Triosidines: novel Maillard reaction products and cross-links from the reaction of triose sugars with lysine and arginine residues

Frederic J. TESSIER^{*1}, Vincent M. MONNIER[†], Lawrence M. SAYRE[‡] and Julia A. KORNFIELD^{*‡}

^{*}Department of Chemical Engineering, California Institute of Technology, 1200 East Colarado Blvd, Pasadena, CA 91125, U.S.A., [†]Departments of Pathology and Biochemistry, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44120, U.S.A., and [‡]Department of Chemistry, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44120, U.S.A.

The role of the highly reactive triose sugars glyceraldehyde and glyceraldehyde-3-phosphate in protein cross-linking and other amino acid modifications during the Maillard reaction was investigated. From the incubation of glyceraldehyde with *N*^ε-acetyl-L-lysine and *N*^ε-acetyl-L-arginine, we isolated four new Maillard reaction pyridinium compounds named ‘triosidines’. Two of them, ‘lys-hydroxy-triosidine’ {1-(5-amino-5-carboxypentyl)-3-[(5-amino-5-carboxypentylamino)methyl]-5-hydroxypyridinium} and ‘arg-hydroxy-triosidine’ {2-(4-amino-4-carboxybutylamino)-8-(5-amino-5-carboxypentyl)-6-hydroxy-3,4-dihydro-pyrido[2,3-*d*]pyrimidin-8-ium} are fluorescent, UV-active Lys–Lys and Lys–Arg cross-links respectively. Their structures were identified by NMR and MS. In addition, two UV-active lysine adducts, ‘trihydroxy-triosidine’ [1-(5-amino-5-carboxypentyl)-3,4-dihydroxy-5-(hydroxymethyl)pyridinium] and ‘triosidine carbaldehyde’ [1-(5-amino-5-carboxypentyl)-3-formylpyridinium] were tentatively identified by MS. All structures involve six sugar-derived carbons as part of the

heterocyclic ring. Of the two novel cross-links, only arg-hydroxy-triosidine was formed by glyceraldehyde-3-phosphate, an intermediate metabolite of the glycolytic pathway. Lys-hydroxy-triosidine and arg-hydroxy-triosidine were detected in human and porcine corneas treated with glyceraldehyde. The HPLC-fluorescence identification was confirmed by MS. Triosidines were also formed from dihydroxyacetone, a widely used artificial sun-tanning agent. Triosidines are expected to be useful tools in tissue engineering, where the utilization of highly reactive sugars is needed to stabilize the loose matrix. In addition, they are expected to be present in selected biological conditions, such as on consumption of a high fructose diet, and syndromes associated with high glyceraldehyde excretion, such as Fanconi Syndrome, fructose-1,6-diphosphatase deficiency and tyrosinaemia.

Key words: dihydroxyacetone, glycation, glyceraldehyde, glyceraldehyde-3-phosphate, tissue engineering.

INTRODUCTION

The Maillard reaction [1] is a series of non-enzymic reactions that lead to the modification of proteins in the presence of reducing sugars. The major consequence of the Maillard reaction is the formation of covalently cross-linked [2–6] and non-cross-linked [7–10] amino acids, many of which are chromophores or fluorophores. The reaction is initiated by non-enzymic glycosylation, also called early-glycation, between primary amino groups in proteins and reducing sugars to form the reducing ‘Amadori product’. The Amadori product undergoes a further series of reactions to form stable molecules, called advanced glycation end-products (AGEs). Among other matrix proteins, collagen has a long biological half-life, which explains the observed accumulation of AGEs during normal aging [2,11,12]. It has been proposed that the gradual structural and functional modification of protein contributes to the pathophysiology of aging and diabetes in which sugar levels are elevated [13].

In recent years, it was demonstrated that AGEs are formed not only from glucose, but also from dicarbonyl compounds and short chain sugars, such as glycolaldehyde. Sugar reactivity was found to be proportional to the percentage of sugar present in the open chain form, i.e. inversely proportional to the length of

the carbon chain of the sugar [14]. Although considerable progress has been achieved in our understanding of AGE structures derived from hexoses, pentoses, threose and oxoaldehydes [9,15,16,17], relatively little attention has been given so far to triose-derived structures. Previous studies have shown that glyceraldehyde, a key intermediate of fructose metabolism [18], is a potent protein cross-linking agent, and a precursor for arg-pyrimidine [9,19]. Its phosphorylated form is also a source of *N*^ε-(carboxymethyl)lysine and *N*^ε-(carboxyethyl)lysine [8].

Glyceraldehyde has been found to be potentially useful for several biological applications. For instance, it was used both as an anti-cancer [20] and anti-sickling agent [21]. More recently, based on the work of Girton et al. [22], it is also conceivable that, like glucose and ribose, glyceraldehyde might be used to increase tissue stiffening for tissue engineering purposes. One company, for example, has patented a process utilizing glyceraldehyde for the treatment of keratoconus, a disease characterized by severe laxity of the corneal stroma (www.kcenter.org/past_articles/2.html). Finally, owing to its high reactivity, glyceraldehyde is likely to be a useful tool for the *in vitro* and *in vivo* mapping of protein cross-links.

In the present study we report on the structure and mechanism of formation of three novel AGEs that are formed from the

Abbreviations used: acetyl-arginine, *N*^ε-acetyl-L-arginine; acetyl-lysine, *N*^ε-acetyl-L-lysine; AGE, advanced glycation end-product; CBZ-lysine, benzyloxycarbonyl- α -L-lysine; DHA, 1,3-dihydroxyacetone; DHAP, 1,3-dihydroxyacetone phosphate; DTPA, diethylenetriaminepenta-acetic acid; MS/MS, tandem MS; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail fred01@caltech.edu).

reaction between lysine and glyceraldehyde, and between lysine, arginine and glyceraldehyde. We have also determined their formation rates in corneas treated with glyceraldehyde *in vitro*.

EXPERIMENTAL

Materials

All reagents were of the highest grade available. *N*^ε-Acetyl-L-lysine (acetyl-lysine), *N*^ε-acetyl-L-arginine (acetyl-arginine), *N*^ε-benzyloxycarbonyl-L-lysine (CBZ-lysine), glycolaldehyde, glyoxal, methylglyoxal, DL-glyceraldehyde (95% pure), glyceraldehyde-3-phosphate, threose, D-xylose, D-ribose, D-fructose, D-glucose, collagen type I from bovine achilles tendon, BSA and diethylenetriaminepenta-acetic acid (DTPA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [1-¹³C]Glyceraldehyde (99%) and [1,2,3-¹³C]glyceraldehyde (99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Hydrochloric acid was from Fisher Scientific Optima (Fair Lawn, NJ, U.S.A.). Human corneas were obtained from the Cincinnati Eye Bank (Cincinnati, OH, U.S.A.). Porcine eyes were obtained from Sierra Medical Science (Santa Fe Springs, CA, U.S.A.). Argpyrimidine standard was kindly donated by Dr M. Glomb (Institute for Food Chemistry, Technical University of Berlin, Germany).

Incubation of CBZ-lysine and glyceraldehyde with or without *N*^ε-acetyl-L-lysine

CBZ-lysine (50 mM) or CBZ-lysine plus acetyl-lysine (each 25 mM) were incubated with glyceraldehyde (30 mM) in 200 mM phosphate buffer (pH 7.4), 1 mM DTPA with 25% (v/v) aqueous methanol for 4 days at 37 °C.

Reaction of glyceraldehyde with *N*^ε-acetyl-L-lysine with or without *N*^ε-acetyl-L-arginine

Acetyl-lysine (55 mM) in the presence or absence of acetyl-arginine (27.5 mM) was incubated with glyceraldehyde (27.5 mM) at 37 °C in 200 mM phosphate buffer (pH 7.4), 1 mM DTPA with or without 25% (v/v) aqueous methanol for 50 days.

Incubation of α-amino-protected lysine and arginine with glyceraldehyde at three different pHs

CBZ-lysine (59 mM) was incubated with glyceraldehyde (42 mM) at 37 °C in 200 mM phosphate buffer (pH 5, 7.4 and 10.5), 1 mM DTPA with 25% (v/v) aqueous methanol for 4 and 15 days. A mixture of acetyl-lysine and acetyl-arginine (each 55 mM) was incubated with glyceraldehyde (41.2 mM) at 37 °C in 200 mM phosphate buffer (pH 5, 7.4 and 10.5), 1 mM DTPA with 25% (v/v) aqueous methanol for 4 and 15 days. Samples were kept frozen at -80 °C until required for HPLC analysis.

Reaction of 99% [1,2,3-¹³C]glyceraldehyde with *N*^ε-acetyl-L-lysine and *N*^ε-acetyl-L-arginine

Acetyl-lysine (25 mM) and acetyl-L-arginine (25 mM) were co-incubated with 99% [1,2,3-¹³C]glyceraldehyde (25 mM) at 37 °C in 200 mM phosphate buffer (pH 7.4), 1 mM DTPA with 25% (v/v) aqueous methanol for 4 days.

HPLC

A Waters HPLC system, including an in-line degasser, a 600 controller, a 717 autosampler, a 996 photodiode array detector, a 474 scanning fluorescence detector (monitored at 380 nm with

an excitation wavelength of 330 nm), and Millennium software (Waters Corporation, Milford, MA, U.S.A.) were used. Separations were made on a 250 mm × 4.6 mm Discovery C18 column, 3 μm (Supelco, Bellefonte, PA, U.S.A.) protected by a 20 mm × 4.0 mm Supelguard Discovery C18 column, 5 μm. Peak collection was performed using a FRAC-100 fraction collector (Amersham Biosciences). Acetyl-lysine incubated samples, acid hydrolysates and human or animal tissues were analysed using a linear gradient of 5–30% acetonitrile from 0 to 25 min with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min (gradient A). CBZ-lysine-incubated samples were analysed with a linear gradient of 20–52% acetonitrile from 0 to 35 min with 0.1% TFA at the same flow rate. Each sample was filtered with a 0.45 μm cellulose acetate filter (Costar Spin-X, Corning Incorporated, NY, U.S.A.) prior to injection into the HPLC system.

Synthesis and purification of peaks 1 and 2 (Figure 1) from a synthetic system

Acetyl-lysine (118 mM) was incubated with glyceraldehyde (110 mM) at 37 °C in 200 mM phosphate buffer (pH 10.5), 1 mM DTPA with 25% (v/v) aqueous methanol for 9 days. Fresh glyceraldehyde, 65 and 49 mg, was added to the incubation mixture (to a total volume of 9 ml) after 3 and 6 days respectively [4]. The pH was checked at the end of the incubation time. Peaks 1 and 2 were isolated following repetitive injection of incubated acetyl-lysine (140 injections of 0.78 mg; 110 mg total) into the HPLC system described above, using the same solvents as before with a linear gradient of 10–30% acetonitrile from 0 to 25 min. Both peaks were collected, evaporated in a Speed-Vac concentrator (Savant Instruments, NY, U.S.A.) and kept frozen at -80 °C until NMR analysis. An aliquot of peak 2 was acid hydrolysed in 0.5 ml of 6 M HCl for 16 h at 110 °C, evaporated and purified by the same HPLC system for mass analysis. A minor second product of acid hydrolysis was detected and collected. In addition, the compound in peak 2 was also synthesized with [1-¹³C]glyceraldehyde using the same protocol.

Synthesis and purification of peak 3 (Figure 1) from a synthetic system

Acetyl-lysine (55 mM) and acetyl-arginine (55 mM) were incubated with glyceraldehyde (55 mM) at 37 °C in 200 mM phosphate buffer (pH 10.5), 1 mM DTPA with 25% (v/v) aqueous methanol for 6 days. Glyceraldehyde (40 mg) was added to the solution (8 ml) after 3 days to compensate for degradation. The pH was readjusted to 10.5 after 3 days by addition of 10 M NaOH. Peak 3 was purified from 110 mg of incubated acetyl-lysine, with acetyl-arginine injected into the HPLC system using the same solvents as before with a linear gradient of 5–25% acetonitrile from 0 to 20 min. Peak 3 was collected, evaporated and acid hydrolysed in 0.5 ml of 6 M HCl for 16 h. The acid was evaporated and peak 3 was purified by the same HPLC system. Peak 3 and a second fluorescent peak were collected. Both adducts were evaporated and kept at -80 °C until structural analysis. In addition, the compound in peak 3 was also synthesized with [1-¹³C]glyceraldehyde using the same protocol.

Synthesis and purification of acetyl-peak 4 from a synthetic system

Acetyl-lysine (55 mM) was incubated with glyceraldehyde (55 mM) at 37 °C in 200 mM phosphate buffer (pH 5), 1 mM DTPA with 25% (v/v) aqueous methanol for 12 days. Fresh glyceraldehyde aliquots, i.e. 81, 63 and 45 mg, were added to the

Figure 1 Two dimensional and single C-18 HPLC profiles from incubation mixtures of *N*²-acetyl-L-lysine and glyceraldehyde with (A, B and C) or without (D and E) *N*²-acetyl-L-arginine

Detection was monitored with a photodiode array detector, with a full spectrum (A), absorption at 260 nm (B and D), and with a fluorescence detector at wavelengths of 330 and 380 nm, excitation and emission respectively (C and E). The dashed lines in panels (B)–(E) represent samples incubated without methanol. Peaks of interest were labelled 1, 2, 3 and 4. Peak 'a' was identified as argpyrimidine.

incubation mixture (total volume of 20 ml) after 3, 6 and 9 days respectively. The buffer capacity was sufficient to hold the pH constant throughout the incubation period. Peak 4 was purified by injection of 200 mg of incubated acetyl-lysine into the HPLC system using the same solvents as before and a linear gradient of 15–23% acetonitrile from 0 to 20 min. Peak 4 was collected and treated as described above for peak 2.

Acid hydrolysis of peaks 1, 2, 3 and 4

Samples of 15 nmol of peaks 2 and 3 and unknown concentrations of peaks 1 and 4 were acid hydrolysed individually in 1 ml of 6 M HCl at 110 °C for 16, 18 and 20 h. After evaporation and reconstitution in water, each sample was analysed by HPLC with gradient A and chromatograms were compared with those of non-hydrolysed samples.

Spectroscopy

Absorption spectra were recorded at pH 2.5 both with a D.U. 640 spectrophotometer (Beckman) and a photodiode array detector (Waters Corporation, Milford, MA, U.S.A.). Fluorescence spectra were obtained with a PTI spectrofluorimeter (Photon Technology International, NJ, U.S.A.). Comparisons of the fluorescence intensity were measured at equal concentrations using either the PTI spectrofluorimeter or the Waters 474 scanning fluorescence detector.

Solution state NMR

Samples for NMR spectroscopy were exchanged three times with 99% deuterium oxide (²H₂O). Samples containing 8.20 mg of peak 2 or 7.13 mg of peak 3 in 300 μl of 99.9% ²H₂O were transferred to a 5 mm Advanced Microtube (Shigemi, Allison Park, PA, U.S.A.) and scanned for ¹H-NMR, ¹³C-NMR and hetero-correlation spectra at 25 °C with a 500 MHz Varian Spectrometer. Two other samples containing 17.5 mg of peak 2 and 19.0 mg of peak 3 synthesized with 99% [¹³C]glyceraldehyde were scanned for ¹H-NMR and ¹³C-NMR.

MS

MS was performed with the LCQ Ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.). Samples were dissolved at an approx. concentration of 20 μM in 50% (v/v) aqueous acetonitrile with 0.025% TFA for infusion into the instrument at a flow rate of 3 μl/min. Each new adduct was also analysed after proton exchange with ²H₂O three times and final dissolution in a ²H₂O–C²H₃CN mixture (50% of each) with 0.025% TFA.

Formation of peak 2, peak 3 and argpyrimidine in human corneas incubated with 99% [¹³C]glyceraldehyde

Three fragments of human cornea (120 mg wet weight) were incubated with 0.45 mg of 99% [¹³C]glyceraldehyde in 90 μl of 40 mM phosphate buffer at 37 °C for 40 h. Chloroform and toluene (1 μl of each) were added to prevent any bacterial growth [24]. The treated corneas were acid hydrolysed in 3 ml of 6 M HCl for 16 h at 110 °C. The acid was evaporated and leucine equivalent amino acids were measured by the ninhydrin assay in sample reconstituted in water. Peak 2, peak 3 and argpyrimidine were quantified from an injection of 70 μmol of leucine equivalent into the HPLC system (gradient A). Chromatographic peaks of interest were collected for MS analysis.

Formation of peak 2 and peak 3 from porcine corneas treated with 99% [¹³C]glyceraldehyde

Fresh porcine cornea fragments (60 ± 1 mg) were incubated with 0.3 and 0.6 mg of 99% [¹³C]glyceraldehyde dissolved in 30 μl of 50 mM phosphate buffer, pH 7.4, containing chloroform and toluene (1 μl/ml each) to prevent bacterial growth. After 2 days at 37 °C (and also 6 days for the highest concentration of glyceraldehyde), each cornea was acid hydrolysed individually in 1 ml of 6 M HCl at 110 °C for 16 h. After evaporation, reconstitution in water and leucine equivalent amino acid measurement, each sample (70 μmol of leucine equivalent) was analysed by HPLC with gradient A.

To investigate potential artifacts due to acid hydrolysis, non-incubated cornea fragments (40 mg) were acid hydrolysed in the presence of increasing concentrations of glyceraldehyde (0, 0.3 and 0.6 mg/ml) and analysed as described above.

Incubation of sugars, oxoaldehydes and glyceraldehyde-3-phosphate with acetyl-lysine and acetyl-arginine *in vitro*

Sugars (glycolaldehyde, glyceraldehyde, threose, xylose, ribose, fructose and glucose 25 mM), 25 mM oxoaldehydes (glyoxal and methylglyoxal), 25 mM 1,3-dihydroxyacetone (DHA) and 18 mM glyceraldehyde-3-phosphate were incubated with acetyl-lysine (50 mM) and acetyl-arginine (25 mM) in 200 mM phosphate buffer (pH 7.5) with 1 mM DTPA or 10 μ M CuSO₄, after sterile filtration through a 0.2 μ m Acrodisc filter (Gelman Sciences, Ann Arbor, MI, U.S.A.). After 13 days at 37 °C, acetyl-peaks 1 to 4, acetyl-argpyrimidine and acetyl-pentosidine were directly quantitated in each sample by our HPLC system with gradient A. Acetyl-peak 1 was detected by UV absorbance at 275 nm, acetyl-peak 2 and acetyl-peak 4 were detected at 268 nm, acetyl-argpyrimidine at 330 nm, and acetyl-peak 3 and acetyl-pentosidine at 330 nm using fluorescence detection at wavelengths of 330 and 380 nm (excitation and emission respectively). Standards of each molecule were also injected into the HPLC system. Furthermore, each chromatographic peak of interest was collected, concentrated and analysed by MS.

Kinetic studies on peaks 2–4 and argpyrimidine formation from *N*^ε-acetyl-L-lysine and *N*^ε-acetyl-L-arginine incubated with glyceraldehyde with increasing concentrations of methylglyoxal

Mixtures of acetyl-lysine (100 mM), acetyl-arginine (50 mM) and glyceraldehyde (25 mM) were incubated at 37 °C with 0, 5, 15 and 25 mM methylglyoxal in 200 mM phosphate buffer (pH 7.4), 1 mM DTPA with 25% (v/v) aqueous methanol. Aliquots were withdrawn after 1, 2 and 4 days of incubation and frozen at –80 °C until HPLC analysis.

RESULTS

Detection of four new adducts from the reaction between glyceraldehyde and *N*^ε-acetyl-L-lysine with or without *N*^ε-acetyl-L-arginine

In order to facilitate the identification of Lys–Lys from Lys–Arg cross-links or simple Lys or Arg adducts with glyceraldehyde, a strategy of comparing peaks resulting from the incubation of glyceraldehyde with *N*^ε-acetyl-L-lysine (acetyl-lysine), *N*^ε-acetyl-L-arginine (acetyl-arginine), or the combination of the two was adopted. Furthermore, the utilization of CBZ-lysine and quantification at 257 nm was used to identify Lys–Lys cross-links.

When glyceraldehyde (27.5 mM) was incubated with acetyl-lysine (55 mM) for 50 days at 37 °C in 25% aqueous methanol, four major chromophores were observed (Figure 1). No major UV-active or fluorescent peaks were detectable before 13 min. Peaks 1, 2 and 4 were formed from glyceraldehyde and lysine and were not fluorescent at wavelengths of 330 and 380 nm, excitation and emission respectively (Figures 1D and 1E). Peak 3 was highly fluorescent at the same wavelengths and was isolated only in the presence of glyceraldehyde, lysine and arginine (Figure 1, A–C). Acetyl-argpyrimidine was also detected in this incubation mixture based on published UV and fluorescence spectra, and *m/z* of 297.2 [9] (Figures 1A and 1B, peak a). Incubation without methanol showed the same chromatographic profiles but with lower intensity, possibly due to decreased solubility of the reactants.

Figure 2 HPLC chromatograms of 100% CBZ-lysine (solid line) or CBZ- α -L-lysine and *N*^ε-acetyl-L-lysine mixture (50% of each; dashed line) incubated with glyceraldehyde

Peaks 1, 2 and 4 were detected by their specific UV spectrum. Peak areas (arbitrary units in bold numbers) are indicated.

Figure 3 Absorption spectra at pH 2.5

(A) Peak 1, trihydroxy-triosidine, (B) peak 2, lys-hydroxy-triosidine, (C) peak a, argpyrimidine, (D) peak 4, triosidine-carbaldehyde and (E) peak 3, arg-hydroxy-triosidine. Pentosidine spectrum (F) is shown as a comparison.

Analysis by HPLC-UV-MS of the reaction mixture of glyceraldehyde with acetyl-arginine in the presence of poly-L-lysine as a catalyst revealed that only acetyl-argpyrimidine and acetyl-5-hydro-5-methylimidazolone [10], but not peak 3, formed under these conditions (results not shown).

Identification of lysine–lysine cross-links

Except for peak 3, which was identified as a lysine–arginine cross-link, the three other adducts were characterized as follows. Reaction mixtures of glyceraldehyde with 100% CBZ-lysine or a CBZ-lysine/acetyl-lysine mixture (50% of each) were injected into the HPLC system and peak areas for hydrophobic peaks eluting after 7 min were compared (Figure 2). A 75% reduction of the peak areas between the two samples indicated the presence of a lysine–lysine cross-link, whereas a reduction of 50%

Figure 4 Effect of pH on the synthesis of the triosidines (peaks 1, 2, 3 and 4) and argpyrimidine (peak a)

The area was measured at 257 nm for peaks 1, 2 and 3, and at 330 nm for peaks 4 and 'a'.

indicated a single lysine adduct. With this method, peak 2 was presumed to be a lysine–lysine cross-link, and peaks 1 and 4 were suggestive of single lysine adducts (peak area ratios = 1080/504 and 3930/1993 respectively).

UV absorbance of the four adducts

Each UV spectrum was measured during the HPLC separation using the photodiode array detector, and confirmed using a spectrophotometer for the four purified adducts. The four spectra differed from each other and the well-known spectra of argpyrimidine and pentosidine (Figure 3). Peaks 1 and 3 each had one absorbance maximum at 275.7 and 330.3 nm respectively. Peak 2 had three maxima at 232.2, 268.7 and 349.3 nm. Finally, peak 4 had a maximum at 297 nm with a shoulder at 260 nm. The molar absorption coefficients for the purified peaks 2 and 3 (see below) were determined to be 5853 (ϵ_{269}) and 2089 (ϵ_{349}), and 10576 (ϵ_{330}) litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ in H $_2$ O respectively.

Influence of pH and incubation time on the modification of lysine and arginine by glyceraldehyde

A set of samples was incubated to determine the optimal pH for the formation of each compound. The yield of peak 2 and peak 3 increased dramatically from pH 5 to 10.5 (Figure 4). The opposite was observed for peak 4 with a maximum at pH 5. Peak 1 was pH independent, and argpyrimidine formed optimally at pH 7.5. HPLC analysis after 4 and 15 days of incubation indicated no major change in the yield of each adduct, except for the argpyrimidine concentration, which increased during this time. The CBZ-lysine/glyceraldehyde (molar ratio = 59/42) incubations showed that only 26% of the lysine was glycosylated at pH 5, whereas 54% and 60% was glycosylated at pH 7.5 and 10.5 respectively (theoretical maximum = 71%).

Synthesis and isolation of the four chromophores

Acetyl-peak 2 was synthesized at pH 10.5 by incubation of acetyl-lysine with glyceraldehyde. After 9 days at 37 °C, the pH was still above 10, and peak 2 was purified by HPLC; 8.2 mg of its acetyl form was obtained and used for NMR spectroscopy. A sample was acid hydrolysed and analysed by HPLC (Figures

Figure 5 HPLC chromatograms of peak 3 and peak 2 after acid hydrolysis of their pure acetyl forms

Detection was monitored by UV at wavelengths of 268 nm (A) and 330 nm (C) and by fluorescence at wavelengths of 354/440 nm (B) and 330/380 nm (excitation and emission respectively) (D) for peaks 2 and 3 respectively. The first peaks represent 57% and 75% of the initial acetyl-peak 2 and 3 respectively. The second peaks (35% and 12%) were identified as the same products with an increased mass of 18.

5A and 5B). Two peaks with the same UV spectrum as the acetyl adduct were detected, a major one at 5.9 min and a more hydrophobic molecule at 14.0 min. Samples from both peaks were used separately for mass characterization.

Acetyl-peak 1 was purified from the same incubation mixture and HPLC run as acetyl-peak 2. Acetyl-peak 1 was collected (Figure 6A) at a lower retention time than acetyl-peak 2 and kept frozen until mass analysis.

Peak 3 was synthesized at pH 10.5 by incubation of equimolar concentrations of acetyl-lysine, acetyl-arginine and glyceraldehyde. After 6 days at 37 °C, peak 3 was purified by HPLC, hydrolysed to remove the acetyl protecting groups and re-purified by HPLC (Figures 5C and 5D). The result of the acid hydrolysis showed again a major peak at 7.6 min and a minor peak at 15.7 min. Both had the same UV spectrum as the original acetyl compound. The first peak (7.13 min) was collected and used for structural analysis. The second compound was characterized only by MS.

Acetyl-peak 4 synthesis was carried out at pH 5 with acetyl-lysine and glyceraldehyde. After 12 days at 37 °C, acetyl-peak 4 was purified by HPLC (yield 14.85 mg) and kept frozen until analysis (Figure 6B).

Figure 6 HPLC chromatograms of pure acetyl forms of peak 1 (A) and peak 4 (B)

Detection was monitored by UV at 275 (A) and 295 (B) nm for peaks 1 and 4, respectively. Different linear gradients were used: 10–30% (A) and 15–23% (B) acetonitrile in 0.1% TFA from 0 to 20 min.

Structural characterization of the synthetic lysine–lysine cross-link 'lys-hydroxy-triosidine' (peak 2)

The synthetic lysine–lysine cross-link was characterized by MS and NMR spectroscopy (^1H , ^{13}C , and ^1H – ^{13}C heterocorrelation). Electrospray ion-trap MS gave an ion of m/z 383 in the positive ion mode (Figure 7B). The tandem M/S (MS/MS) fragmentation indicated m/z losses of NH_3 (366), COOH (338), norleucine (254), lysine (236), norleucine + COOH (209), norleucine + $\text{COOH} + \text{NH}_3$ (192) and 2 norleucine residues (125). Pure acetyl-peak 2 synthesized with 99% $[1,2,3\text{-}^{13}\text{C}]$ glyceraldehyde gave an m/z of 473 corresponding to $383 + (2 \times 2_{\text{acetyl}}) + 6$ (results not shown). This experiment proved that six carbons from glyceraldehyde are implicated in the cross-link. Eight exchangeable protons were observed when peak 2 was solubilized in 1:1 $^2\text{H}_2\text{O}/\text{C}^2\text{H}_3\text{CN}$ instead of $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (results not shown). In addition to the two carboxylic acid protons and the four α -amino protons, two more exchangeable protons were located either on a ring or on an ϵ -amino group. The formula of $\text{C}_{18}\text{H}_{31}\text{O}_5\text{N}_4$ was confirmed by NMR spectroscopy. In order to prevent any acid degradation of this new cross-link, the NMR spectrum was obtained with the acetyl form. The proton NMR (Figure 8A) showed three ^1H aromatic singlets (a1, c1 and a2) at 7.94, 7.88 and 7.54 p.p.m., a 2H c2-carbon-linked singlet at 4.71 p.p.m., two 2H ϵ -carbon-linked triplets ($\epsilon 1$ and $\epsilon 2$) at 4.43 and 3.21 p.p.m., two overlapping α -carbon-linked protons ($\alpha 1$ and $\alpha 2$) at 4.32 p.p.m., two acetyl 3H methyl singlets at 2.00 and 2.02 p.p.m., and twelve aliphatic lysyl protons between 1.37 and 2.04 p.p.m. The ^{13}C -NMR spectrum (Figure 8B) showed 2 carbons at 179.12 and 178.80 p.p.m. from the carboxylic groups of lysine, two ketone-carbons at 177.32 p.p.m. (superimposed peaks) attributed to the acetyl groups, 5 carbons between 120 and 160 p.p.m. from the aromatic ring (a1, b1, c1, b2 and a2), one CH_2 carbon (c2) at 62.31 p.p.m., two ϵ -carbons ($\epsilon 1$ and $\epsilon 2$) at 64.66 and 45.30 p.p.m., two α -carbons ($\alpha 1$ and $\alpha 2$) at 55.78 and 55.46 p.p.m., and six carbons from the two aliphatic lysyl chains between 24.70 and 33.26 p.p.m. The ^{13}C -NMR spectrum obtained when peak 2 was synthesized with $[1\text{-}^{13}\text{C}]$ glyceraldehyde

showed a 100% increase in the signal of the carbons at 129.1 and 132.5 p.p.m. (Figure 8B). Based on the NMR and mass data, the structure of the molecule was determined to be 1-(5-amino-5-carboxypentyl)-3-[(5-amino-5-carboxypentylamino)methyl]-5-hydroxypyridinium. We named this product lys-hydroxy-triosidine (Figure 9B). The structure was confirmed by the NMR ^1H – ^{13}C heterocorrelation spectrum (Figure 8C).

Structural characterization of the synthetic lysine–arginine cross-link 'arg-hydroxy-triosidine' (peak 3)

The LCQ mass spectrometer gave an ion of m/z 409 [M^+] (Figure 7C). The MS/MS fragmentation indicated losses of H_2O (391), COOH (364), $\text{H}_2\text{O} + \text{COOH}$ (346), 4-amino-4-carboxybutane (294), norleucine (280), lysine (262), norleucine + COOH (235), norleucine + $\text{COOH} + \text{H}_2\text{O}$ (217), and a loss of norleucine and one 4-amino-4-carboxybutane (165). The infusion of a pure acetyl-peak 3 synthesized with 99% $[1,2,3\text{-}^{13}\text{C}]$ glyceraldehyde gave an m/z of 499 (results not shown) which corresponds to $409 + (2 \times 42_{\text{acetyl}}) + 6$. A total of six carbons from glyceraldehyde were also involved in this cross-link. Nine labile protons were observed when peak 3 was solubilized in 1:1 (v/v) $^2\text{H}_2\text{O}/\text{C}^2\text{H}_3\text{CN}$ (results not shown). In addition to the 2 carboxylic acid protons and the 4 α -amino protons, 3 more exchangeable protons were located either on a ring or on an ϵ -amino group of lysine or arginine. Based on these data, the most likely elemental composition for this molecule is $\text{C}_{18}\text{H}_{31}\text{O}_5\text{N}_4$. To complete the structural characterization, NMR spectroscopy was performed on the free α -amino form. The proton NMR spectrum (Figure 8D) showed two ^1H aromatic singlets (a1 and c1) at 7.97 and 7.80 p.p.m., a c2-carbon-linked 2H singlet at 4.72 p.p.m., an ϵ -carbon-linked 2H triplet ($\epsilon 1$) at 4.56 p.p.m., two α -carbon-linked protons ($\alpha 1$ and $\alpha 2$) at 4.10 and 4.03 p.p.m., a δ -carbon-linked 2H triplet ($\delta 2$) at 3.59 p.p.m. and ten aliphatic lysyl/arginyl protons between 1.46 and 2.10 p.p.m. The ^{13}C -NMR spectrum (Figure 8E) showed 2 overlapping carbons at 175.69 p.p.m. from the carboxylic groups of lysine and arginine, six carbons between 120 and 165 p.p.m. attributed to the two rings (a1, b1, c1, b2, a2 and the tri-amino-linked-carbon from arginine), one ring carbon (c2) at 64.18 p.p.m., one ϵ -carbon ($\epsilon 1$) at 56.55 p.p.m., two α -carbons ($\alpha 1$ and $\alpha 2$) at 56.30 p.p.m. (overlapping peaks), and five carbons from the two aliphatic lysyl and arginyl chains between 24.70 and 32.78 p.p.m. The ^{13}C -NMR spectrum obtained when peak 3 was synthesized with $[1\text{-}^{13}\text{C}]$ glyceraldehyde showed a 100% signal increase for the two carbons at 134.1 and 154.9 p.p.m. (Figure 8E). Taken together, the NMR and mass data indicated that the structure of this molecule is 2-(4-amino-4-carboxybutylamino)-8-(5-amino-5-carboxypentyl)-6-hydroxy-3,4-dihydropyrido[2,3-*d*]pyrimidin-8-ium. This adduct was named 'arg-hydroxy-triosidine' (Figure 9C). The structure was confirmed by the NMR ^1H – ^{13}C heterocorrelation spectrum (Figure 8F). It should be noted that an alternative isomeric structure involving fusion of the dihydropyrimidine ring to positions 3 and 4 of the pyridinium ring is also compatible with the NMR and MS results. However, this isomer was considered less likely to form, based on the proposed mechanism of formation (see Scheme 1).

Partial structural characterization of the synthetic non-cross-link lysine adducts 'trihydroxy-triosidine' and 'triosidine-carbaldehyde' (peaks 1 and 4)

The acetyl-peak 1 was characterized by electrospray MS, giving an m/z of 313 [M^+] (Figure 7A). The MS/MS fragmentation indicated losses of H_2O (295), acetyl (271), acetyl + $\text{CO} + \text{H}_2\text{O}$

Figure 7 Electron spray ionization-MS and MS/MS spectra of four synthetic trisidines

Panels (A)–(D) are full MS and MS/MS of *N*^α-acetyl-trihydroxy-trisidine (MS/MS at 313.2), lys-hydroxy-trisidine (MS/MS at 383.4), arg-hydroxy-trisidine (MS/MS at 409.4) and *N*^α-acetyl-trisidine-carbaldehyde (MS/MS at 279.2) respectively.

(225), acetyl + NH₃ + CO + H₂O (208), a loss of acetyl-norleucine (142), and a loss of acetyl-lysine (126). After acid hydrolysis, the peak 1 *m/z* value appeared to be 271 (results not shown). The infusion of an acetyl-peak 1 synthesized with 99% [1,2,3-¹³C]-

glyceraldehyde gave an *m/z* of 319 (results not shown), representing 313 + 6. This experiment proved that 6 carbons from glyceraldehyde are implicated in the glycosylated lysine. Five exchangeable protons were observed when acetyl-peak 1 was

Figure 8 NMR spectra of lys-hydroxy-triosidine and arg-hydroxy-triosidine from *N*^ε-acetyl-L-lysine, *N*^ε-acetyl-L-arginine and glyceraldehyde mixture

¹H (A and D), ¹³C (B and E) and hetero-correlation (C and E) NMR spectra of lys-hydroxy-triosidine (8.2 mg in 300 μl of ²H₂O; A to C) and arg-hydroxy-triosidine (7.1 mg in 300 μl of ²H₂O; D to E) from *N*^ε-acetyl-L-lysine, *N*^ε-acetyl-L-arginine and glyceraldehyde mixture. The thick arrows indicate chemical shifts that increased in intensity when lys-hydroxy-triosidine and arg-hydroxy-triosidine were synthesized with [1-¹³C]glyceraldehyde.

Figure 9 Structures of trihydroxy-triosidine (A), lys-hydroxy-triosidine (B), arg-hydroxy-triosidine (C) and triosidine-carbaldehyde (D)

Figure 10 Acid hydrolysis stability of trihydroxy-triosidine, lys-hydroxy-triosidine and arg-hydroxy-triosidine

Each bar corresponds to the sum of the pure triosidine molecule (lower hatched bars) plus the '+18mass' form (upper white bars).

solubilized in deuterated solvents (results not shown). In addition to the 2 exchangeable protons from the acetyl-norleucine, 3 more exchangeable protons were located on a ring. Taken together, these data suggested the formula of $C_{12}H_{19}O_5N_2$ for the non-acetylated peak 1, and the structure 1-(5-amino-5-carboxypentyl)-3,4-dihydroxy-5-(hydroxymethyl)pyridinium was proposed (Figure 9A). It was named 'trihydroxy-triosidine'. Here again, we cannot completely exclude the 2,5-dihydroxy-3-(hydroxymethyl) isomer, although the structure shown is more compatible with the proposed mechanism of formation (Scheme 1A). NMR spectroscopic studies using $^{13}C_1$ -labelled glyceraldehyde will be needed to resolve this issue.

Mass analysis of acetyl-peak 4 gave an m/z of 279 [M^+] (Figure 7D). The MS/MS fragmentation gave similar losses, as did acetyl-peak 1 (H_2O 261; $CO + H_2O$ 233; acetyl + $CO + H_2O$ 191; acetyl + $NH_3 + CO + H_2O$ 174; acetyl-NH-CH-COOH 162 and acetyl-norleucine 108). The analysis of acetyl-peak 1 synthesized with 99% $[1,2,3-^{13}C]$ glyceraldehyde gave an ion of 285 (results not shown), which indicated that 6 carbons from glyceraldehyde were bonded to the acetyl-lysine. A total of three exchangeable protons were observed on acetyl-peak 1 solubilized in deuterated solvents (results not shown). In addition to the two exchangeable protons from the acetyl-norleucine, one more exchangeable proton was expected on a ring. From these data we suggested the formula of $C_{12}H_{17}O_5N_2$ for the non-acetylated peak 4 and proposed the structure 1-(5-amino-5-carboxypentyl)-3-formylpyridinium, also named triosidine-carbaldehyde (Figure 9D).

Acid stability of trihydroxy-triosidine, lys-hydroxy-triosidine, arg-hydroxy-triosidine and triosidine-carbaldehyde

Only trihydroxy-triosidine, lys-hydroxy-triosidine and arg-hydroxy-triosidine were acid resistant in 6 M HCl at 110 °C. However, the latter two gave a non-identified side-product that was detected by HPLC at a higher retention time (Figure 5) and had an increase of mass of 18, possibly corresponding to a hydrated form of the molecule. The spectroscopic properties of the two side-products appeared to be identical to their respective native product. Furthermore, a small percentage, corresponding with 5 to 22%, 8 to 21% and 13 to 16% of trihydroxy-triosidine,

Figure 11 Fluorescence excitation and emission spectra at pH 3.3, 7.5, 10.5 and 12.5 of lys-hydroxy-triosidine and arg-hydroxy-triosidine

lys-hydroxy-triosidine and arg-hydroxy-triosidine respectively, was destroyed over a period of 16 to 20 h (Figure 10).

Fluorescence characteristics of lys-hydroxy-triosidine and arg-hydroxy-triosidine: comparison with other fluorophores of the Maillard reaction

Lys-hydroxy-triosidine and arg-hydroxy-triosidine fluorescence spectra revealed excitation/emission maxima at 354/440 and 331/380 nm respectively (Figure 11). For both, fluorescence intensity decreased with an increase in pH, and to a greater extent for lys-hydroxy-triosidine. Fluorescence intensities at the maximum wavelength of lys-hydroxy-triosidine, arg-hydroxy-triosidine, argpyrimidine and pentosidine were compared at the same pH and concentration. Arg-hydroxy-triosidine appeared to be 3 times more fluorescent than pentosidine, 160 times more fluorescent than argpyrimidine and 200 times more fluorescent than lys-hydroxy-triosidine. No fluorescence was detected in trihydroxy-triosidine or in triosidine-carbaldehyde, although some fluorescence (at wavelengths of 350 and 440 nm, excitation and emission respectively) was detected in the samples after 2 months; this most likely reflects degradation products.

Mechanism of formation of trihydroxy-triosidine, lys-hydroxy-triosidine, arg-hydroxy-triosidine and triosidine-carbaldehyde

Acetyl-lysine (50 mM) and acetyl-arginine (25 mM) were incubated with various sugars (glycolaldehyde, glyceraldehyde, threose, xylose, ribose, fructose and glucose), oxoaldehydes (glyoxal and methylglyoxal) and glyceraldehyde-3-phosphate and

Table 1 The effects of incubation of various sugars, oxoaldehydes and glyceraldehyde-3-phosphate with *N*^ε-acetyl-L-lysine (ac-lys) and *N*^ε-acetyl-L-arginine (ac-arg) for 13 days at 37 °C on the formation of the four triosidines, argpyrimidine and pentosidine

Amounts are given as nmol of AGE formed with 1 mmol of acetyl-lysine and 0.5 mmol of acetyl-arginine. Trihydroxy-triosidine (THT) was detected at 275 nm, lys-hydroxy-triosidine (LHT) and triosidine-carbaldehyde (TC) at 268 nm, argpyrimidine, arg-hydroxy-triosidine (AHT) and pentosidine at 330 nm. RT, retention time (min) from the HPLC system described in the Experimental section; ND, not detected; OL, overlapped with an unknown peak with $\lambda_{\max}$ at 287 nm.

Sugar (25 mM)	Amount [nmol/mmol (ac-lys + 0.5 mmol ac-arg)]						Peak area (arbitrary units)	
	CuSO ₄ (10 μ M)	DTPA (1 mM)	LHT RT17.25	Argpyrimidine RT17.4	AHT RT17.8	Pentosidine RT18.6	THT RT14.6	TC RT20.17
Glycolaldehyde	+	—	ND	ND	ND	ND	ND	ND
	—	+	ND	ND	ND	ND	ND	ND
Glyoxal	+	—	ND	ND	ND	ND	ND	ND
	—	+	ND	ND	ND	ND	ND	ND
Methylglyoxal	+	—	ND	28 899	ND	ND	OL	ND
	—	+	ND	26 476	ND	ND	OL	ND
Glyceraldehyde	+	—	1032	3407	3195	ND	1185	564
	—	+	898	4552	3395	ND	1566	659
Glyceraldehyde 3-phosphate	+	—	ND	17 444	258	ND	OL	1270
	—	+	ND	15 400	281	ND	OL	888
DHA	+	—	182	2026	1170	ND	469	323
	—	+	292	4828	1343	ND	2280	715
Threose	+	—	ND	ND	ND	ND	ND	ND
	—	+	ND	ND	ND	ND	ND	ND
Xylose	+	—	ND	ND	ND	5141	ND	ND
	—	+	ND	ND	ND	117	ND	ND
Ribose	+	—	ND	ND	ND	18 289	ND	ND
	—	+	ND	ND	ND	1829	ND	ND
Fructose	+	—	ND	ND	ND	ND	ND	ND
	—	+	ND	ND	ND	ND	ND	ND
Glucose	+	—	ND	ND	ND	ND	ND	ND
	—	+	ND	ND	ND	ND	ND	ND

DHA with or without Cu²⁺ (10 μ M) for 13 days at 37 °C. Table 1 shows the four triosidines that were formed specifically from glyceraldehyde, glyceraldehyde-3-phosphate, and DHA, a glyceraldehyde isomer. None of the other sugars or oxoaldehydes were able to form any of the new glycated molecules. However the lack of formation of trihydroxy-triosidine by methylglyoxal or glyceraldehyde-3-phosphate was not proven, since another unknown peak suspected to be methylglyoxal-derived ($\lambda_{\max}$ 287 nm) had the same retention time and might have covered the peak of interest. The two well known AGEs, argpyrimidine and pentosidine, were totally separated with our HPLC method and were quantified. The first one is formed with all three 3-carbon molecules, though we cannot rule out the possibility that there are traces of methylglyoxal in each sample of carbonyl compound used. In our incubation conditions, the pentosidine could be detected only with pentoses and could not be formed from glyceraldehyde. UV spectra MS were used to confirm the identity of each adduct.

Molar concentrations of lys-hydroxy-triosidine, arg-hydroxy-triosidine, argpyrimidine and pentosidine were calculated by comparison with their respective standards. The results indicated that after 13 days of incubation with glyceraldehyde, arg-hydroxy-triosidine and argpyrimidine were formed at approx. the same level, whereas the lys-hydroxy-triosidine concentration was three times lower. From the data listed in Table 1, based on the products that could be identified and quantitated, the relative reactivity of all sugars and oxoaldehydes in the Cu²⁺-containing buffer appeared to be methylglyoxal > ribose ~ glyceraldehyde-3-phosphate > glyceraldehyde > xylose ~ DHA. In the absence of Cu²⁺, methylglyoxal remained the most reactive molecule, with ribose and xylose becoming at least 10 times less reactive than in the presence of Cu²⁺, and less reactive than glyceraldehyde.

Since the four new glycated amino acids were not formed with methylglyoxal alone, we wanted to evaluate the possible effect of the co-presence of this 3-carbon oxoaldehyde on the reaction of lysine, arginine and glyceraldehyde. The results in Figure 12 did not demonstrate a catalytic effect of methylglyoxal, but instead competition was observed, in that argpyrimidine increased at the expense of lys-hydroxy-triosidine, arg-hydroxy-triosidine and triosidine-carbaldehyde. Competition with trihydroxy-triosidine formation was not easy to demonstrate since an unidentified methylglyoxal-derived molecule appeared at the same retention time as trihydroxy-triosidine.

Formation of lys-hydroxy-triosidine, arg-hydroxy-triosidine and argpyrimidine during the reaction of glyceraldehyde with corneas

In human corneas treated *in vitro* with 50 mM glyceraldehyde for 40 h at 37 °C, lys-hydroxy-triosidine, arg-hydroxy-triosidine and argpyrimidine were detected in the acid hydrolysate at a concentration of 1298, 171 and 2627 pmol/mg of protein respectively. The identity of each peak in the HPLC chromatogram was confirmed by their UV spectrum and *m/z* value.

Porcine corneas were analysed by the same HPLC procedure and the results are shown in Table 2. After 2 days of incubation, formation of lys-hydroxy-triosidine, arg-hydroxy-triosidine and argpyrimidine increased linearly with the concentration of glyceraldehyde added to the corneal tissue. Comparison of 2 and 6 days of incubation at 37 °C indicated no further change in the triosidines and argpyrimidine concentrations. Of the three adducts detected, argpyrimidine was the most abundant: lys-hydroxy-triosidine and arg-hydroxy-triosidine were generated at levels 2.5 and 30 times lower respectively.

Up to 1.1 % of the lysines (70 lysines per 1000 amino acids in type I collagen) in collagen were modified into lys-hydroxy-

Figure 12 Effect of methylglyoxal on trihydroxy-triosidine (A), lys-hydroxy-triosidine (B), arg-hydroxy-triosidine (C), triosidine-carbaldehyde (D) and argpyrimidine (E) formation

Increasing concentrations of methylglyoxal (0, 5, 15 and 25 mM) were initially added to the *N*^ε-acetyl-L-lysine (100 mM), *N*^ε-acetyl-L-arginine (50 mM) and glyceraldehyde (25 mM) mixture.

Table 2 Levels of lys-hydroxy-triosidine, arg-hydroxy-triosidine and argpyrimidine in porcine corneas treated with glyceraldehyde

Means $\pm$ S.D. were calculated from 5 independent samples for each incubation condition. Column headings denote the amount of glyceraldehyde added (mg) and the incubation period (days).

	0 mg – 2 days	0.3 mg – 2 days	0.6 mg – 2 days	0.6 mg – 6 days
Lys-hydroxy-triosidine				
pmol/mg protein	358 $\pm$ 273	1427 $\pm$ 89	2515 $\pm$ 575	2189 $\pm$ 98
pmol/nmol lysine	2.4 $\pm$ 1.8	9.6 $\pm$ 0.6	16.8 $\pm$ 3.8	14.7 $\pm$ 0.6
Arg-hydroxy-triosidine				
pmol/mg protein	0	129 $\pm$ 31	206 $\pm$ 45	312 $\pm$ 42
pmol/nmol lysine	0	0.9 $\pm$ 0.2	1.4 $\pm$ 0.3	2.1 $\pm$ 0.3
pmol/nmol arginine	0	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1
Argpyrimidine				
pmol/mg protein	0	2547 $\pm$ 236	6011 $\pm$ 1085	5779 $\pm$ 631
pmol/nmol arginine	0	5.0 $\pm$ 0.5	11.9 $\pm$ 2.1	11.5 $\pm$ 1.2

triosidine, whereas only 0.1% were transformed to arg-hydroxy-triosidine. Argpyrimidine and arg-hydroxy-triosidine accounted for up to 1.2% and 0.5% of the arginine in collagen respectively (150 arginines per 1000 amino acids in type I collagen) [25].

DISCUSSION

This study is, to our knowledge, the first to describe structures resulting from the reaction of trioses with lysine and arginine residues. All of the new Maillard reaction products involve two glyceraldehyde molecules. Arg-hydroxy-triosidine and lys-

hydroxy-triosidine are the major fluorescent products detected. The first is an arginine-lysine cross-link and the second is a lysine-lysine cross-link. The structures of the lysyl adducts, trihydroxy-triosidine and the labile triosidine-carbaldehyde, will need to be confirmed by NMR analysis. A possible mechanism of formation for the four novel structures is shown in Scheme 1.

Briefly, the reaction cascade starts with condensation of glyceraldehyde with a lysine residue to give the 3-hydroxy-2-oxopropyl Amadori product, presumed by previous workers [26] to afford protein cross-links directly by Schiff base formation. However, such simple cross-links would neither be hydrolytically stable nor fluorescent. Based on this mechanism, which is

Scheme 1 Proposed mechanism of formation of trihydroxy-triosidine (A), lys-hydroxy-triosidine (B), arg-hydroxy-triosidine (C) and triosidine-carbaldehyde (D) from the reaction of glyceraldehyde with N^ϵ -protected-L-lysine (L-NH₂) and N^ϵ -protected-L-arginine [A-NH⁺-C(NH₂)=NH]

supported by the data with [1-¹³C]glyceraldehyde, the initial Amadori product condenses with a second molecule of glyceraldehyde to give a bis-Amadori product that is a common intermediate to all of the proposed structures. Intramolecular aldol condensation followed by a series of dehydrations and tautomerizations (keto-enol and Amadori rearrangement) leads to triosidine-carbaldehyde (Scheme 1, compound D), whereas a two-electron oxidation of a 1,2-dihydropyridine tautomer leads to trihydroxy-triosidine (Scheme 1, compound A). On the other hand, keto-enol tautomerization of the bis-Amadori product followed by condensation with an arginine residue or a second lysine residue, then cyclization, and further tautomerizations and dehydrations, leads to the two cross-link structures B and C, the latter requiring a two-electron oxidation of a 1,2-dihydropyridine penultimate intermediate. Although other reaction mechanisms can be given that lead to the proposed structures, those shown in Scheme 1 serve to illustrate the required stoichiometries and involve straightforward chemistry. As shown in Scheme 1, 3-hydroxypyridinium compounds B and C probably exist as the 3-oxido zwitterions at neutral pH. In contrast, the 4-hydroxypyridinium compound A can exist as a neutral 4-pyridone (except at low pH). There is precedent for the 4-pyridone structure of A being associated with the observed UV absorption at 276 nm [27].

Incubation with other sugars and oxoaldehydes indicates that the four triosidines are glyceraldehyde- and dihydroxyacetone-specific. Glyceraldehyde-3-phosphate, the glycolytic intermediate, is also a precursor of arg-hydroxy-triosidine and triosidine-carbaldehyde. Contrary to previous data [19,28], we did not detect any pentosidine in glyceraldehyde incubation mixtures (shown by HPLC-fluorescence-MS). Even though Chellan and Nagaraj indicated that the glyceraldehyde-derived product exhibited proton NMR and UV characteristics compatible with those reported for ribose-derived pentosidine [19], it is possible that these workers actually were looking at arg-hydroxy-triosidine, which has some spectral features in common with pentosidine. We also confirmed that argpyrimidine, which is mainly produced by methylglyoxal, can also be synthesized from glyceraldehyde, glyceraldehyde-3-phosphate and DHA.

The formation of argpyrimidine from trioses and the previously reported susceptibility of glyceraldehyde to easily oxidize into α -ketoaldehyde and hydroxypyruvaldehyde in the presence of metals [29] raises the question of whether the structures described above could be due to contaminants present in commercial batches of glyceraldehyde, or autoxidation products generated during the incubation reaction. An investigation by GC/MS of the nature of the contaminants present in the 1-¹³C-labelled and unlabelled batches of glyceraldehyde revealed a peak equivalent to 90–95% glyceraldehyde, whereby the trace contaminating peaks in the two batches were non-identical (results not shown). Since the triosidines were synthesized similarly from both batches, it is thus unlikely that they could be derived from contaminants. While *in vitro* synthesis of argpyrimidine from glyceraldehyde has been explained by the non-enzymic degradation of glyceraldehyde into methylglyoxal [30] and by the trace contamination of methylglyoxal in commercial preparations of glyceraldehyde [31], no methylglyoxal was detected in any of the batches used in this study. Based on these findings, and the fact that the triosidine chemistry could not be explained by methylglyoxal or any other sugar tested in Table 1, we conclude that the proposed structures are best explained by a mechanism involving glycation by glyceraldehyde, as depicted in Scheme 1. Nonetheless, from the same glyceraldehyde incubation mixtures, both *N*^ε-(carboxymethyl)lysine and *N*^ε-(carboxyethyl)lysine were detected by LC-MS (results not shown), suggesting that autoxidation was active

in our incubation system. Finally, no new glyceraldehyde-specific adducts were found from the incubation of glyceraldehyde with arginine.

The purpose of the present study was also to determine whether the four triosidines could form in protein incubated with glyceraldehyde. After glyceraldehyde incubation of porcine corneas and acid hydrolysis, arg-hydroxy-triosidine, lys-hydroxy-triosidine (acid-stable triosidines) and argpyrimidine were quantified. The analytical system used was not sensitive enough to detect trihydroxy-triosidine despite the fact that it is also an acid stable adduct. HPLC analysis of these showed that argpyrimidine appears to be the major modification of amino acids. Considering that the corneal stroma consists mainly of type I collagen [32], we estimated that 1% and 0.05% of the total arginine was modified to argpyrimidine and arg-hydroxy-triosidine respectively. The relative amount of argpyrimidine compared with the Arg–Lys cross-link is consistent with the natural proportion of arginine that is in proximity to lysine in type I collagen (approx. 1/10). We similarly estimate that 1.5% of the lysine was modified to lys-hydroxy-triosidine and 0.15% to arg-hydroxy-triosidine. Here again, the proportion of lys-hydroxy-triosidine and arg-hydroxy-triosidine formed is consistent with the natural ratio of lysine–lysine adjacency to lysine–arginine adjacency (5/1) in the collagen type I triple helix.

Although hexoses have been thought to play a primary role in glycation and cross-linking *in vivo*, several studies indicate that a number of sugars in addition to glucose can form AGEs. Short chain sugars or oxoaldehydes have been described as highly reactive in terms of glycation [14]. Glyceraldehyde-3-phosphate and 1,3-dihydroxyacetone phosphate (DHAP) are two intermediate metabolites of the glycolytic pathway. Glyceraldehyde is an intermediate of fructose metabolism [18] of particular interest because of the high consumption of fructose (which has increased ten-fold since 1975 in the U.S.A.) (www.winningmag.com/training/bod/bod18September1998.shtml) and the recommendation of a fructose diet for type II diabetics [34]. Several studies have shown that glyceraldehyde-3-phosphate, DHAP, and their dephosphorylated forms, glyceraldehyde and DHA, are potent glycation and cross-linking agents that can generate yellow fluorophores and chromophores [26,35,36]. Prabhakaram and Ortwerth [37] have shown that the cross-linking abilities of glyceraldehyde and DHA were three times higher than those of erythrose and threose, and eight times higher than that of ribose. Similarly, the increase in fluorescence and the loss of lysine and arginine residues mediated by these sugars occurs at a higher rate compared with other sugars [38,39]. Furthermore, glyceraldehyde-3-phosphate has been described as a 'potential toxic triose' which inhibits different enzyme activities [35,40].

For more than a decade, glycation of peptides and proteins by glyceraldehyde has also been studied for its therapeutic applications. Among the extensive reports on the anti-sickling effect of glyceraldehyde on erythrocytes, the sites of haemoglobin glycation by glyceraldehyde [41], the factors that influence the reaction [42] and the deleterious effects of glyceraldehyde on protein and cell structure and function have been described [43]. The latter study indicates that, at some concentrations, glyceraldehyde induces cross-linking of haemoglobin and erythrocyte membrane proteins. Not only is the local application of glyceraldehyde to collagen-rich tissue being evaluated to improve the mechanical strength of the cornea (www.kcenter.org/past_articles/2.html) but external application of its isomer, DHA, is being used in cosmetology approved by the FDA (www.cfsan.fda.gov/~dms/cos-220.html). Thus, the potential endogenous formation and therapeutic applications of triosidines in selected biomedical states, as well as the wide-spread cosmetic

utilization of trioses, warrant a detailed understanding of the non-enzymic glycation chemistry of short chain sugars.

Bunn and Higgins noted that glyceraldehyde-3-phosphate was the most reactive glycation intermediate found to be elevated in diabetic cells [45]. Since a fructose load has been found to increase the glyceraldehyde concentration in the liver [46], the combination of diabetes and fructose load may lead to accumulation of glyceraldehyde at a rate that exceeds the capacity to phosphorylate it [18]. In these conditions, and in certain rare medical conditions that are associated with increased secretion of glyceraldehyde in the urine [47,48,49], triosidines may thus become useful markers of disease. In this regard, the finding of glyceraldehyde-specific immunological AGE epitopes in serum protein of diabetics is of the highest interest [50]. Finally, the chronic utilization of artificial sun-tanning agents that are DHA-based suggests that triosidines are responsible for the effect, but may also act as photosensitizers and potential carcinogens.

In summary, the discovery of triosidines as a novel class of advanced Maillard reaction compounds is expected to offer new insight of biomedical importance for disorders of triose metabolism, biomedical engineering and in the study of the biophysical and toxic effects of triose-based sun-tanning agents.

We thank Dr Marcus Glomb for his gift of argpyrimidine standard and Dr Robert Salomon (Department of Chemistry, Case Western Reserve University, OH, U.S.A.) for assistance with computerized systematic nomenclature. We also thank Drs David Sell and Tiberiu G. Mustata (Institute of Pathology, Case Western Reserve University, OH, U.S.A.) for help with determination of glyceraldehyde contaminants in commercial batches. This work was supported by a grant from the National Institute of Aging (AG 18629) and in part by the National Eye Institute (EY07099).

REFERENCES

- Hodge, J. E. (1953) Dehydrated foods, chemistry of browning reactions in model systems. *J. Agric. Food Chem.* **1**, 928–943
- Sell, D. R. and Monnier, V. M. (1989) Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J. Biol. Chem.* **264**, 21597–21602
- Brinkmann, E., Wells-Knecht, K. J., Thorpe, S. R. and Baynes, J. W. (1995) Characterization of an imidazolium compound formed by reaction of methylglyoxal and *N*^ε-hippuryllysine. *J. Chem. Soc. Perkin Trans I*, **1**, 2817–2818
- Wells-Knecht, K. J., Brinkmann, E. and Baynes, J. W. (1995) Characterization of an imidazolium salt formed from Glyoxal and *N*^ε-hippuryllysine: A model for Maillard reaction crosslinks in proteins. *J. Org. Chem.* **60**, 6246–6247
- Tessier, F., Obrenovich, M. and Monnier, V. M. (1999) Structure and mechanism of formation of human lens fluorophore LM-1. Relationship to vesperlysine A and the advanced Maillard reaction in aging, diabetes, and cataractogenesis. *J. Biol. Chem.* **274**, 20796–20804
- Nagaraj, R. H., Portero-Otin, M. and Monnier, V. M. (1996) Pyrraline ether crosslinks as a basis for protein crosslinking by the advanced Maillard reaction in aging and diabetes. *Arch. Biochem. Biophys.* **325**, 152–158
- Ahmed, M. U., Thorpe, S. R. and Baynes, J. W. (1986) Identification of *N*^ε-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.* **261**, 4889–4894
- Ahmed, M. U., Brinkmann-Frye, E., Degenhardt, T. P., Thorpe, S. R. and Baynes, J. W. (1997) *N*^ε-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem. J.* **324**, 565–570
- Shipanova, I. N., Glomb, M. A. and Nagaraj, R. H. (1997) Protein modification by methylglyoxal: chemical nature and synthetic mechanism of a major fluorescent adduct. *Arch. Biochem. Biophys.* **344**, 29–36
- Lo, T. W., Westwood, M. E., McLellan, A. C., Selwood, T. and Thornalley, P. J. (1994) Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with *N*^ε-acetylarginine, *N*^ε-acetylcysteine, and *N*^ε-acetyllysine, and bovine serum albumin. *J. Biol. Chem.* **269**, 32299–32305
- Dunn, J. A., McCance, D. R., Thorpe, S. R., Lyons, T. J. and Baynes, J. W. (1991) Age-dependent accumulation of *N*^ε-(carboxymethyl)lysine and *N*^ε-(carboxymethyl)-hydroxylysine in human skin collagen. *Biochemistry* **30**, 1205–1210
- Shamsi, F. A., Partal, A., Sady, C., Glomb, M. A. and Nagaraj, R. H. (1998) Immunological evidence for methylglyoxal-derived modification *in vivo*. *J. Biol. Chem.* **273**, 6928–6936
- Monnier, V. M., Kohn, R. R. and Cerami, A. (1984) Accelerated age-related browning of human collagen in diabetes mellitus. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 583–587
- Syrový, I. (1993) Glycation of albumin: reaction with glucose, fructose, galactose, ribose or glyceraldehyde measured using four methods. *J. Biochem. Biophys. Meth.* **28**, 115–121
- Grandhee, S. K. and Monnier, V. M. (1991) Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors. *J. Biol. Chem.* **266**, 11649–11653
- Biemel, K. M., Reihl, O., Conrad, J. and Lederer, M. O. (2001) Formation pathways for lysine-arginine cross-links derived from hexoses and pentoses by Maillard processes: unraveling the structure of a pentosidine precursor. *J. Biol. Chem.* **276**, 23405–23412
- Glomb, M. A. and Monnier, V. M. (1995) Mechanism of protein modification by glyoxal and glyceraldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **270**, 10017–10026
- Sillero, M. A., Sillero, A. and Sols, A. (1969) Enzymes involved in fructose metabolism in liver and the glyceraldehyde metabolic crossroads. *Eur. J. Biochem.* **10**, 345–350
- Chellian, P. and Nagaraj, R. H. (2001) Early glycation products produce pentosidine cross-links on native proteins. Novel mechanism of pentosidine formation and propagation of glycation. *J. Biol. Chem.* **276**, 3895–3903
- Eng, C. P., Bhatnagar, M. K. and Morgan, J. F. (1972) Inhibition of mouse ascites tumors by carbohydrate combined with immunization. *Can. J. Physiol. Pharmacol.* **50**, 156–163
- Benjamin, L. J. and Manning, J. M. (1986) Enhanced survival of sickle erythrocytes upon treatment with glyceraldehyde. *Blood* **67**, 544–546
- Girton, T. S., Oegema, T. R. and Tranquillo, R. T. (1999) Exploiting glycation to stiffen and strengthen tissue equivalents for tissue engineering. *J. Biomed. Mater. Res.* **46**, 87–92
- Reference deleted
- Young, L. Y. and Mitchell, R. (1973) Negative chemotaxis of marine bacteria to toxic chemicals. *Appl. Microbiol.* **25**, 972–975
- Miller, E. J. and Gay, S. (1982) Collagen: an overview. *Methods Enzymol.* **82**, 3–32
- Acharya, A. S., Cho, Y. J. and Manjula, B. N. (1988) Cross-linking of proteins by aldotoxose: reaction of the carbonyl function of the keto amines generated *in situ* with amino groups. *Biochemistry* **27**, 4522–4529
- Umeda, H., Nakamura, F. and Suyama, K. (2001) High-performance liquid chromatographic quantification of allysine as bis-p-cresol derivative in elastin. *Arch. Biochem. Biophys.* **385**, 209–219
- Dyer, D. G., Blackledge, J. A., Thorpe, S. R. and Baynes, J. W. (1991) Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine *in vivo*. *J. Biol. Chem.* **266**, 11654–11660
- Thornalley, P., Wolff, S., Crabbe, J. and Stern, A. (1984) The autoxidation of glyceraldehyde and other simple monosaccharides under physiological conditions catalysed by buffer ions. *Biochim. Biophys. Acta* **797**, 276–287
- Ohmori, S., Mori, M., Shiraha, K. and Kawase, M. (1989) Enzymology and Molecular Biology of Carbonyl Metabolism 2 (Weiner, H. and Flynn, T. G., eds.), pp. 397–412, A.R. Liss, New York
- Taguchi, T. and Miwa, I. (1996) Carbonyl contaminants in commercially available preparations of D-glyceraldehyde. *Med. Sci. Res.* **24**, 781–783
- Fatt, I. and Weissman, B. A. (1992) Cornea 1: Form, swelling pressure, transport processes and optics. In *Physiology of the eye: An Introduction to the Vegetative Functions*, 2nd edn, pp. 97–204, Butterworth-Heinemann, Boston
- Reference deleted
- Endres, J., Poon, S. W. and Welch, P. (1989) Diabetics in long-term care. Effect of sweetness on dietary intake. *Ann. N.Y. Acad. Sci.* **561**, 157–161
- Vander Jagt, D. L., Robinson, B., Taylor, K. T. and Hunsaker, L. A. (1992) Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications. *J. Biol. Chem.* **267**, 4364–4369
- Fitzgerald, C., Swearingin, T. A., Yeagans, G., McWhorter, D., Cucchetti, B. and Seidler, N. W. (1999) Non-enzymatic glycosylation (or glycation) and inhibition of the pig heart cytosolic aspartate aminotransferase by glyceraldehyde 3-phosphate. *J. Enzyme Inhib.* **15**, 79–89
- Prabhakaram, M. and Ortwerth, B. J. (1994) Determination of glycation crosslinking by the sugar-dependent incorporation of [¹⁴C]lysine into protein. *Anal. Biochem.* **216**, 305–312
- Stevens, A. (1995) The effectiveness of putative anti-cataract agents in the prevention of protein glycation. *J. Am. Optom. Assoc.* **66**, 744–749
- Lee, K. W., Simpson, G. and Ortwerth, B. (1999) A systematic approach to evaluate the modification of lens proteins by glycation-induced crosslinking. *Biochim. Biophys. Acta* **1453**, 141–151
- Murata, T., Miwa, I., Toyoda, Y. and Okuda, J. (1993) Inhibition of glucose-induced insulin secretion through inactivation of glucokinase by glyceraldehyde. *Diabetes* **42**, 1003–1009

-
- 41 Acharya, A. S. and Manning, J. M. (1980) Reactivity of the amino groups of carbonmonoxyhemoglobin S with glyceraldehyde. *J. Biol. Chem.* **255**, 1406–1412
- 42 Bai, Y., Ueno, H. and Manning, J. M. (1989) Some factors that influence the nonenzymatic glycation of peptides and polypeptides by glyceraldehyde. *J. Protein Chem.* **8**, 299–315
- 43 Nigen, A. M. and Manning, J. M. (1978) Effects of glyceraldehyde on the structural and functional properties of sickle erythrocytes. *J. Clin. Invest.* **61**, 11–19
- 44 Reference deleted
- 45 Bunn, H. F. and Higgins, P. J. (1981) Reaction of monosaccharides with proteins: possible evolutionary significance. *Science* **213**, 222–224
- 46 Heinz, F. and Junghanel, J. (1969) Metabolite model in rat liver following application of fructose. *Hoppe Seylers Z. Physiol. Chem.* **350**, 859–866
- 47 Jonas, A. J., Lin, S. N., Conley, S. B., Schneider, J. A., Williams, J. C. and Caprioli, R. C. (1989) Urine glyceraldehyde excretion is elevated in the renal Fanconi syndrome. *Kidney Int.* **35**, 99–104
- 48 Nakai, A., Shigematsu, Y., Liu, Y. Y., Kikawa, Y. and Sudo, M. (1993) Urinary sugar phosphates and related organic acids in fructose-1,6-diphosphatase deficiency. *J. Inherited Metab. Dis.* **16**, 408–414
- 49 Tomer, K. B., Rothman, R., Yudkoff, M. and Segal, S. (1977) Unusual pattern of metabolites in the urine of child with tyrosinemia: glyceraldehyde. *Clin. Chim. Acta* **81**, 109–117
- 50 Takeuchi, M., Makita, Z., Bucala, R., Suzuki, T., Koike, T. and Kameda, Y. (2000) Immunological evidence that non-carboxymethyllysine advanced glycation end-products are produced from short chain sugars and dicarbonyl compounds *in vivo*. *Mol. Med.* **6**, 114–125
-

Received 26 April 2002/23 September 2002; accepted 14 October 2002

Published as BJ Immediate Publication 14 October 2002, DOI 10.1042/BJ20020668