of complex pathways and the process has been speculated to also play a role in 'diabetes induced early aging' and to account for changes in structural proteins such as type IV collagen, laminin, and fibronectin [16,17]. The occurrence of AGE-linked DNA has been demonstrated predominantly 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 [18]. In contrast to DNA, RNA molecules have a shorter half-life; consequently, we speculate that the extent in RNA glycation to be generally less pronounced than DNA in patients with diabetes.

Recent studies in our laboratory demonstrated that DL-glyceraldehyde is a more effective glycating agent than D-glucose and that the molecule can readily react with proteins, purines, and pyrimidines to form AGEs [9,10]. The higher reactivity of DL-glyceraldehyde relative to D-glucose was also demonstrated in this study with GTP and we speculate it to be caused by GA existing in the open chain reactive free aldehyde form in solution. This is in contrast to D-glucose which when in solution predominantly exists in the form of a hemiacetal ring with little in the free open chain reactive aldehyde. The greater suscepti-

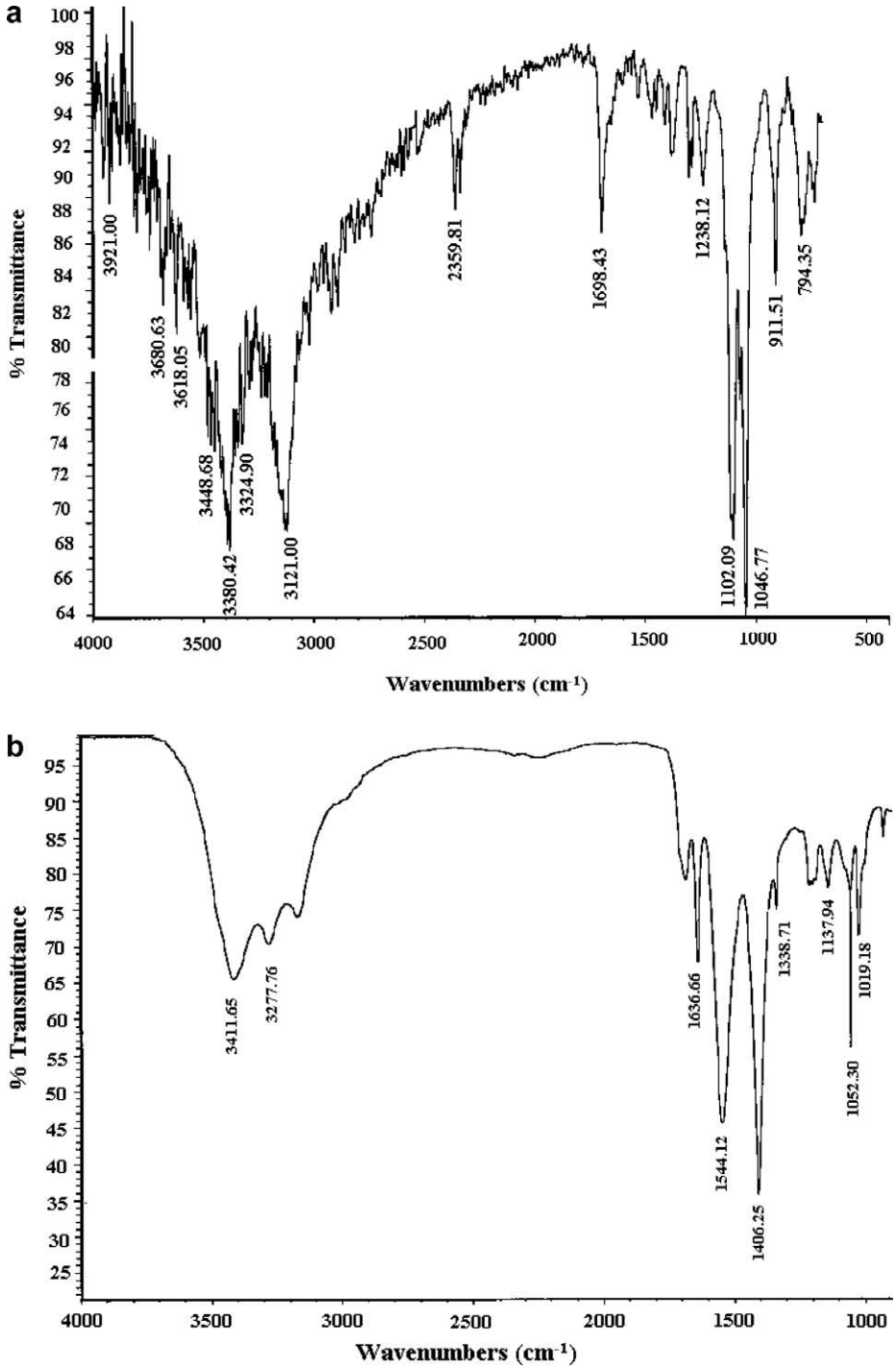

Fig. 5. Infrared spectral analysis of a freshly prepared GA (20 mM) and GTP (5 mM) mixture (a) and after incubation of the mixture for 5 days at 37 °C (b).

bility of GTP to nonenzymatic glycation may be also attributed to its lower oxidation potential, a condition which can predispose a RNA molecule rich in guanine residues, to sugar attack [19–21].

In a previous study, focusing on the nucleosides of DNA, our laboratory demonstrated that deoxyguanosine was more reactive than deoxyadenosine, deoxycytidine, and deoxythymidine in its reaction with D-galactose, D-glucose, or DL-glyceraldehyde [9]. This increased susceptibility to sugar attack was found to be affected by several factors, of which the most important included the concentration of reactants, ionic strength, temperature, and time of incubation [9]. Our findings also demonstrated that the AGEs of deoxyguanosine with glyceraldehyde exhibited a different HPLC profile relative to the AGEs of GTP with glyceraldehyde, a finding that we speculate to have resulted from the presence of ribose and the phosphate groups on GTP.

Fig. 5a and b show the respective infrared spectral analysis of a freshly prepared GA and GTP mixture, and of the same solution after 5 days at 37 °C. The disappearance of the carbonyl band at 1698 cm^{-1} clearly demonstrates the involvement of the carbonyl groups of GA in nonenzymatic reactions. Other findings revealed that that extent in the heterogeneity of AGEs was dependent on temperature and pH of the incubation mixtures and on the concentration of GA and GTP.

The reaction of sugars with GTP warrants further investigation as these reactions may occur *in vivo* affecting such functions as cGMP formation, signal transduction, RNA, and protein synthesis.

Acknowledgments

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