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Formation of Pentosidine during Nonenzymatic Browning of Proteins by Glucose

IDENTIFICATION OF GLUCOSE AND OTHER CARBOHYDRATES AS POSSIBLE PRECURSORS OF PENTOSIDINE *IN VIVO**

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A fluorescent compound has been detected in proteins browned during Maillard reactions with glucose *in vitro* and shown to be identical to pentosidine, a pentose-derived fluorescent cross-link formed between arginine and lysine residues in collagen (Sell, D. R., and Monnier, V. M. (1989) *J. Biol. Chem.* 264, 21597–21602). Pentosidine was the major fluorophore formed during nonenzymatic browning of ribonuclease and lysozyme by glucose, but accounted for <1% of non-disulfide cross-links in protein dimers formed during the reaction. Pentosidine was formed in greatest yields in reactions of pentoses with lysine and arginine in model systems but was also formed from glucose, fructose, ascorbate, Amadori compounds, 3-deoxyglucosone, and other sugars. Pentosidine was not formed from peroxidized polyunsaturated fatty acids or malondialdehyde. Its formation from carbohydrates was inhibited under nitrogen or anaerobic conditions and by aminoguanidine, an inhibitor of advanced glycation and browning reactions. Pentosidine was detected in human lens proteins, where its concentration increased gradually with age, but it did not exceed trace concentrations (≤5 μmol/mol lysine), even in the 80-year-old lens. Although its precise carbohydrate source *in vivo* is uncertain and it is present in only trace concentrations in tissue proteins, pentosidine appears to be a useful biomarker for assessing cumulative damage to proteins by nonenzymatic browning reactions with carbohydrates.

Glycation (nonenzymatic glycosylation) and Maillard (nonenzymatic browning) reactions of proteins by reducing sugars are thought to contribute to the aging and cross-linking of tissue proteins (1–3). Acceleration of these reactions as a result of hyperglycemia in diabetes and the consequent increase in structural and functional modification of long-lived proteins are implicated in the pathogenesis of the chronic complications of diabetes (4). Since the extent of glycation of protein is relatively constant with age (5–7), a major challenge in research on the Maillard reaction *in vivo* is to identify specific products formed in tissue proteins during later stages of the Maillard reaction and to evaluate their possible role in the development of complications. Fluorescent products have provided a focus for this work since fluorescence increases in proteins during browning reactions *in vitro* (8, 9), and the fluorescence of long-lived proteins, such as lens crystallins and tissue collagens, increases with age and at an accelerated rate in diabetes (10). Increased collagen-linked fluorescence is also correlated with the appearance of complications in diabetes (11).

In 1989, Sell and Monnier isolated and characterized the fluorescent cross-link, pentosidine (Fig. 1), in human collagens and other tissue proteins. Pentosidine was found to accumulate in tissue collagens with age and at an accelerated rate in diabetic patients with renal complications (13). Since it was readily formed in synthetic reactions between pentoses, arginine, and lysine, Sell and Monnier (12) proposed that this compound was also formed spontaneously by reaction between pentoses and proteins *in vivo*. At the same time our laboratory had identified a fluorescent compound, named Maillard Fluorescent Product 1 (MFP-1),[1] which was formed in browning reactions of glucose with model proteins *in vitro* and was also observed to accumulate in tissue proteins with age and in diabetes (14–16). Because of their similar chromatographic properties, absorbance maxima (328 nm) and fluorescence spectra (excitation and emission maxima of 328 and 378 nm, respectively), we considered that MFP-1 and pentosidine might be related compounds, perhaps differing by a single carbon atom, *i.e.* containing six and five carbohydrate-derived carbons, respectively. In this report we show, however, that MFP-1 and pentosidine are the same compound and that pentosidine is formed by reaction of proteins with glucose, pentose, and numerous other carbohydrates and Maillard reaction products under aerobic conditions *in vitro* (Fig. 1). We also present evidence that pentosidine accounts for <1% of the total cross-links formed in proteins during browning reactions with glucose *in vitro*. Although its precise carbohydrate source is uncertain and it is present in only trace concentrations in tissue proteins, the results presented below indicate that pentosidine should be useful as a biomarker for assessing cumulative damage to proteins by nonenzymatic browning reactions with carbohydrates.

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

The Origin of Pentosidine in Tissue Proteins—The glyca-

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[1] The abbreviations used are: MFP-1, Maillard fluorescent product 1, shown in this paper to be pentosidine; CML, N^{ϵ}-(carboxymethyl)lysine; CMhL, N^{ϵ}-(carboxymethyl)hydroxylysine; FL, fructoselysine; HPLC, high performance liquid chromatography.

[2] Portions of this paper (including "Materials and Methods," "Results," Figures 2–8 and Tables I–IV) are presented in miniprint at

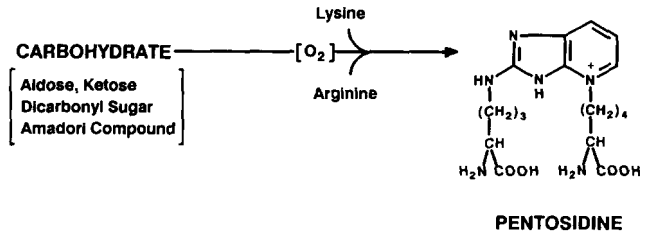

FIG. 1. **Generalized pathway for formation of pentosidine.** In addition to arginine and lysine, this scheme includes several possible carbohydrate precursors of pentosidine identified in this study. Oxygen is required for the formation of pentosidine from these precursors.

tion hypothesis proposes that the pathogenesis of long-term complications in diabetes is a consequence of chemical, structural, and functional modifications of proteins resulting from non-enzymatic reactions with glucose. The glucose or Maillard theory of aging (1, 3) also treats aging as a natural consequence of cumulative chemical modification of proteins via the Maillard reaction and considers diabetic complications to be, in part, an expression of accelerated age-related pathology resulting from hyperglycemia. The chemical modifications and damage to proteins and other biomolecules are thought to be produced during the late or browning stages of the Maillard reaction, however, evidence for age-dependent accumulation of Maillard products in tissue proteins is still limited to only three compounds: (i) N^{ϵ}-(carboxymethyl)lysine (CML) in lens proteins (5) and skin collagen (7), (ii) N^{ϵ}-(carboxymethyl)hydroxylysine (CMhL) in skin collagen (7), and (iii) pentosidine in lens proteins (Fig. 8) and collagens (12). Levels of CML, CMhL, and pentosidine are also increased in diabetic, compared to non-diabetic skin collagen (12),[3] consistent with the hypothesized role of the Maillard reaction in the accelerated aging of proteins in diabetes.

There is increasing uncertainty about the origin(s) of Maillard products in tissue proteins. CML and CMhL, which were originally observed as products of oxidative cleavage of glucose-derived Amadori compounds *in vitro* (19), may also be derived from reactions of proteins with ascorbate (31), as well as glucose (19), ribose, threose, and probably other sugars *in vivo* (31). Thus, while CML and CMhL can be measured as indicators of carbohydrate-derived damage, the exact identity of the carbohydrate source remains uncertain. Similarly, while Sell and Monnier (12, 13) characterized pentosidine as a pentose-derived fluorophore in proteins, the results of the present study suggest that glucose itself, or Amadori adducts of glucose to protein, can also be sources of pentosidine, thus explaining the increase in pentosidine in tissue proteins in diabetes. However, the origin of pentosidine from ascorbate, fructose, pentose, and lower sugars cannot be rigorously excluded, either during the normal aging process or in diabetes, and glucose may be only one of many sources of Maillard reaction damage to tissue proteins.

Measurements of the pentoses in non-diabetic serum indicate that they are present in micromolar concentrations (reviewed in Ref. 12), but, considering the relative reactivity of glucose and pentoses *in vitro* (Table II), this would still be sufficient to support a significant role for pentoses in the formation of pentosidine *in vivo*. However, there is no evidence that pentose concentrations are increased in diabetes, as might be expected if ribose were derived directly from glucose or indirectly from Amadori adducts. The resolution of this question may be complicated since pentoses could be a major source of the age-dependent increase in pentosidine in non-diabetic tissue, while the incremental increase in pentosidine in diabetic tissue could be attributed to glucose.

Mechanism of Formation of Pentosidine—The requirement for oxygen in the formation of pentosidine from glucose, ribose, ascorbate, and Amadori compounds provides evidence that oxidation is required at some stage in the formation of pentosidine. The requirement for the Amadori rearrangement is less certain. Pentosidine is formed from Amadori compounds, such as 1-deoxy-1-morpholino-D-fructose (Table IV) and N^{α}-formyl-N^{ϵ}-fructoselysine (data not shown), and from 3-deoxyglucosone (Table II) which is formed on rearrangement and dissociation of Amadori adducts (33). However, kinetic experiments, such as in Fig. 6, show at most a short lag-phase in the formation of pentosidine from glucose. Thus, oxidative fragmentation of Schiff base adducts or eneaminol intermediates (34) cannot be excluded, since these are formed rapidly from sugars and amines and may also be formed from Amadori compounds by reversal of the Amadori rearrangement (19). Numerous oxidation and cleavage products are known to be formed on oxidation of carbohydrates (35), but only trace amounts of the critical intermediate(s) would be required for formation of pentosidine at the yields obtained in our experiments. Among the likely candidates for pentosidine precursors are dicarbonyl sugars (35), which should react readily with arginine (36). Pentoses and 5-carbon dicarbonyl sugars may also be intermediates in the formation of pentosidine from 6-carbon and smaller precursors. The 5-carbon sugars could be formed from larger sugars by oxidative fragmentation (35) or from other sugars by condensation and/or reverse aldol reactions.

Significance of Pentosidine in Tissue Proteins—Our estimate of the pentosidine content of skin collagen (up to 7 pmol pentosidine/mg collagen at 70 years of age) appears to be about 5-fold lower than reported by Sell and Monnier (~32 pmol/mg at age 70) (12, 13), but there is essential agreement that pentosidine is present at only trace concentrations. Our measurements indicate approximately 2 mmol pentosidine/mol triple-stranded collagen and 25 μmol pentosidine/mol lens crystallin (assuming crystallin M_r = 20,000) at age 70, with at most a 2-fold increase in diabetes.[3] We think it unlikely that this level of pentosidine would have a major impact on collagen or lens protein structure, function, or turnover. However, even in studies on the browning of lysozyme (Fig. 2) or RNase (Fig. 6) by glucose *in vitro*, when the proteins are extensively modified by glucose, pentosidine accounts for only a small fraction of the total modification of lysine residues in the protein and less than 1% of the cross-links in protein dimers formed during the glycation reaction (Figs. 6 and 7). At the same time, the kinetics of pentosidine formation in the proteins during glycation reactions (Fig. 6) reflect the accumulation of more extensive chemical modifications (loss of lysine residues, formation of CML and fluorescent products, cross-linking of the protein) and damage to the protein. Although pentosidine does not discriminate between browning derived from various sugars, it does appear to be specific for carbohydrates since it is not formed on incubation of lysine and arginine with malondialdehyde (Table II) or other products of oxidation of fatty acids. Thus, despite its low concentration in tissues, pentosidine should be useful as a unique biomarker of carbohydrate-dependent damage to protein via the Maillard reaction.

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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.

[3] D. G. Dyer, J. A. Blackledge, S. R. Thorpe, D. R. McCance, T. J. Lyons, and J. W. Baynes, manuscript in preparation.

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Supplementary Material to:

FORMATION OF PENTOSIDINE DURING NONENZYMATIC BROWNING OF PROTEINS BY GLUCOSE: IDENTIFICATION OF GLUCOSE AND OTHER CARBOHYDRATES AS POSSIBLE PRECURSORS OF PENTOSIDINE *IN VIVO*

by

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MATERIALS AND METHODS

Materials - Protease (type XIV, Pronase E), leucine aminopeptidase (type IV-S), hen egg white lysozyme (grade I) and bovine pancreatic ribonuclease A (Type XII-A), were obtained from Sigma Chemical Co., and α-chymotrypsin from Cooper Biomedical. [1-^{14}C], [6-^{14}C] and [U-^{14}C]Glucose were purchased from American Radiolabeled Chemicals and purified by chromatography on an Aminex HPX-87C carbohydrate column (Ca^{++}-form; Bio-Rad) using deionized water as eluent (17,18). When radioactive glucose was diluted with non-radioactive glucose in experiments, the specific radioactivity of the glucose was based on the glucose concentration measured using a glucose oxidase-peroxidase kit (Amresco). N^{α}-formyl-N^{ϵ}-fructoselysine and N^{ϵ}-(carboxymethyl)lysine (CML) were prepared as described previously (19). N^{ϵ}-hexitollysines, a mixture of mannitol- and glucitol-lysine, were prepared by reduction of N^{α}-formyl-N^{ϵ}-fructoselysine with $NaBH_4$, followed by acid hydrolysis to remove the formyl group (19). All other reagents were obtained from Sigma Chemical Co., unless otherwise indicated.

HPLC Analyses - Amino acid analysis was performed by ion exchange HPLC using a pH and salt gradient (NaCl-Na citrate) on a Waters HPLC system equipped with a post-column reactor for measuring amino acids as their o-phthaldialdehyde derivatives (18-20). Reversed-phase HPLC (RP-HPLC) was used for purification of pentosidine and measurement of the pentosidine content of tissue proteins. Chromatography was performed on a Varian 5500 liquid chromatograph using C-18 silica columns, a Supelcosil LC-318 column (250 x 4.6 mm, 5 μm; Supelco) for analytical separations and quantitative analyses, and a Hi-Pore RP-318 column (250 x 10 mm, 5μm; Bio-Rad) for preparative isolations; flow rates were 1 and 4.5 ml/min, respectively. Two different gradient systems were used to optimize various separations, one containing 0.1% trifluoroacetic acid (TFA), and the other, 0.1% heptafluorobutyric acid (HFBA) as organic modifier in each solvent. In both cases Solvent A was H_2O and Solvent B was 50% acetonitrile. The solvent gradients were: for the TFA system, 0 min = 100% A, 15 min = 9% B, 25 min = 9% B, 70 min = 60% B, 80 min = 100% B; and for the HFBA system, 0 min = 10% B, 40 min = 18% B, 60 min = 18% B, 65 min = 100% B. In both cases pentosidine eluted near the end of an isocratic region of the gradient, 25 min in TFA (see Figure 2) and 53 min in HFBA (see Figure 3), and was detected by its fluorescence at Ex/Em = 328/378 nm, using either a Gilford Fluoro-IV or Shimadzu RF-5000 spectrofluorometer, equipped with a continuous flow cell. Peak areas were integrated using a Baseline Chromatography Workstation (Dynamic Solutions).

Synthesis and Quantitation of Radioactive Pentosidine - N^{α}-Acetyl-[G-^{3}H]-arginine) was prepared by adding 25 μmoles arginine in 200 μl acetic acid to 250 μCi [G-^{3}H]arginine (ICN Biomedicals), followed by 200 μl (~2.5 mmol) acetic

anhydride. The reaction was quenched after 5 min by the addition of 1.5 ml of water, and the water and acetic acid removed by rotary evaporation. The recovery of N^{α}-acetyl-[G-^{3}H]arginine was >95% by amino acid analysis. N^{α}-Acetyl-[4,5-^{3}H]lysine was prepared from [4,5-^{3}H]lysine (ICN Biomedicals). Briefly, radioactive lysine (400 μCi) was dried under a stream of N_2 and mixed with 40 μmol lysine, 300 μl (~4 mmol) acetic anhydride, and 300 μl acetic acid at 25°C. The mixture was vortexed and quenched after 45 seconds by the addition of 2 ml of water. N^{α}-acetyllysine and lysine were separated on the amino acid analyzer, and aliquots of eluant fractions counted for radioactivity. The reaction yielded N^{α}-acetyllysine (27%), N^{ϵ}-acetyllysine (3%), N,N'-diacetyllysine (20%) and lysine (50%). The unreacted lysine was recovered and desalted by chromatography on a 2 ml column of Dowex-50-H$^+$ using 2 N NH_4OH as eluant, recycled through the acetylation and purification procedure, and pooled with the original product to yield 19.2 μmol N^{α}-acetyl-[4,5-^{3}H]lysine (48% recovered yield). The specific activity of the radioactive N^{α}-acetyl amino acids was determined experimentally by amino acid analysis with on-line flow-through radioactivity detection (Flo-One\Beta; Radiomatic Instruments).

Radioactive pentosidine, labeled with either arginine, lysine or glucose, was prepared by including the radioactive compound in reaction mixtures (500 μl total volume) containing 100 mM each glucose, N^{α}-acetylarginine and N^{α}-acetyllysine in 200 mM phosphate buffer, pH 9.0. The reactions were incubated for 1 week at 65°C. The pH decreased rapidly during the initial stages of the reaction and was re-adjusted to 8.5 - 9.0 (using narrow-range pH paper) by addition of 2 N NaOH, initially at 6-12 hour intervals, then daily, as required. The reaction mixture was applied to a 1 ml Supelclean solid phase extration tube (SPE; Supelco, Inc.) equilibrated in water, the column washed with 2 ml of water, and the crude di-N-acetyl-pentosidine eluted with 2 ml of 5% acetonitrile. After de-acetylation by heating in 6 N HCl for 1 hr at 110°C, pentosidine was purified by analytical RP-HPLC using sequential HFBA and TFA gradients, as described above. The final specific radioactivity of the purified pentosidine was determined by integration of the pentosidine peak fluorescence and measurements of the specific radioactivity of the precursors and the radioactivity content of the pentosidine fractions (see Figure 5). The standardization of RP-HPLC assays for the pentosidine content of browned proteins and tissue samples was based on the fluorescence and radioactivity content of the pentosidine preparations labeled with amino acids, i.e., 4.6 nmol pentosidine/10^9 area units (see Table I).

Preparative Synthesis and Purification of Pentosidine - An aqueous solution (500 ml, 100 mM N^{α}-acetylarginine, N^{α}-acetyllysine and glucose in 200 mM phosphate buffer, pH 9.0) was heated at 65°C for 3 days, with pH adjustment, as above. The solution was evaporated to a syrup and di-N-acetyl-pentosidine extracted with 100 ml of methanol to precipitate inorganic salts. Methanol was removed by rotary evaporation, the residue dissolved in 80 ml of water, and 20 ml aliquots were applied to a 20 ml preparative C-18 column (LiChroprep RP-18, 40-63 μm, bulk resin; EM Science) equilibrated in water. The column was washed with 80 ml of water to remove salts and browning products, and the di-N-acetyl-pentosidine eluted with 80 ml of 5% acetonitrile. The column eluates were pooled and concentrated to dryness, diluted in 2% HFBA and the di-N-acetyl-pentosidine isolated by semi-preparative RP-HPLC using the HFBA buffer system with fluorescence detection. The di-N-acetyl-pentosidine had fluorescence maxima identical to those of pentosidine, and was pooled, concentrated and repurified using the TFA system. Following deacetylation (see above), the pentosidine was finally purified on the analytical column using the HFBA buffer system. The yield of pentosidine was 5.8 μmol (2.2 mg, ~0.01% overall yield).

Glycation of Proteins and Analysis of Products - Procedures for glycation of proteins were similar to those described previously (18,20). Briefly, lysozyme or ribonuclease (20 mg/ml) was incubated with 250 mM glucose in 0.2 M phosphate buffer at 37°C under an air atmosphere. The reaction mixtures were sterilized by ultrafiltration through a 0.2 μm Acrodisc filter (Gelman Sciences). To discharge Schiff base adducts, the protein was dialyzed overnight at 4°C against 50 mM phosphate buffer, pH 7.4. The extent of glycation of the protein was measured following reduction of the protein for 12 hr with a 50-fold molar excess of $NaBH_4$ over protein, hydrolysis in 6 N HCl for 18 hr at 95°C, and quantitation of hexitollysines by amino acid analysis (18-20). CML, the major product formed on oxidative cleavage of glucose adducts to proteins (19,21), was also measured by amino acid analysis of the $NaBH_4$-reduced protein.

For enzymatic digestion of glycated lysozyme, aliquots were removed from glycation reactions, dialyzed overnight at 4°C against 50 mM phosphate buffer, pH 7.4, and concentrated to dryness by centrifugal evaporation. The protein was then reduced with β-mercaptoethanol in 8 M urea, and S-carboxymethylated with iodoacetic acid (22). After precipitation with acetone and drying under a stream of nitrogen, the protein was digested sequentially in phosphate buffer at 37°C for 12 hr each with chymotrypsin (2% w/w) at pH 8.2, followed by pronase E (2% w/w) and leucine aminopeptidase (1% w/w) at pH 7.4. The enzymes were inactivated after each digestion by heating at 100°C for 2 minutes. Based on amino acid analysis, ≥90 % of the peptide bonds in the protein were cleaved by this digestion procedure. The pentosidine content of acid hydrolyzed or proteolytically digested protein was measured by RP-HPLC with post-column fluorescence detection, then normalized to the valine content of the protein, as determined by acid hydrolysis and amino acid analysis.

Analysis of Pentosidine Content of Tissue Proteins - Whole human lenses were obtained from the Medical College of Georgia Eye Bank (Augusta, GA) and the South Carolina Lions Eye Bank (Columbia, SC) and stored at -70°C until used. Lenses were decapsulated, homogenized in deionized water and dialyzed as described previously (5,6). An aliquot of lens protein homogenate containing 16 mg protein, measured by the biuret method (23), was dissolved in 4 ml 10 mM $NaBH_4$ in 0.1 N NaOH. After incubation for 4 h at room temperature, residual borohydride was discharged and the sample diluted to 2 mg protein/ml by the addition of an equal volume of concentrated HCl. Borohydride reduction was not essential for measurement of pentosidine, but yielded more reproducible analytical results because of decreased fluorescence background in $NaBH_4$-reduced samples. The protein was then hydrolyzed under vacuum for 18 hr at 95°C (18,20). HCl was removed by centrifugal evaporation (SpeedVac Concentrator, Savant Instruments) and the sample dissolved in 1 ml of 1% trifluoroacetic acid. An aliquot was removed for amino acid analysis to determine the lysine content of the hydrolysate. A second aliquot (300 μl, ~5.0 mg protein) was analyzed for pentosidine by RP-HPLC using the TFA gradient system.

Insoluble skin collagen samples were prepared by extraction with salt, organic solvent and acetic acid, as described previously (24). For measurement of pentosidine, 3 mg of collagen (dry weight) was hydrated in 750 ul of deionized water by heating at 65°C for 2 minutes and then reduced by addition of 1.5 ml of 3.3 mM $NaBH_4$ in 0.1 N NaOH, followed by incubation for 18 hr at room temperature. The collagen was hydrolyzed as described above for the lens protein, HCl removed by centrifugal evaporation and the sample dissolved in 1 ml of 1% heptafluorobutyric acid (HFBA). The samples (500 μg collagen) were analyzed in the HFBA gradient system in order to increase the reproducibility and resolution of pentosidine from other minor components in the collagen samples.

Spectroscopy - NMR spectra were recorded on a Bruker AM-500 spectrometer running at 500 and 125 MHz for proton and ^{13}C-NMR, respectively. High resolution fast atom bombardment mass spectrometry was performed on a VG-70SQ spectrometer.

RESULTS

Detection of Pentosidine in Browned Lysozyme - Lysozyme was used as a model protein in preliminary studies on the formation of fluorescent products during browning reactions with glucose. After incubation of lysozyme with 0.25 M glucose in 0.2 M phosphate buffer, pH 7.4, for 28 days at 37°C, the fluorescence spectrum of the dialyzed, browned protein showed emission and excitation maxima at approximately 325 and 400 nm, respectively. The identification of specific glucose-derived fluorophores formed in the browning reaction at physiological pH and temperature was complicated by the fact that acid hydrolysis of the browned protein produced about a 10-fold increase in fluorescence. To avoid the production of artifacts during hydrolysis, the browned protein was reduced and S-carboxymethylated, then digested sequentially with chymotrypsin, pronase and aminopeptidase, as described in "Materials and Methods". The fluorescence of the protein was not significantly increased (<10%) by this procedure. Analysis of the proteolytic digest by RP-HPLC with fluorescence detection at 325/400 nm yielded one major fluorescent product (Figure 2A); this fluorophore had maxima at 328/378 nm and was not present in native lysozyme (data not shown). Aliquots of the proteolytic digest were analyzed on the HPLC system, but by-passing the column, to estimate the total fluorescence in the protein digest at these wavelengths. The fluorophore (eventually identified as pentosidine) accounted for approximately 80% of the fluorescence at 328/378 nm in the browned protein.

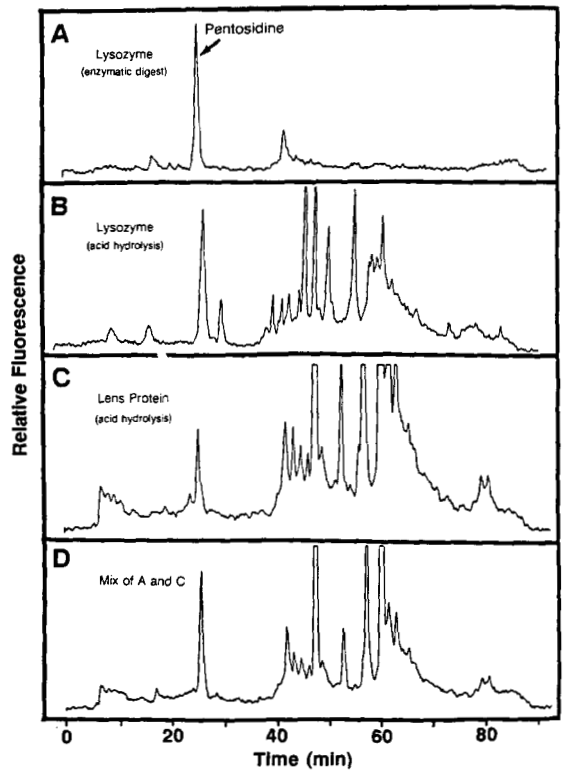

Figure 2. **Detection of pentosidine in glycated lysozyme and in lens proteins.** Lysozyme (20 mg/ml) was glycated for 28 days, then dialyzed, reduced with $NaBH_4$ and either digested with proteases or hydrolyzed in 6 N HCl, all as described in "Materials and Methods". Human lens proteins were isolated from a 78 year old donor and hydrolyzed in 6 N HCl as described in "Materials and Methods". Samples were analyzed by reversed-phase HPLC using an acetonitrile/water gradient containing 0.1% TFA, and pentosidine detected by its fluorescence at Ex = 328 nm and Em = 378 nm with the Gilford Fluoro-IV spectrofluorometer. **A**, Analysis of an enzymatic digest of glycated lysozyme (125 μg protein), showing pentosidine at 25 min. The peak at 42 min was identified as tryptophan. **B**, Analysis of an acid hydrolysate of glycated lysozyme (125 μg). **C**, Analysis of acid hydrolysate of lens protein (2 mg). **D**, Analysis of mixture of 62 μg of enzymatically digested, glycated lysozyme (A) and 1 mg of hydrolyzed lens protein shown in (C).

We observed that reduction by $NaBH_4$ at neutral or alkaline pH or treatment with 6 N HCl for 24 hr at 110°C had no effect on the elution position or recovery of the fluorescent compound. Thus, the fluorophore was readily detected in the acid hydrolysate of browned lysozyme (Figure 2B), and the yield from the acid hydrolysate agreed well with that obtained by proteolytic digestion of the protein (Figure 2A vs. 2B). Additional fluorescent products formed during acid hyrolysis, but not present in the original browned protein, are apparent by comparison of the chromatograms in Figures 2A and 2B. The presence of the fluorophore in human lens proteins is illustrated in Figure 2C and supported by the mixing experiment shown in Figure 2D. Similar results were obtained in mixing experiments with the samples in Figure 2A and 2B, and in Figure 2B and 2C. The chromatograms of the acid hydrolysates of browned lysozyme and natural lens proteins (Figure 2B and 2C) show a number of coincident peaks, but many of these products appear to be formed during the acid hydrolysis since they are not present in the proteolytic digest of browned lysozyme (Figure 2A). Most of these peaks are thought to originate from the degradation of tryptophan since they were also not detected in an acid

hydrolysate of browned RNase A, a protein which does not contain tryptophan (data not shown).

Synthesis of Pentosidine from Glucose - The fluorophore isolated from browned lysozyme and lens proteins had fluorescence properties similar to those of pentosidine, the pentose-derived fluorescent compound described by Sell and Monnier (12), i.e., an absorbance maximum at 328 nm, fluorescence maxima at 328/378 nm, and a minimum in fluorescence intensity at pH 9. We prepared pentosidine from N^{α}-acetylarginine, N^{α}-acetyllysine and ribose, using conditions similar to those described by Sell and Monnier (12) (see legend to Figure 3). The glucose-derived fluorophore was prepared using the same conditions, but substituting glucose for ribose in the reaction mixture. Since pentosidine was originally described in tissue collagens (12), we also compared the RP-HPLC chromatographic behavior of the fluorescent product recovered in hydrolysates of skin collagen with those prepared synthetically from glucose or ribose. As shown in Figure 3, the glucose-derived fluorophore and pentosidine had identical chromatographic properties (also using the TFA solvent system and various gradient modifications), which, coupled with the similarities in absorbance and fluorescence properties, strongly suggested that the glucose-derived fluorophore was, in fact, pentosidine. The yield of the fluorophore from ribose reaction mixtures was typically 500-1000 fold greater than that from glucose (legend to Figure 3, and Table II, discussed below). To exclude possible ribose or pentose contaminants in the glucose used for the protein browning and synthetic reaction mixtures, the pentose content of the glucose was determined by measuring glucose and pentoses as their alditol acetates (25) by selected ion monitoring gas chromatography-mass spectometry. These analyses yielded less than 10 ppm ribose or pentose contamination in the glucose preparation, a level 100 fold lower than that required to explain the yield of pentosidine from ribose contaminants.

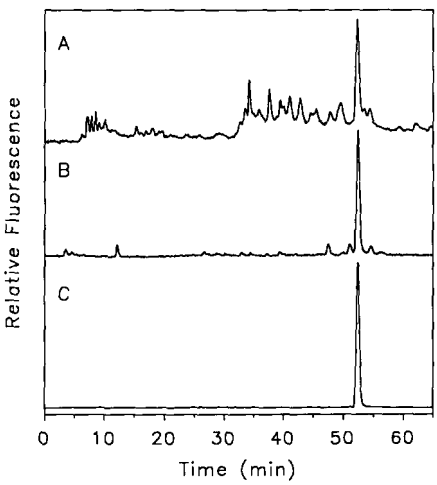

Figure 3. **Identification of pentosidine in human skin collagen (A) and in reactions of glucose (B) or ribose (C) with N^{α}-acetylarginine and N^{α}-acetyllysine.** Samples were analyzed using the HFBA gradient system with the Shimadzu RF-5000 spectrofluorophotometer. **A**, Pentosidine in skin collagen sample (500 µg) from a 20 year old donor. **B and C**, Glucose or ribose (100 mM) were incubated with 100 mM N^{α}-acetylarginine and N^{α}-acetyllysine in 0.2 M phosphate buffer, pH 9, for 6 hours at 60°C. The samples were reduced with $NaBH_4$ and deacetylated by heating in 6 N HCl for 2 hr at 100°C. The volume injected from the glucose reaction (C) was equivalent to 1000 times the volume injected from the ribose reaction (B).

The experimental data suggested that pentosidine was formed from glucose, with loss of one carbon in the process, but it was conceivable that the glucose and ribose products were different, but structurally related compounds which were not separable by RP-HPLC analysis. For this reason we decided to proceed with the preparative synthesis and characterization of the glucose fluorophore, as described in "Materials and Methods". Analysis of the product by ^1H NMR (Figure 4A) indicated that the glucose-derived fluorophore was, in fact, pentosidine (Figure 1). The ^{13}C NMR spectrum in Figure 4B confirmed the presence of the 6 aromatic carbons in the imidazo[4,5-b]pyridinium ring. INEPT analysis (26) also confirmed the presence of 3 quaternary and 3 singly protonated aromatic carbons in the compound, consistent with the structural assignment of Sell and Monnier (12). High resolution fast atom bombardment mass spectroscopy also yielded m/z = 379.2089 compared to the calculated mass of 379.2094 (1.3 ppm error) for pentosidine, $C_{17}H_{27}N_6O_4$. These results established that the glucose-derived fluorescent compound which we had originally identified as MFP-1 (14-16) was, in fact, pentosidine, and indicated that pentosidine could be formed from glucose.

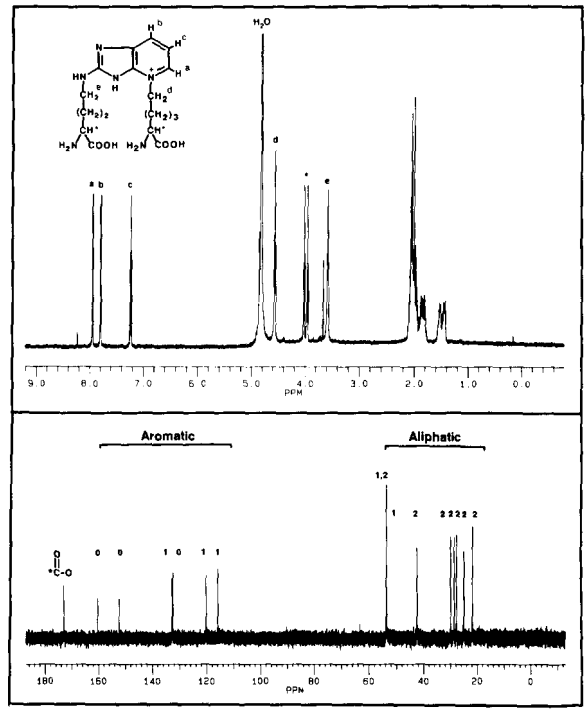

Figure 4. **Proton and ^{13}C NMR spectra of pentosidine.** Pentosidine (1.2 mg) was synthesized from glucose, N^{α}-acetylarginine and N^{α}-acetyllysine, and purified as described in "Materials and Methods". NMR spectra were recorded in deuterium oxide (1 ml for ^{13}C, and 2 ml for proton). The proton assignments in the upper frame are based on those of Sell and Monnier (12). The ^{13}C-NMR spectrum in the lower frame was resolved into unprotonated (0), singly protonated (1) and doubly protonated (2) carbons by INEPT analysis. At higher resolution, the tall peak at 53 ppm was also resolved into two separate peaks, one of which was separated in singly and doubly protonated carbons by INEPT analysis. The presence of three unprotonated and three singly protonated aromatic carbons is consistent with the proposed structure of pentosidine. The singly protonated α-carbons of the amino acids resonate at 53 ppm and the seven backbone carbons of the amino acids at 22-53 ppm.

Synthesis of Pentosidine from Radioactive Precursors - Pentosidine prepared from N^{α}-acetyl-[G-^{3}H]arginine and N^{α}-acetyl-[4,5-^{3}H]lysine yielded similar specific activities (mol amino acid/fluorescence area unit; Table I), consistent with incorporation of equimolar amounts of arginine and lysine per mol pentosidine. As shown in Figure 5 and summarized in Table I, pentosidine could also be labeled with [U-^{14}C] and [6-^{14}C] and [1-^{14}C]glucose. The consistent, ~10% lower specific activity obtained with [U-^{14}C]glucose, compared to amino acids (Table I), is consistent with loss of 1 carbon from uniformly-labeled glucose during formation of pentosidine. (The theoretical specific activity for pentosidine synthesized from [U-14C]glucose would be 3.8 nmol/10^9 fluorescence area unit, i.e., 83% that of pentosidine labeled with amino acids.) One possible route to formation of pentosidine from glucose would be the formation of a 5-carbon intermediate by oxidative elimination of C-1 from glucose. However, the comparable specific radioactivity obtained from [U-^{14}C] and [6-^{14}C] glucose and the decreased specific activity obtained with [6-^{14}C]glucose, compared to amino acid labels (Table I), suggests that some pentosidine may be formed by pathways involving elimination of C-6, or rearrangement and cleavage of the glucose chain. Partial elimination of C-6 is also supported by the experiment in Figure 5C, in which pentosidine is labeled, albeit to lower specific radioactivity, with [1-^{14}C]glucose. Although the uniformity or specificity of labeling of the various glucose preparations used in these experiments was not confirmed experimentally, the [1-^{14}C]glucose was synthesized by the cyanohydrin procedure (27), so that significant labeling at other than C-1 is unlikely. Thus, the labeling of pentosidine by [1-14C]glucose indicates that as much as 25% of pentosidine formation from glucose may result from fragmentations not involving the loss of the C-1 carbon.

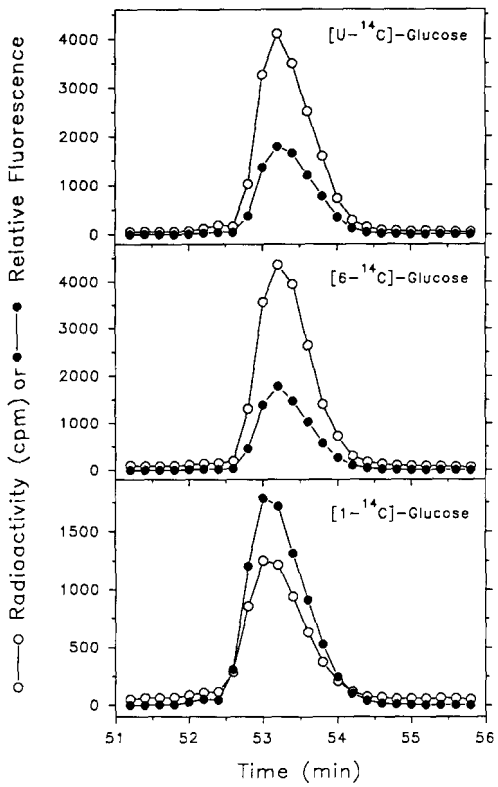

Figure 5. **Characterization of radioactive pentosidine by RP-HPLC.** Pentosidine was synthesized from radioactive precursors and purified as described in "Materials and Methods". This figure shows chromatograms obtained in the final purification step by RP-HPLC using the HFBA gradient system. The specific radioactivity of the various preparations is described in Table I.

TABLE I

Summary of the synthesis and quantitation of radioactive pentosidine.

Radiochemical	Specific Radioactivity
	nmol/10^9 area units[a,b]
Arginine, [G-^{3}H]	4.6 ± 0.1 (n=2)
Lysine, [4,5-^{3}H]	4.6 (n=1)
Glucose, [U-^{14}C]	4.2 ± 0.3 (n=3)
Glucose, [6-^{14}C]	4.2 ± 0.3 (n=3)
Glucose, [1-^{14}C]	1.3 ± 0.2 (n=2)

[a] Mean ± SD.
[b] Expressed as nmol of radiochemical per 10^9 area units as determined from the integration of the pentosidine peak from the HPLC chromatogram. Quantitation of fluorescence and radioactivity were as described in "Materials and Methods" and Figure 5.

Formation of Pentosidine from Other Carbohydrates - Table II summarizes the results of a number of experiments in which various sugars were evaluated as possible precursors of pentosidine. These experiments indicate that while pentoses are the most efficient source of pentosidine, other compounds present in biological systems may also be precursors of pentosidine, including hexoses (both aldoses and ketoses), ascorbic acid, 3-deoxyglucosone and simpler sugars, such as tetroses and trioses. In each case, the pentosidine was identified by its RP-HPLC elution position, its fluorescence maxima and decrease in fluorescence at pH 9, compared to pH 2 (12). The yield of pentosidine from non-pentose sugars was typically less than 10% of that from the pentoses, but in all cases the presence of significant amounts (>10 ppm) of pentose was excluded by analysis for pentoses as their alditol acetates (25) by gas chromatography-mass spectrometry. The fact that pentosidine is formed from such a variety of sugars suggests that a common intermediate may be formed by a variety of pathways. Aldol condensation reactions must be involved in the formation of pentosidine *in vitro* from sugars with less than 5 carbons, although the biological significance of this pathway is questionable because of the low concentrations of tetrose and triose sugars *in vivo*. In all cases the yield of pentosidine was significantly decreased under vacuum, indicating an essential role for oxygen in the oxidation, and possibly the oxidative fragmentation, of the sugars or of some precursor to pentosidine. Surprisingly, the transition metal chelator, diethylenetriaminepentaacetic acid (DTPA), which is often used to inhibit autoxidative reactions of sugars, was not an efficient inhibitor of pentosidine formation under air, although formation of CML was more than 90% inhibited. In fact, DTPA consistently stimulated the formation of pentosidine from ribose. Although measurable amounts of pentosidine are detected in all of the reactions conducted *in vacuo*, the presence of trace amounts of oxygen cannot be rigorously excluded. It is also possible, however, that the sugars could be oxidized anaerobically by intermolecular redox reactions. Notably, as shown near the bottom of Table II, among the compounds tested, malondialdehyde, a product of peroxidation of unsaturated lipids, did not form pentosidine. We were also unable to detect pentosidine in reactions which included linoleic, linolenic or arachidonic acids peroxidized by treatment with H_2O_2 in the presence of ferrous iron (data not shown).

TABLE II

The formation of pentosidine from various compounds and the effects of anaerobic incubation conditions.

Compounds were incubated with 100 mM N^g-acetylarginine, 100 mM N^g-acetyllysine and 200 mM phosphate, pH 9.0, at 60°C for 6 hr, under air or vacuum. Pentosidine was quantitated by reversed phase HPLC as described in "Materials and Methods" and legend to Figure 3.

Compound	Pentosidine (air)	Pentosidine (vacuum)	% of Air[a]	% of Ribose[b]
	nmol/ml	*nmol/ml*		
D-Glucose	0.11	0.01	9.0	0.2
D-Glucose + 2 mM DTPA	0.08	0.007	8.8	0.2
D-Fructose	0.37	0.01	3.0	0.8
D-Galactose	0.67	0.02	3.5	1.4
L-Ascorbic Acid	0.06	0.002	3.9	0.1
D-Gluconic Acid	ND[c]	-[d]		
3-Deoxyglucosone[e]	0.48	-		1.4
D-Ribose	48.0[f]	1.5	3.2	100.0
D-Ribose + 2 mM DTPA	55.6	1.6	2.9	116.0
D-Xylose	22.1	1.1	4.8	46.0
D-Erythrose	4.7	0.33	7.0	9.3
D-Threose	1.1	0.08	7.3	2.2
DL-Glyceraldehyde	3.1	0.14	4.5	6.4
Dihydroxyacetone	12.6	0.53	4.2	26.2
Malondialdehyde	ND	-		
Formaldehyde	ND	-		

[a] Expressed as a percentage of the yield from the parallel incubation under air.
[b] Expressed as a percentage of the yield from the ribose incubation under air.
[c] ND, not detected; < 0.4 pmol/ml.
[d] -, not determined.
[e] The concentration of the amino acids and sugar in this experiment was 25 mM; the % of ribose is based on a parallel incubation with 25 mM ribose.
[f] The % yield of pentosidine based on ribose is 0.05%.

Inhibition of pentosidine formation by aminoguanidine (AG) - AG has been identified as a potent inhibitor of the browning, crosslinking and development of fluorescence of collagen *in vivo* (28) and *in vitro* (9). Table III illustrates that AG also inhibits the formation of pentosidine from hexose and pentose precursors in a dose-dependent fashion. Notably, AG was effective at concentrations significantly lower than that of the starting sugar, consistent with its role in trapping either a product of oxidation of the sugar or a sugar-amino acid adduct formed during the Maillard reaction. The experiments summarized in Table IV suggest that pentosidine precursors may also be formed by decomposition of Amadori adducts to protein in biological systems, since pentosidine is formed from the commercially available Amadori compound, 1-deoxy-1-morpholino-D-fructose in the presence of N^g-acetylarginine and N^g-acetyllysine at pH 7.4 and 37°C. In this case, too, AG was an effective inhibitor of pentosidine formation.

TABLE III

The effect of aminoguanidine (AG) on the formation of pentosidine from glucose and ribose.

An aqueous solution of 100 mM each sugar, N^g-acetylarginine and N^g-acetyllysine and 200 mM phosphate, pH 9.0, was heated at 60°C for 6 hr in the absence or presence of varying concentrations of aminoguandine (AG). Quantitation of pentosidine was as described in "Materials and Methods" and legend to Figure 3.

Conditions	Pentosidine	% Inhibition[a]
	pmol/ml	
Glucose control	110	0
Glucose + 1 mM AG	66	40
Glucose + 10 mM AG	32	71
Glucose + 20 mM AG	17	84
Ribose control	39300	0
Ribose + 1 mM AG	38500	2
Ribose + 10 mM AG	28700	27
Ribose + 20 mM AG	18400	53

[a] Compared to glucose or ribose control.

TABLE IV

The effects of various reagents on the formation of pentosidine from the Amadori rearrangement product, 1-deoxy-1-morpholino-D-fructose (DMF).

Aqueous solutions containing 10 mM DMF in 200 mM phosphate buffer, pH 7.4, were incubated at 37°C for 14 days in the absence or presence of various additives (N^G-acetylarginine, AcArg; N^6-acetyllysine, AcLys; aminoguanidine, AG). Quantitation of pentosidine was as described in "Materials and Methods" and legend to Figure 3.

Additive	Pentosidine	Remarks
	pmol/ml	
None	ND[a]	
AcArg	ND	
AcLys	ND	
AcArg + AcLys	220	0.02% yield
AcArg + AcLys + AG	35	84% inhibition

[a] ND, not detected; < 0.4 pmol/ml.

Kinetics and Products of Glycation and Browning of Protein *in vitro* - Because lysozyme gradually precipitates from solution during glycation reactions, we used RNase as a model protein for studying the kinetics of modification of lysine residues, formation of pentosidine and development of fluorescence during long-term browning reactions. Glycation of RNase increased rapidly during the first few weeks of reaction (Figure 6A), reaching a steady state of approximately 1.8 mol glucose/mol protein for the remainder of the 5 month period. CML, formed on oxidative cleavage of the Amadori compound, fructoselysine, also appeared early in the reaction and continued to accumulate (Figure 6A), exceeding the concentration of fructoselysine after about 2 months. The kinetics of disappearance of lysine (Figure 6A), measured by amino acid analysis, were consistent with the cumulative rate of formation of fructoselysine and CML, although there was an increasing discrepancy of up to 2 mol lysine modified (unrecovered during amino acid analysis) per mol RNase, starting from the first week of incubation. At the conclusion of the experiment, fructoselysine and CML accounted for ~4 out of 10 mol lysine/mol RNase, and ~70% of the total loss of lysine residues in the protein, as measured by amino acid analysis, although the possible regeneration of lysine from some products during acid hydrolysis cannot be excluded.

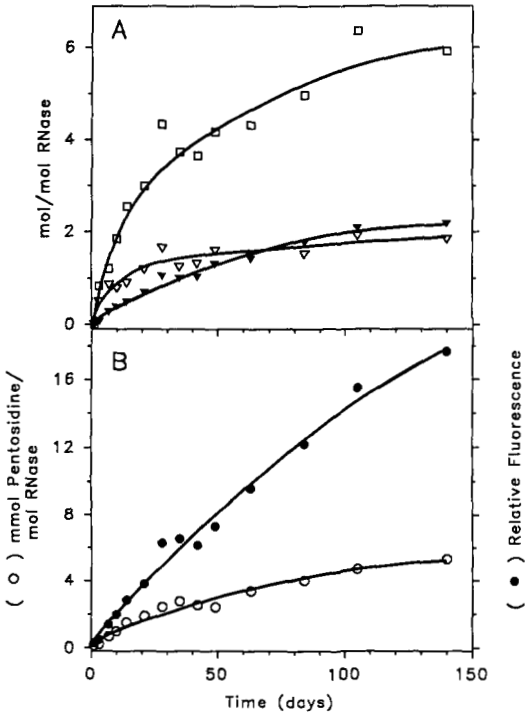

Figure 6. **Formation of browning and oxidation products during glycation of RNase.** RNase A (20 mg/ml) was glycated as described in "Materials and Methods". Aliquots were removed at various times, reduced with $NaBH_4$, and hydrolyzed in 6 N HCl. A, Lysine modification (open squares), hexitollysines (open triangles) and CML (closed triangles) were measured by amino acid analysis. B, Pentosidine (open circles) was measured by RP-HPLC, as described in "Materials and Methods". A separate aliquot was dialyzed at 4°C against several changes of 0.5 M acetic acid, diluted to 250 µg/ml with 0.5 M acetic acid, pH 2, and the fluorescence measured at Ex = 328nm and Em = 378 nm (closed circles). Note the 1000-fold difference in vertical scales in frames A and B.

As shown in Figure 6B, pentosidine, like CML, appeared early in the reaction and followed a kinetic course similar to that of CML. However, even at the end of 5 months, pentosidine accounted for only a small fraction of the total modification of lysine residues in the protein, i.e., <0.01 mol pentosidine/mol RNase, or <0.1% of the 10 lysine residues in RNase. Total fluorescence and pentosidine in the protein increased in parallel, but the fractional contribution of pentosidine to the total fluorescence at 328/378 nm declined from ~90% of the total fluorescence at 1 day, to ~50% after 14 days and 30% at 140 days of incubation. Although these results indicate that other fluorophores are formed during the course of the reaction and contribute to the overall fluorescence of the protein, there were no significant differences in either the fluorescence maxima or the 3-dimensional fluorescence surface plots of the protein glycated for 28 days vs. 140 days (data not shown). Fractionation of the browned RNase by gel exclusion chromatography on Sephadex G-75 (Pharmacia), shown in Figure 7, revealed that fluorescence was not significantly enriched in dimer compared to monomer. The pentosidine content of the peak fractions was also similar, ~0.005 mol pentosidine/mol RNase monomer (see also Figure 6B), indicating that pentosidine accounts for <1% of the total glucose-derived crosslinks in the protein. Crosslinking of the RNase was also observed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and mercaptoethanol (29), excluding a significant contribution of disulfide bonds to the crosslinking of the protein. These results are consistent with previous studies on dimerization of lysozyme by glucose, in which pentosidine accounted for only about 0.1% of the total intermolecular crosslinks in lysozyme dimer (9,16).

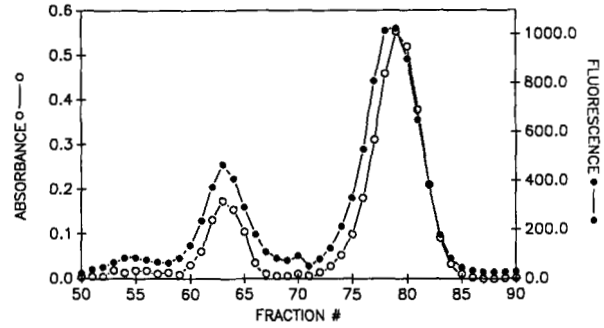

Figure 7. **Distribution of fluorescence in RNase monomer and dimer fractions.** An aliquot of RNase (20 mg, 1 ml) was removed at 60 days from the reaction mixture described in Figure 6, dialyzed overnight against 10 mM phosphate buffer, pH 7.4, then chromatographed on Sephadex G-75 superfine (1.5 x 165 cm column) using 50 mM phosphate buffer, pH 7.4, as eluent. Fractions (1.5 ml) were assayed for protein (A_{280}) and fluorescence (Ex/Em = 328/378 nm).

Fluorescence and Pentosidine in Human Tissues - The changes occurring during glycation and browning of protein *in vitro* (Figure 6) show interesting parallels to processes which occur naturally in tissue proteins. Thus, in the model system, the level of the Amadori adduct, fructoselysine, reaches a steady state, while the level of the oxidation product, CML, increases gradually with time (Figure 6). Similarly, levels of fructoselysine are relatively constant with age in both lens protein (5,6,9) and skin collagen (7,9), while levels of CML increase gradually with age in both tissues (5,7,9). The concentration of pentosidine in lens proteins, shown in Figure 8, also increases gradually with age, consistent with the accumulation of pentosidine in proteins *in vitro* (Figure 6), and mirroring the accumulation of pentosidine in tissue collagens (12). The approximate 4-fold increase in pentosidine in lens protein between 20 and 80 years of age agrees well with the relative increase in pentosidine in dura matter collagen through the same age range (12). Pentosidine accounted for modification of <0.01% of lysine residues in lens proteins in the oldest sample shown in Figure 9, i.e., or ~25 µmol pentosidine/mol lens crystallin, thus it clearly does not have a major role in the age-dependent aggregation and crosslinking of lens proteins.

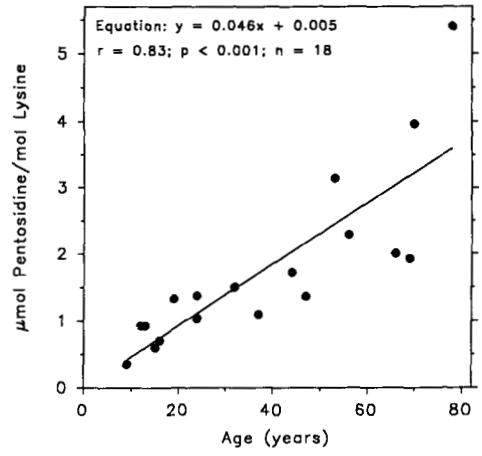

Figure 8. **Age-dependent accumulation of pentosidine in human lens proteins.** Lens proteins were reduced with $NaBH_4$ and hydrolyzed as described in "Materials and Methods". Samples (5 mg protein) were analyzed by reversed-phase HPLC, as described in Figure 2.