

# Mechanism of Formation of the Maillard Protein Cross-link Pentosidine

GLUCOSE, FRUCTOSE, AND ASCORBATE AS PENTOSIDINE PRECURSORS\*

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Pentosidine is a recently discovered fluorescent protein cross-link from human extracellular matrix that involves lysyl and arginyl residues in an imidazo(4,5b)pyridinium ring. Pentosidine could be synthesized *in vitro* by the reaction of ribose, lysine, and arginine. The potential biological significance of the molecule prompted us to investigate its mechanism of formation from D-ribose and key Maillard intermediates, as well as from other potential precursor sugars. The yield of pentosidine from *N*<sup>ε</sup>-*t*-Boc-lysine, *N*<sup>ε</sup>-*t*-Boc-arginine, and D-ribose was highest at pH 9.0 and 65 °C, but was unaffected by reactant ratios at alkaline pH suggesting an important role for base catalysis. Ribated Boc-lysine on incubation with *N*<sup>ε</sup>-*t*-Boc-arginine afforded a fluorescent compound with UV, fluorescence, <sup>1</sup>H NMR, and MS properties identical with those from native or synthetic pentosidine. 3-Deoxypentosone, however, was not a major pentosidine precursor. Pentosidine became slowly detectable in bovine serum albumin incubated with 0.25 M and 1.0 M glucose and reached, at 30 days, 13.2 and 17 pmol/mg bovine serum albumin, respectively. Spectroscopical properties of glucose-derived pentosidine were identical with those from ribose-derived pentosidine. Pentosidine formed from glucated Boc-lysine with *N*<sup>ε</sup>-*t*-Boc-arginine in higher yields than from glucose under standard conditions. Fructose, and unexpectedly ascorbate, also formed pentosidine in similar yields as glucose. The discovery that pentosidine can form not only from pentoses but also from hexoses and ascorbate raises major new questions concerning biochemical pathways of the Maillard reaction *in vivo*.

The Maillard or nonenzymatic glycosylation reaction (1) has been postulated to explain age-related changes like cross-linking and yellowing in long-lived proteins such as collagen and lens crystallins. Recent progress from our laboratory led to the structure elucidation of a fluorescent cross-link molecule named pentosidine from aging extracellular matrix (2, 3). In view of the fact that pentosidine also formed in cell cultures, ribose emerged as the likely major precursor *in vivo*. However, preliminary data<sup>1</sup> suggested that plasma ribose levels are very low even in the presence of diabetes. This obser-

vation and the necessity to understand the mechanism of pentosidine synthesis led us to initiate such a study and to search for other carbohydrates as potential precursors of pentosidine.

## RESULTS AND DISCUSSION<sup>2</sup>

The effects of varying reaction parameters like reactant ratios, temperature, and pH on pentosidine synthesis from D-ribose, Boc-lysine, and Boc-arginine were studied. Both tripling the amount of Boc-arginine (Fig. 1) and lowering the temperature from 80 to 65 °C led to a 2–2.5-fold increase in the yield (Fig. 2). Alkaline pH (pH 9) also had a substantial effect on the initial reaction rate (Fig. 3) as well as the use of phosphate salts in the buffer (not shown).

**Role of the Amadori Product and 3-Deoxypentosone in the Formation of Pentosidine**—Ribated lysine (0.1 M) incubated with Boc-arginine (0.1 M) under the standard conditions (65 °C, pH 7.4, 48 h) led to the formation of a fluorescent HPLC<sup>3</sup> peak with the same retention time as that of pentosidine, while no such peak was observed in incubation mixtures of ribated lysine alone. The identity of the fluorophore with pentosidine was confirmed by purifying the compound from large scale incubation mixtures of ribated lysine and Boc-arginine using a combination of ion-exchange chromatography and reverse-phase HPLC. The purified fluorescent compound was identical with original pentosidine in terms of UV, fluorescence, and proton NMR (not shown). Its MS/MS spectrum shown in Fig. 4A was identical with pentosidine synthesized from ribose (3). Pentosidine formed from ribated lysine five to six times faster at pH 9 than from D-ribose (yield 12.5%) while hardly any synthesis was observed at low pH (Fig. 5A). Acid hydrolysis did not play a role in pentosidine formation. It was observed that the yield of pentosidine was similar in the acid-treated and untreated samples (not shown). Surprisingly, the yield of pentosidine from incubation of 0.1 M 3-deoxypentosone (3-DP) with equimolar concentrations of Boc-lysine and Boc-arginine at 65 °C at various pH was negligible (Fig. 5B) compared to that from the Amadori product (Fig. 5A), suggesting either that 3-DP is not an intermediate or that it is too reactive leading to preferential formation of other products. Furfural, a Maillard product

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<sup>1</sup> D. R. Sell and V. M. Monnier, unpublished data.

<sup>2</sup> Portions of this paper (including "Materials and Methods," Figs. 1–9, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

<sup>3</sup> The abbreviations used are: HPLC, high pressure liquid chromatography; PBS, phosphate buffer saline; MS, mass spectrometry; Boc-arginine, *N*<sup>ε</sup>-*t*-Boc-L-arginine; Boc-lysine, *N*<sup>ε</sup>-*t*-Boc-L-lysine; glucated lysine, *N*<sup>ε</sup>-*t*-Boc-*N*<sup>ε</sup>-(1-deoxy-D-fructos-1-yl)-L-lysine; ribated lysine, *N*<sup>ε</sup>-*t*-Boc-*N*<sup>ε</sup>-(1-deoxy-D-ribulos-1-yl)-L-lysine; 3-DP, 3-deoxypentosone.

formed from pentoses preferentially at low pH also was not a pentosidine precursor when reacted with Boc-lysine and Boc-arginine under standard conditions.

**Role of Other Sugars in Pentosidine Formation: Glucose, Fructose, and Ascorbate as Pentosidine Precursors**—Previous data<sup>1</sup> from our laboratory revealed that long term incubation of proteins with low levels of glucose (<50 mM) did not lead to detectable amounts of pentosidine. However, data in the literature suggested that glucose fragmentation may occur particularly at alkaline pH (8). We therefore investigated more systematically the role of *glucose* and other carbohydrates as potential pentosidine precursors.

Defatted bovine serum albumin (50 mg/ml) was incubated with glucose at 5, 25, 250, and 1000 mM at pH 7.4 for a period of 28 days and analyzed for pentosidine in acid hydrolysate by HPLC. A linear increase in pentosidine formation which reached 13.2 and 17.0 pmol/mg at 28 days was noted at 0.25 and 0.1 M glucose, respectively (Fig. 6). Pentosidine formed also in incubation mixtures of D-glucose with Boc-lysine and Boc-arginine. Confirmation of the structure was obtained by isolating and purifying the fluorophore and by carrying out spectral analysis using UV, fluorescence, high resolution NMR, and MS/MS. FAB high resolution analysis (Fig. 4B) showed a measured mass of  $m/z$  379,2076 similar to that from ribose (379,2053) (3). The fragmentation pattern and the CAD MS/MS of pentosidine from glucose and from the Amadori product of ribose were identical, except for a contaminant at  $m/z$  243. High resolution (400 Mhz) NMR of the compound was identical to pentosidine obtained from ribose (Fig. 7). Comparison of the reactivity of D-ribose, D-glucose, and their Amadori products at 65 °C showed the following reactivities: ribated lysine>ribose>glucated lysine>glucose (Fig. 8). During these experiments, it was noted that incubation mixtures of glucose/lysine as well as ribose/lysine also showed a fluorescent peak at the same retention time as pentosidine. However, the fluorophore was quenched or destroyed by borohydride treatment or HCl hydrolysis, while pentosidine derived from incubation of glucose, or ribose with lysine and arginine was stable when subjected to similar treatment.

Similar experiments under standard conditions with fructose as pentosidine precursor was investigated. Incubation of fructose with Boc-arginine and Boc-lysine at pH 7.4 and 65 °C for 48 h showed a borohydride and acid-resistant fluorescent peak with retention time identical with pentosidine. Interestingly, the yield of pentosidine from fructose was close to that obtained from glucose (Table I).

Furthermore, both ascorbate and dehydroascorbate which are potent glycation and cross-linking agents of proteins (9) were found to be pentosidine precursors. Incubation of ascorbate and dehydroascorbate with Boc-lysine and Boc-arginine under the standard conditions, however, afforded lower yields of pentosidine than from ribose (Fig. 9).

The experiments described above demonstrate the role of Amadori product in the formation of pentosidine from D-ribose/D-glucose, lysine, and arginine residues. The strong pH dependence, as well as the increased yield with increasing concentration of arginine, suggests that the dehydration of the ribose-derived Amadori compound proceeds via an  $\alpha$ -dicarbonyl intermediate which can cyclize to form I and under base catalysis condense with the guanido group of arginine (Fig. 10). The extremely low yield observed at pH 4 indicates that 3-DP formed by the degradation of the Amadori product is unlikely to be of any significance.

Somewhat surprising was the discovery that other carbohydrates could act as pentosidine precursors. Prolonged incubations of bovine serum albumin with supraphysiological

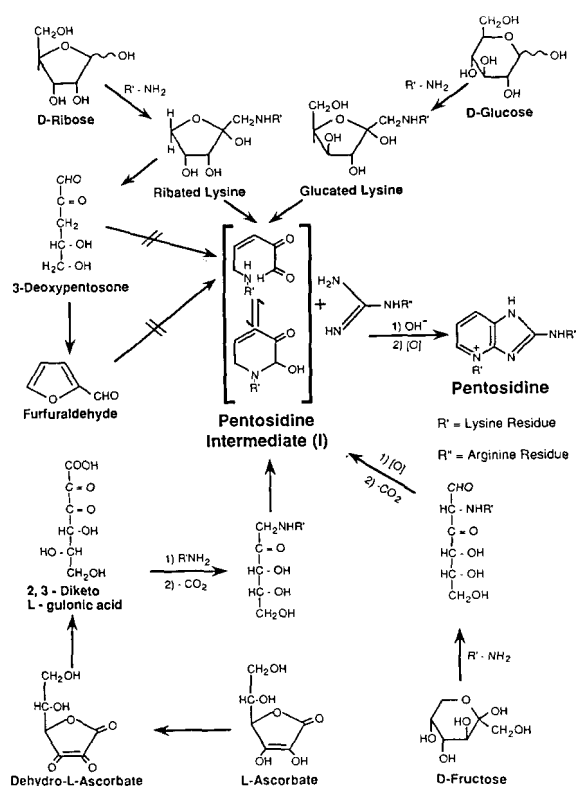


FIG. 10. Proposed mechanism of pentosidine formation from D-ribose, D-glucose, D-fructose, and ascorbate via the common intermediate I.

glucose concentrations were necessary for the formation of pentosidine. The possibility that this would occur as a consequence of contamination of the D-glucose batch by free ribose is unlikely since the Amadori product of glucose formed pentosidine at a higher rate than D-glucose itself. This latter observation and the lag phase seen throughout our experiments suggests that the Amadori product is a necessary intermediate in pentosidine synthesis.

The formation of pentosidine from D-glucose as well as its Amadori product may again proceed through intermediate I. Autooxidation catalyzed by alkali would lead to fragmentation of C<sub>5</sub>-C<sub>6</sub> via formation of a glycolosonic acid (8). However, we cannot exclude a C<sub>1</sub>-C<sub>2</sub> fragmentation of the Amadori product to form a C-5-fragment sugar with ribose-like reactivity. The mechanism of formation of pentosidine from fructose could proceed via a Heyns rearrangement (11) followed by an alkali-catalyzed autooxidation of the C-1 to a carboxylic acid with subsequent decarboxylation. This is of interest, since the implication of fructose in the Maillard reaction is of particular relevance to diabetes, as fructose levels in diabetic patients are increased as a consequence of activation of the aldose reductase pathway. Ascorbate upon reaction with amines loses CO<sub>2</sub> rapidly (12). Thus, it is not too surprising to find that ascorbate and dehydroascorbate can act as precursors of pentosidine, possibly via 2,3-diketogulonic acid. The discovery that pentosidine can form from other sugars raises a number of questions of biological significance. Amadori products of glucose may thus explain in part the elevated levels of pentosidine in diabetic skin collagen (13). However, a role for dehydroascorbic acid is not excluded since its plasma concentrations are at least 2-fold elevated in diabetes (14). Because ascorbate is also found intracellularly, it may serve as pentosidine precursor in cellular systems. Metal-catalyzed glucose autooxidation and the formation of cross-linking agents as



suggested by Wolff *et al.* (15) may explain pentosidine formation in uremia. However, it is also possible that ribonucleotide breakdown products and free pentoses are released as a consequence of increased red blood cell turnover and poorly cleared from the plasma by the diseased kidney.

In summary, pentosidine emerges as an end product of the Maillard reaction common to hexoses, pentoses, and ascorbate. It is likely that its synthesis proceeds through a common intermediate I. The discovery that pentosidine can form from ascorbate opens new insight into oxidative aspects of the Maillard reaction *in vivo*.

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Supplementary Material to

MECHANISM OF FORMATION OF THE MAILLARD PROTEIN CROSSLINK PENTOSIDINE:  
Ribose, Glucose, Fructose and Ascorbate as Pentosidine precursors

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## MATERIALS AND METHODS

### Chemicals

D-glucose, D-ribose, furfural, aminoguanidine and trifluoroacetic acid were purchased from Aldrich (Milwaukee, Wisconsin). Bovine serum albumin (fatty acid and globulin free, Sigma Cat #0281) and heptafluorobutyric acid were purchased from Sigma (St. Louis, MO). Boc-L-lysine and Boc-arginine were purchased from Bachem (Torrance, CA). HPLC grade water, methanol and acetonitrile from Fisher Scientific Co., (Pittsburgh, PA).

### General Methods

<sup>1</sup>H-NMR spectra were recorded with a 400 MHz spectrometer (MSL 400, Bruker instruments Inc., Billerica, MA). Samples for proton NMR were carried out either in deuterated chloroform or water. Tetramethylsilane (TMS) or 3-(trimethylsilyl)propionic acid (DMS) were used as internal standards. Absorption spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer connected to an IBM PC/AT computer (Hewlett-Packard, Inc., Avondale, PA, IBM corp, Boca Raton, FL). Fluorescence spectra were recorded with a J4-8202 Amino-Bowman spectrofluorometer (SLM instruments Inc., Urbana, IL).

Mass spectrometry analyses were performed by Dr Douglas Gage at the National Institutes of Health Mass Spectrometry Facility (Department of Biochemistry, Michigan State University, East Lansing, MI). Molecular weights were determined by fast atom bombardment (FAB) spectroscopy with a JEOL SX 100HF double focusing mass spectrometer. Analysis was initially conducted at low resolution (1000) at accelerating voltage of 10 kV. Samples were dissolved in 0.1% trifluoroacetic acid and mixed with an equal volume of glycerol. Ions were formed by FAB with a 6 keV beam of Xe atoms. FAB-high resolution mass analysis was performed at resolution 20,000 by peak matching on the glycerol matrix ion at m/z 369.

Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel 60 F<sub>254</sub> (0.2 mm Merck). Flash chromatography was performed by the method of Still (4) using 40-65 µm (400-230 mesh) silica gel 60 (E. Merck, No 9385).

### High Performance Liquid Chromatography (HPLC)

HPLC was performed with a Waters HPLC system (Waters Chromatography Div., Milford, MA) equipped with a model 510 dual pump system, 56K manual injector, model 712 WISP automatic injector, and an automated gradient controller. The effluent was monitored with a J4-8202 Amino-Bowman spectrofluorometer (SLM instruments Inc., Urbana, IL) or with a Waters 470 fluorescence detector. Chromatograms were recorded on a Waters 740 data module.

Analytical separations were carried out using 4.6 mm (analytical) Vydac (218TP104) C<sub>18</sub> column by application of a linear gradient of 10-17% of acetonitrile from 0-35 min with 0.01% heptafluorobutyric acid (HFBA) as a counterion at a flow rate of 1 ml/min. Preparative HPLC was achieved with a 2.2 X 25 cm (semi-preparative) Vydac (218TP1010) C<sub>18</sub> column by application of a linear gradient of 0-30% of acetonitrile from 0-65 min with trifluoroacetic acid (0.01%) as counterion and a flow rate of 2 ml/min or with a preparative 2.2 X 25 cm Vydac (218TP1022) C<sub>18</sub> column by application and a linear gradient of 0-30% acetonitrile from 0-50 min with trifluoroacetic acid as counterion and a flow rate of 5 ml/min. Quantitation was performed using pentosidine standards described previously (3).

### Synthesis of Maillard Compounds

#### Synthesis of Pentosidine

Pentosidine was synthesized and purified using a modification of the procedure of Sell and Monnier (3). 7.49 g of D-Ribose, 11.35 g of Boc-L-lysine and 15.5 g of Boc-arginine were dissolved in 200 ml of sodium phosphate buffer (0.5M). The pH of the solution was raised to pH 9 by the addition of a few drops of 5N NaOH. The reaction mixture was heated and maintained at 65 °C for 48 h. The solution was cooled, concentrated and loaded onto a 60 X 5 cm ion exchange column filled with Dowex 50X-400 ion exchange resin (Aldrich Chemical Co., Milwaukee, Wisconsin) equilibrated according to the conditions of Boas (5). The column was sequentially washed with distilled water (1000 ml), 1M pyridine (2000 ml) and 1M pyridine acetate (2000 ml) pH 6. Fractions of 10 ml were collected and spotted on filter paper to check for ninhydrin positive material and analyzed for fluorescence (335/385 nm). Pentosidine eluted with the pyridine acetate buffer in fractions 26-30. These were pooled and concentrated to dryness. The residue was treated with 50 ml of trifluoroacetic acid and left to stand overnight, dried and loaded on to a column of neutral alumina (90X6 cm) that was eluted with 1:1 methanol:water. Fractions containing pentosidine were pooled, dried and subjected to preparative HPLC analysis.

A crude separation step was achieved using reverse phase C<sub>18</sub> preparative column with water/acetonitrile solvent system and trifluoroacetic acid as counter-ion. Final purification was achieved with repetitive injections onto an analytical C<sub>18</sub> reverse phase column using the same solvents as those for preparative HPLC. Yield 50 mg (0.23%).

#### N<sup>ε</sup>-t-Boc-N<sup>ε</sup>-(1-deoxy-D-ribulos-1-yl)-L-lysine (Ribated lysine)

Ribated lysine was synthesized from D-ribose and Boc-lysine using a modified procedure according to Finot and Mauron (6). A suspension of 1.32 g (0.005 mol) of Boc-lysine and 6.1 g (0.04 mol) of D-ribose in 100 ml of methanol was refluxed for 45 min. Methanol was removed using rotary evaporator to yield a dark gum. The residue was dissolved in 10 ml of water and loaded onto a Dowex 50W x 4 column (75 x 2.5 cm). The column was eluted with 0.2M pyridine formate (pH 3.25) and the effluent was tested for the presence of Ribated-lysine by thin layer chromatography using solvent system containing 50:6:10 butanol:acetic acid:water and ninhydrin spray. Fractions corresponding to ribated lysine were combined, concentrated and chromatographed using flash column chromatography (20cm x 5 cm) with silica gel. Elution with 3:1 methanol:ethyl acetate afforded the Anadori product in 45% yield as a white sticky solid. TLC RF in butanol:acetic acid:water 50:6:10 was 0.33. NMR absorptions <sup>1</sup>H-NMR(D<sub>2</sub>O) δ 1.29(s, 9H, -CH<sub>3</sub>), δ 1.61(m, 4H, -CH<sub>2</sub>-CH<sub>2</sub>), δ 3.1 (t, J 10.2H, -CH<sub>2</sub>N), δ 3.21 (s, 2H, -COCH<sub>3</sub>), δ 3.7 (s, 4H, 7H, CH<sub>2</sub>OH), δ 3.8-3.9 (m, aryl), δ 3.9-3.0 (m, 4H, -5, -5') δ 3.20(s, water) and δ 3.2-6.0 (m, 2-3').

#### 3-deoxy-D-glycero-pentose-2-ulose bis(benzoylhydrazine) (3-deoxypentose benzoylhydrazine 3DPH)

This compound was synthesized following the general method of El Khadem et al (7). A solution of D-ribose (15 g) and p-toluidine (6 g) in a mixture of 95% ethanol (400 ml) and acetic acid (10 ml) was boiled under reflux for 30 min. To this solution was added benzoyl hydrazine (19.2 g) and the reflux was continued for 4h. The solution was allowed to cool to 25 °C with stirring. The product was separated after 32 h was collected by filtration, washed with methanol (3 X 100 ml) and air dried to give 11.4 g of material. Recrystallization from 95% ethanol (1L) gave a product with a m.p. 239-240 °C. Yield 55% <sup>1</sup>H-NMR (Dimethyl sulfoxide-d<sub>6</sub>) δ 11.9 & 11.8 (2s NH, disappears on addition of triethylamine and D<sub>2</sub>O), δ 8.05(s, H-1), δ 8.3-7.6 (m, aryl), δ 3.9-3.0 (m, 4H, -5, -5') δ 3.20(s, water) and δ 3.2-6.0 (m, 2-3').

#### 3-deoxy-D-glycero-pentose-2-ulose (3-deoxypentose 3-DP)

This compound was synthesized using the procedure for 3-deoxypentose (7). A suspension of the 3-deoxypentose benzoylhydrazine (2g) in ethanol (60 ml) water (100 ml) acetic acid (2.4 ml) and freshly distilled benzaldehyde (3.2 ml) was boiled under reflux. After an hour, benzaldehyde hydrazine began to precipitate. Reflux was continued for an additional 30 min with the concurrent addition of water (100 ml). Later, the reaction mixture was concentrated and 50 ml of distillate was collected in less than 30 min. The mixture was cooled and filtered, the filtrate concentrated, washed with six 25 ml portions of ether, clarified with charcoal and evaporated under reduced pressure to a thick syrup. A solution of this product in water (10 ml) and hot 100% ethanol (50 ml) was filtered and stirred with a mixture of Amberlite IR-120(H<sup>+</sup>) and Amberlite IR-4B(OH<sup>-</sup>) ion-exchange resin. The mixture was filtered and concentrated under reduced pressure to dryness to yield a syrup. Further purification was achieved by flash chromatography using silica. Eluting with 6:1 ethyl acetate:methanol afforded as a light yellow gum, yield 0.53 g (70 %) TLC RF 0.53 in 3:1 ethyl acetate:methanol. Further characterization was achieved by conversion to its bisbenzoylhydrazine derivative m.p. 226-237 °C. The melting point of the compound was not depressed on a mixed melting point with authentic 3-deoxypentose bisbenzoylhydrazine.

#### N<sup>ε</sup>-t-Boc-N<sup>ε</sup>-(1-deoxy-D-fructos-1-yl)-L-lysine (Glycated lysine)

Glycated lysine was synthesized by the method of Finot and Mauron (7). The compound was obtained in 50% yield as a white fluffy solid. The purity was determined by TLC and HPLC.

#### Kinetic studies on pentosidine formation from D-Ribose, Boc-lysine and Boc-arginine

##### Reaction conditions

Unless otherwise specified, incubation of D-ribose with Boc-Lysine and Boc-arginine were carried out in phosphate buffer (0.5M in sodium) at pH 4, 7.4 or 9 in sealed 10 ml tubes and were heated in a Reacti-therm heating block (Pierce Chemical Co., Rockford, IL). Reactions were generally carried out at 65°C to shorten the reaction time. Aliquots were withdrawn at appropriate intervals and frozen at -4°C until analyzed for pentosidine. For quantitation, samples were thawed at room temperature, reduced for 2h at room temperature by addition of 200-300 fold molar excess of sodium borohydride to inactivate potential pentosidine precursors. The reaction was terminated by the slow addition of 6N HCl to destroy excess borohydride. The reduced samples were subjected to 6N HCl hydrolysis at 110°C for 10 min in order to free the α-amino group. Samples were

evaporated to dryness using a Speedvac concentrator (Savant, Farmingdale, NY) and the resulting residue was dissolved in 1 ml of HPLC solvent and filtered through 0.45  $\mu$ m nylon 66 membrane filters (Rainin Instrument company Inc., Woburn MA 01801). Pentosidine was quantitated by HPLC as described above.

#### Effect of Ratio, Temperature, and pH

The effect of reactant ratio on yield of pentosidine was studied by incubation of D-ribose with Boc-arginine and Boc-lysine at 1:1:1, 3:1:1, 1:3:1 and 1:1:3 ratio, whereby the lower concentration was always 0.25M.

The effect of temperature was investigated by incubating the reactant solutions of D-ribose, Boc-lysine and Boc-arginine at equimolar concentrations of 0.25M at 37°C, 65°C and 80°C in phosphate buffer (0.5M in sodium). Effect of pH was investigated at pH 4, 7.4 and 9 using 0.5M phosphate buffers prepared with various ratios of mono and dibasic 0.5M phosphate buffers.

#### Role of Amadori products and 3-deoxypentose in pentosidine synthesis:

Solutions of Ribated lysine (0.1M), Glucated lysine (0.1M) and 3-deoxypentose (0.1M) were incubated with Boc-arginine at 65°C at pH 4, 7.4 and 9 and processed as described above.

#### Role of acid hydrolysis in Pentosidine formation

0.1M solution of ribated lysine was deprotected with trifluoroacetic acid (3 ml) overnight at room temperature. The trifluoroacetic acid was removed under vacuo and the free Amadori product was incubated with unprotected L-arginine (0.1M) in phosphate buffer (0.5M) at pH 7.4 and 65°C for 48 h. After incubation, the reaction mixture was divided into two portions. One portion was left untouched while the other was hydrolyzed with 6N HCl at 110°C for 10 min. Both portions were dried down and reconstituted in equal volume of solvent and HPLC analysis was carried out under identical analytical conditions.

#### Role of other carbohydrates in pentosidine synthesis

##### Incubation of Bovine Serum Albumin with D-glucose:

Defatted bovine serum albumin (BSA) was dissolved in phosphate buffer (PBS 50 mg/ml) at pH 7.4 and incubated at 37°C with 0.5, 2.5, 250 and 1000 mM D-glucose for 0, 1, 6, 12, 20 and 30 days. The mixtures were sterilized by filtration over 0.2  $\mu$ m filter discs (Gelman Acrodisc 4182) into sterile conical tubes. Aliquots of 0.5 ml were withdrawn using sterile pipetted tips on days 1, 6, 12 and 30 days and stored at -80°C. Prior to analysis, the samples were dialyzed for 48h against 2x3 liters of PBS at -4°C using Spectrapor dialysis tubing (Spectrum Medical Industries, Inc. Los Angeles, CA) with a molecular weight cutoff of 3000 daltons. After dialysis, all volumes were equalized to a final concentration of 24 mg/ml. One hundred microliters of these solutions (2.4 mg protein) were hydrolyzed with 6N HCl at 110°C for 18 hours prior to pentosidine analysis by HPLC.

#### Comparative study of formation of pentosidine from D-glucose, D-ribose, Glucated-lysine and Ribated-lysine.

0.1M solutions of D-ribose, D-glucose, Glucated-lysine and Ribated-lysine were incubated with 0.1M of Boc-lysine and Boc-arginine in phosphate buffer (0.5M) at pH 7.4 and 65°C for 48 h. Aliquots were removed at regular intervals and refrigerated (-4°C). After 48h, the samples were analyzed for pentosidine by HPLC after the usual workup.

#### Comparative study of formation of pentosidine from D-ribose, D-glucose, ascorbic acid and dehydroascorbic acid:

0.1M solutions of D-ribose, D-glucose, ascorbic acid and dehydroascorbic acid were incubated with 0.1M of N<sup>ε</sup>-Boc-L-lysine and N<sup>ε</sup>-Boc-L-arginine in phosphate buffer (0.5M) at pH 7.4 and 65°C for 48h. Aliquots were removed at regular intervals and refrigerated (-4°C). After 48h, the samples were analyzed for pentosidine by HPLC after the usual workup.

TABLE I

Summary of pentosidine yields at plateau levels using equimolar concentrations of reactants at 65°C and 48 hrs

Reactants	pH	Yield%
Ribated Lysine & Arginine	9	12.4
	7.4	2.42
	4	0.01
D-Ribose, Lysine & Arginine	9	3.9
	7.4	2.75
	4	.005
Glucated Lysine & Arginine	7.4	.99
	9	.41
	7.4	.25
D-Fructose, Lysine & Arginine	7.4	.29
	9	.139
	7.4	.033
3-DP, Lysine & Arginine	7.4	.014
	9	.08
	7.4	.079
Ascorbate, Lysine & Arginine	7.4	.08
Dehydroascorbate, Lysine & Arginine	7.4	.079

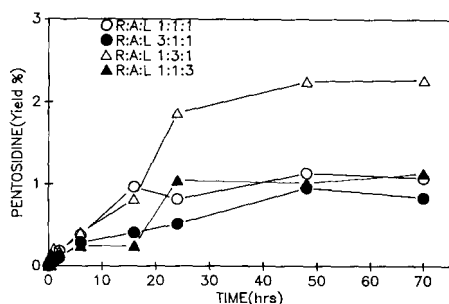


Fig 1 Effect of varying the ratio of D-ribose(R), Boc-arginine(A) and Boc-lysine(L) on the formation of pentosidine at pH 7.4 and 80°C. All concentrations are in the multiple of 0.25M, i.e. R:A:L 1:1:1, R:A:L 3:1:1, R:A:L 1:3:1, R:A:L 1:1:3. The yield represents the mol percentage of pentosidine formed per mol of theoretical yield.

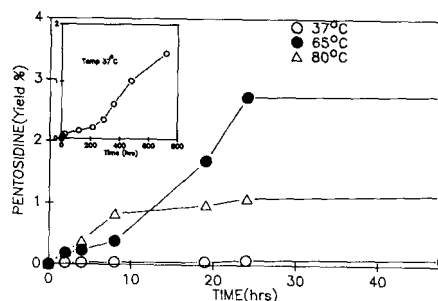


Fig 2 Effect of Temperature on the formation of pentosidine from equimolar concentrations of D-ribose, Boc-arginine and Boc-lysine. The reaction conditions are identical with those described in Fig 1.

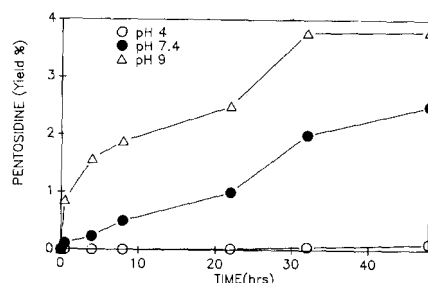


Fig 3 Effect of pH on the condensation of equimolar quantities (0.1M) of D-ribose, Boc-Arginine and Boc-Lysine at 65°C and pH 4.0, 7.4 and 9.0.

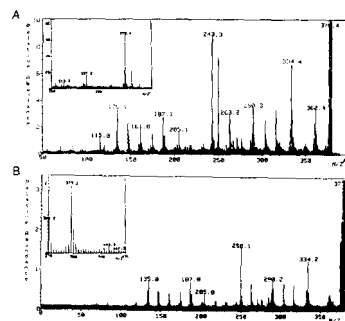


Fig 4 Fast Atom Bombardment Mass spectra of pentosidine obtained from Boc-lysine Amadori products of D-ribose (upper) and D-glucose (lower) reacted with equimolar concentrations of Boc-arginine at 65°C for 48 hrs.

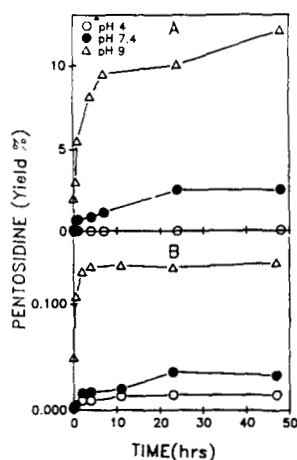


Fig. 5: Comparative rates of formation of pentosidine from ribated lysine with Boc-arginine (upper) and from 3-deoxypentoseone reacted with equimolar concentrations of Boc-arginine and Boc-lysine (lower), at various pH of 4.0, 7.4 and 9.0.

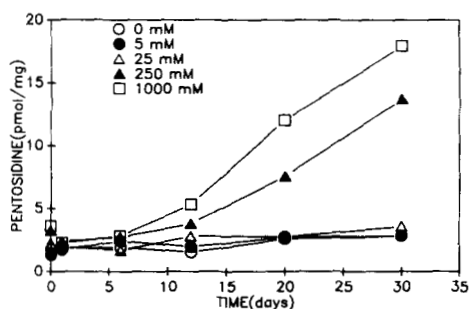


Fig. 6: Formation of pentosidine in bovine serum albumin incubated upto 30 days with D-glucose in phosphate buffer ed saline at concentrations of 0 mM, 5 mM, 25 mM, 250 mM and 1000 mM.

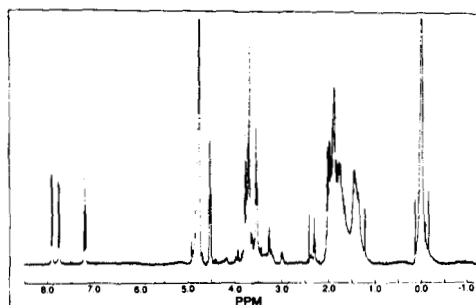


Fig. 7: 400 Mhz <sup>1</sup>H-NMR spectrum of pentosidine obtained from the reaction of D-glucose with Boc-lysine and Boc-arginine. NMR solvent was D<sub>2</sub>O.

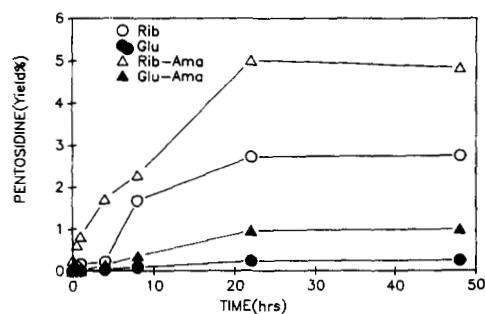


Fig. 8: Comparative rates of formation of pentosidine from D-ribose and D-glucose incubated with Boc-lysine and Boc-arginine, ribated lysine (Rib-Ama) and glucated lysine (Glu-Ama) with equimolar concentration of Boc-arginine at pH 7.4 and 65°C.

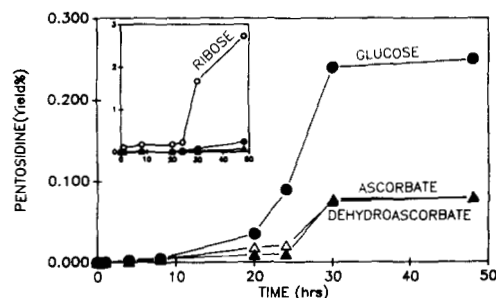


Fig. 9: Comparative rates of formation of pentosidine from D-ribose, D-glucose, ascorbate and dehydroascorbate at pH 7.4 and 65°C.

TABLE I

Summary of pentosidine yields at plateau levels using equimolar concentrations of reactants at 65°C and 48 hrs

Reactants	pH	Yield%
Ribated Lysine & Arginine	9	12.4
	7.4	2.42
	4	0.01
D-Ribose, Lysine & Arginine	9	3.9
	7.4	2.75
	4	.005
Glucated Lysine & Arginine	7.4	.99
D-Glucose, Lysine & Arginine	9	.41
	7.4	.25
	4	0
D-Fructose, Lysine & Arginine	7.4	.29
	9	.139
	7.4	.033
2-OP, Lysine & Arginine	9	.014
	7.4	.08
	4	.079
Ascorbate, Lysine & Arginine	7.4	.08
Dehydroascorbate, Lysine & Arginine	7.4	.079