

# 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\*

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Human lens crystallins become progressively yellow-brown pigmented with age. Both fluorescent and non-fluorescent protein adducts and cross-links are formed, many of which result from the advanced Maillard reaction. One of them, LM-1, is a blue fluorophore that was earlier tentatively identified as a cross-link involving lysine residues (1). A two-step chromatographic system was used to unequivocally identify and quantitatively prepare a synthetic fluorescent cross-link with lysine residues that had identical UV, fluorescent, and chromatographic properties with both acetylated and non-acetylated LM-1. Proton,  $^{13}\text{C}$  NMR, and molecular mass of the synthetic compound were identical with vesperlysine A, a fluorescent cross-link discovered by Nakamura *et al.* (2). The fragmentation patterns of vesperlysine A and LM-1 were identical as determined by NMR/mass spectrometry. Lenticular levels of vesperlysine A increase curvilinearly with age and reach 20 pmol/mg at 90 years. Levels correlate with degree of lens crystallin pigmentation and fluorescence and are increased in diabetes, in contrast to  $N^{\epsilon}$ -(carboxymethyl)lysine and pentosidine. Ascorbate, D-pentoses, and D-threose, but neither D-glucose under oxidative conditions, DL-glyceraldehyde, methylglyoxal, glyoxal, nor glycolaldehyde, are precursors. However, addition of C-2 compounds greatly catalyzes vesperlysine A formation from ribose. Thus, vesperlysine A/LM-1 is a novel product of the advanced Maillard reaction *in vivo* and a specific marker of a diabetic process in the lens that is different from glyco- and lipoxidation.

LM-1 is a fluorescent protein modification that was originally discovered in and isolated from acid hydrolysate of human cataractous lens crystallins (1). The blue fluorophore had excitation emission maxima at 370 and 440 nm. It was found to increase with age and levels correlated with the overall degree of lens pigmentation except for a decrease in so-called brunescient lenses, *i.e.* a rare form of advanced cataract characterized by extreme browning and cross-linking. LM-1 was also increased in diabetic lenses.

Further studies showed a LM-1-like fluorescent compound could be synthesized from bovine serum albumin reacted with ascorbic acid or its oxidation products, or from ribose, but not glucose or fructose (1). Considerable efforts in our laboratory were devoted toward obtaining sufficient quantities of LM-1 for structural identification. Unpublished data were suggestive of one or two lysine residues linked to a heterocyclic ring. However, further studies were hampered by our unexpected inability to prepare the compound from L-lysine and D-ribose and its presence in very low concentrations in the lens. In a more recent collaborative study with Graham and Nagaraj (3), LM-1 was found to co-chromatograph, by RP-HPLC<sup>1</sup> and TLC, with a fluorescent cross-link synthesized from L-lysine and D-ribose that had been originally identified as pentodilysine in the “cross-linking region” of the chromatogram obtained with diphenylboronic acid derivatives. However, despite extensive spectroscopic data, unequivocal structural identification of pentodilysine was not achieved.

The enormous efforts that had already gone into our attempts to elucidate the structure of LM-1 combined with the unknown intrinsic value of LM-1 as a marker of the advanced Maillard reaction *in vivo* would have made us abandon the project. However, when LM-1 was quantitated in lenses from dogs that had been diabetic for 5 years (4), the surprising observation was made that LM-1 was elevated in dogs with moderate glycemic control, whereas pentosidine, a glycoxidation product, was elevated in only in lenses from poorly controlled animals. This suggested that LM-1 was a unique marker for a diabetic process reflecting mild hyperglycemia.

This promising ability of LM-1 to reflect mild hyperglycemia in the lens, together with the availability of newer LC/MS instruments, such as the LCQ ion-trap instrument, led us to pursue the project further. We present below our approach and data that have led us to conclude that LM-1 is identical with vesperlysine A, a recently discovered lysine cross-link of the advanced Maillard reaction (2). Its mechanism of formation and relevance to the biochemistry of the aging lens in comparison to glycation and other advanced glycation products are described.

## EXPERIMENTAL PROCEDURES

**Materials**—All reagents were of the highest grade available. Poly-L-lysine HBr ( $M_n = 30,000$ –70,000),  $N^{\epsilon}$ -acetyl-L-lysine, L-lysine, ribose, glucose, ascorbic acid, arabinose, xylose, threose, glyceraldehyde, and glycolaldehyde were obtained from Sigma. Glyoxal and methylglyoxal were purchased from Fluka (St Louis, MO). Human lenses were ob-

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<sup>1</sup> The abbreviations and trivial names used are: RP-HPLC, reverse phase-high performance liquid chromatography; CML,  $N^{\epsilon}$ -(carboxymethyl)lysine; HPLC, high performance liquid chromatography; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; WI, water-insoluble; WS, water-soluble.

tained from the National Disease Research Interchange (Philadelphia, PA), classified into type I–IV and brunescent based on pigmentation using Pirie classification (5). Dr. R. H. Nagaraj also kindly provided brunescent lenses. Lenses were stored at  $-70^{\circ}\text{C}$  until used.

**Reaction of Ribose with Poly-L-lysine or *N*<sup>α</sup>-Acetyl-L-lysine or L-Lysine**—Ribose (150 mM) and poly-L-lysine (10 g/liter) or *N*<sup>α</sup>-acetyl-L-lysine (60 g/liter) or L-lysine (60 g/liter) were dissolved in 200 mM sodium phosphate buffer, pH 7.4, filtered through 0.2-μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI), and incubated for 93 h at  $37^{\circ}\text{C}$  for the poly-L-lysine or for 48 h at  $50^{\circ}\text{C}$  for the amino acids. The poly-L-lysine/ribose mixture was dialyzed against  $4 \times 4000$  ml of water for 48 h, using Spectra/Por membrane tubing with a molecular weight cut-off of 12,000–14,000 (Spectrum Medical Industries, Los Angeles, CA). The dialyzed poly-L-lysine and the *N*<sup>α</sup>-acetyl-L-lysine were hydrolyzed with 6 N HCl at  $110^{\circ}\text{C}$  for 18 h, at a concentration of 5 mg of poly-L-lysine or 30 mg of *N*<sup>α</sup>-acetyl-L-lysine/ml of HCl.

**Isolation of a LM-1-like Fluorophore from the Reaction Mixtures**—The fluorophore with excitation and emission maxima at 370–440 nm was isolated using a combination of reverse phase and ion exchange chromatography. The poly-L-lysine acid hydrolysate was evaporated in a Speed Vac concentrator (Savant Instruments, Hicksville, NY), and 63 mg was dissolved in 8 ml of 0.2 M pyridine formate (pH 3.25) and loaded onto a cation exchange column ( $3 \times 15$  cm, Dowex 50WX8–400). Fractions of 5 ml were collected at a flow rate of 1 ml/min. Fractions 1–40 were eluted with 0.2 M pyridine formate (pH 3.25), fractions 41–80 with 0.4 M pyridine formate (pH 5.75), and fractions 81–120 with 1 M pyridine formate (pH 8.4). Some fractions were evaporated in a Speed-Vac concentrator, concentrated to 1 ml of water, filtered through a 0.45-μm cellulose acetate centrifuge tube filter (Costar, Corning Inc., Corning, NY) and analyzed by high performance liquid chromatography (HPLC) in order to detect a fluorescent compound with retention time similar to LM-1. Initially, the retention time of LM-1 was determined from human lens proteins (see below). Five microliters of selected fractions were injected into a C-18 reverse-phase analytical column (Vydac 218TP104, 4.6 mm  $\times$  25 cm, 10 μm; The Separation Groups, Hesperia, CA). A Waters HPLC instrument (Waters Chromatography Div., Milford, MA) with model 510 pumps, automatic injector (model 712 WISP), and a model 680 controller were used. The column was eluted at a flow rate of 0.7 ml/min with 1% acetic acid for 20 min, and washed with an increasing gradient of acetonitrile (0–45%) for 40 min. The column eluent was monitored with an on-line fluorescence detector (Waters 470 scanning fluorescence detector) at 370 and 440 nm for the excitation and the emission, respectively. The chromatograms were recorded with a chromatography software (Borwin, JMBS Developpements, Le Fontanil, France). Fractions 100–120, which showed a major fluorescent peak similar to LM-1, were evaporated in a Speed-Vac concentrator and reconstituted in water.

The *N*<sup>α</sup>-acetyl-L-lysine hydrolysate was centrifuged at  $1000 \times g$  for 10 min to remove a black pellet resulting from the acid hydrolysis. The HCl was evaporated using a Rotavapor. Methanol was used to wash the dry pellet. The pellet was dissolved in 600 ml of 0.4 M pyridine formate (pH 5.25) and loaded onto a cation exchange column ( $10 \times 15$  cm, Dowex 50WX8–400). The column was eluted at a flow rate of 2.9 ml/min with two different solutions. Five hundred fractions of 20 ml were collected: fractions 1–200 with 0.4 M pyridine formate (pH 5.25) and fractions 201–500 with 1 M pyridine formate (pH 9). Fractions were analyzed by C-18 analytical HPLC, and those containing the fluorescent peak of interest were pooled. The pyridine formate was evaporated. The substrate was washed twice with methanol and evaporated. In order to purify the fluorophore of interest, the substrate was solubilized in 25 ml of 0.4 M pyridine formate (pH 5.25) and reloaded on an identical but smaller cationic column ( $3 \times 15$  cm). Forty fractions of 5 ml were collected with the same solvent system as before. After analysis by HPLC fluorescence, the fractions that contained the fluorescent compound were pooled, evaporated, and reconstituted in water. The same purification procedure was applied to the fluorophore from the L-lysine and D-ribose mixture.

**HPLC Purification**—The fluorophore was purified by HPLC with three different columns. The samples were first injected into a C-18 reverse-phase semi-preparative column (Vydac 218TP1010, 10 mm  $\times$  25 cm, 10 μm; The Separation Groups) using the same solvents and gradient as before, but with a higher flow rate: 2.5 ml/min. The fluorophore, which had a retention time of 14.2 min, was collected, evaporated, dissolved in water, and injected into a cation exchange column (Vydac 400VHP575, 7.5  $\times$  50 mm, 5 μm; The Separation Groups). Elution was at a flow rate of 0.9 ml/min with a gradient starting at 100% ammonium acetate (4 mM, pH 4.2, containing 5% acetonitrile) to 100% 10 mM ammonium acetate (pH 6.7, containing 5% acetonitrile) at

40 min. Under these conditions, the fluorophore eluted between 22 and 23 min. Finally, this compound was purified using the C-18 reverse-phase analytical column described above and eluted with 0.1%  $\text{CH}_3\text{COOH}$  at a flow rate of 0.7 ml/min.

The eluent from the column was monitored with an on-line scanning fluorescence detector (Waters, model 470) with excitation and emission wavelengths set at 370 and 440 nm, respectively, and with an on-line UV detector (Waters, model 486) at 220 nm. After the third HPLC purification, the fluorescent compounds from poly-L-lysine, *N*<sup>α</sup>-acetyl-L-lysine, and L-lysine were dried and weighed.

**Purification of LM-1 from Human Lens Proteins**—A total of 46 human lenses (age range: 17–85 years; including 12 from diabetic donors with unknown diabetes duration) were processed individually for further analysis. Water-soluble (WS) and water-insoluble (WI) proteins were extracted as described previously (1). WI proteins were solubilized by proteolytic digestion with Pronase E (2% w/w) in 50 mM phosphate buffer (pH 7.4), at  $37^{\circ}\text{C}$  with shaking for 5 h. Some WI or WS fractions, selected from the oldest human lenses ( $n = 22$ , age =  $66 \pm 13$  years, including 7 diabetics) were pooled for extraction and purification of LM-1. The pools of WI or WS were hydrolyzed with 6 M HCl for 18 h at  $110^{\circ}\text{C}$ . Acid hydrolysates were dried, taken into 6 ml of water, and filtered. LM-1 was purified by repetitive injections and peak collection of the hydrolyzed proteins using the same three HPLC systems described before. The final pure LM-1 fractions from WS and WI proteins were dried and kept at  $-70^{\circ}\text{C}$ .

**Quantification of LM-1 in Human Lenses**—For increased accuracy, LM-1 was quantified using a two step procedure. Fractions from each WS and WI protein ( $3 \pm 1$  mg/ml) were acid-hydrolyzed with 6 N HCl at  $110^{\circ}\text{C}$  for 18 h, and acid was evaporated in a Speed-Vac concentrator. Leucine equivalent amino acids were measured by the ninhydrin method in each residue reconstituted in water. Protein corresponding to 3–7 μmol of leucine equivalent was injected into a C-18 analytical column as described above. The material corresponding to LM-1 was collected between 7 and 8 min, dried, resolubilized, and injected into cation exchange HPLC as described above. The concentration of LM-1 was determined by comparing the integrated area of the fluorescent peak (370–440 nm) with a standard of the synthetic fluorophore (made from *N*<sup>α</sup>-acetyl-L-lysine and ribose) of known concentration.

**NMR**—The samples were dissolved and evaporated three times in 100% deuterium oxide (pD 7.0). The samples containing 1.7 mg/ml synthetic fluorophores and 0.02 mg/ml LM-1 were transferred to a 5-mm NMR tube.  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, and heterocorrelation spectra were run at  $25^{\circ}\text{C}$  with a 600-MHz Varian VXR spectrometer.

**Mass Spectrometry**—Mass spectrometry was performed with the LCQ ion-trap instrument available at the Veterans Administration Medical Center mass spectrometry facility (Dr. C. Hoppel). Samples were dissolved in 0.5% acetic acid with 50% methanol for direct infusion into the instrument.

**Spectroscopy**—Fluorescence spectra were recorded with a SLM-Aminco spectrofluorometer (model 8100; Aminco-Bowman, Rochester, NY). Absorption spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard, Inc., Avondale, PA) connected to an IBM PC-AT computer (IBM Corp., Boca Raton, FL).

**In Vitro Incubation of Poly-L-lysine with Sugars, Oxaldehydes, or Ascorbic Acid**—Sugars (glyceraldehyde, threose, xylose, ribose, arabinose or glucose), oxaldehydes (glyoxal or methylglyoxal), or ascorbic acid were incubated with poly-L-lysine in 200 mM sodium phosphate buffer (pH 7.4). After 7 days at  $37^{\circ}\text{C}$ , the samples were dialyzed against  $2 \times 4000$  ml of water for 48 h, using Spectra/Por membrane tubing with a molecular weight cut-off of 12,000–14,000. After lyophilization, they were acid-hydrolyzed in 6 N HCl for 18 h at  $110^{\circ}\text{C}$ . Following evaporation of the acid, the material was reconstituted in water and filtered. Vesperlysin A concentration was determined by HPLC.

**Kinetic Studies on LM-1/Vesperlysin A Formation from Poly-L-lysine Incubated with D-Ribose with Increasing Concentrations of Oxaldehydes, Glyceraldehyde, or Glycolaldehyde**—Incubations of poly-L-lysine with ribose were carried out in 200 mM phosphate buffer, pH 7.4, with 0, 1, 5, and 10 mM glyoxal, methylglyoxal, glyceraldehyde, or glycolaldehyde. After 1, 2, and 4 days at  $37^{\circ}\text{C}$ , samples were analyzed for the concentration of vesperlysin A.

## RESULTS

### Isolation of Fluorescent Material from D-Ribose and Poly-L-lysine or *N*<sup>α</sup>-Acetyl-L-lysine

Based on previous experiments suggesting involvement of L-lysine and D-ribose in LM-1 formation (3), we incubated poly-

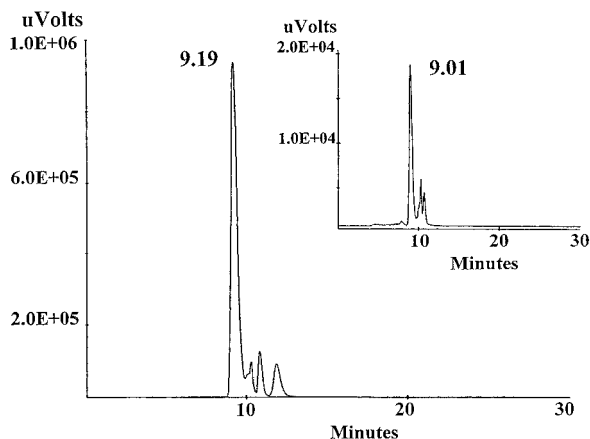


FIG. 1. C-18 RP-HPLC profile of the fraction from cation exchange chromatography containing the fluorophore of interest (major peak at 9.19 min) isolated from the 93-h incubation mixture of poly-L-lysine (or  $N^{\alpha}$ -acetyl-L-lysine in insert) and D-ribose at 37 °C. The eluent was monitored for fluorescence at 370/440 nm.

L-lysine with D-ribose for 93 h and acid-hydrolyzed the reaction products. The mixture was passed over Dowex 50W, which was eluted with three different pyridine formate buffers. A major fluorescent material at 370/440 nm was eluted by HPLC reverse phase chromatography on C-18 column at 9–11 min (Fig. 1). This material was found to elute from the Dowex column between fractions 100 and 120 (data not shown). A similar fluorescent peak was obtained from  $N^{\alpha}$ -acetyl-L-lysine and D-ribose (Fig. 1, inset). In subsequent work, all studies were performed with the material prepared from  $N^{\alpha}$ -acetyl-L-lysine.

The fluorescent peak isolated from  $N^{\alpha}$ -acetyl-L-lysine was rechromatographed on a smaller Dowex column and then sequentially chromatographed with semipreparative C-18 HPLC column, cation exchange column, and C-18 analytical column. Detection was carried out both at 370/440 nm for fluorescence and UV at 220 nm. From 15 g of  $N^{\alpha}$ -acetyl-L-lysine hydrochloride, 500  $\mu$ g of chromatographically pure fluorescent compound were obtained (yield 0.0033%).

The chromatographic properties of the pure compound were compared with LM-1 from human lenses, isolated by a combination of C-18 as described by Nagaraj and Monnier (1) and cation exchange chromatography. Co-chromatography between the synthetic and the native fluorophore was observed in both systems (Fig. 2, A and B).

#### Structural Characteristics of the Synthetic Fluorophore

The synthetic compound was analyzed by proton,  $^{13}\text{C}$  NMR, and mass spectrometry. The proton NMR (Fig. 3A) showed four single aromatic protons (a–d) at 8.31 (s), 8.12 (s), 7.97 (d), and 6.9 (d) ppm, two  $\epsilon$ -carbon linked protons at 4.76 (t) and 4.41 (t) ppm ( $\epsilon$  and  $\epsilon'$ ), two overlapping  $\alpha$ -carbon-linked protons at 3.80 ppm ( $\alpha$  and  $\alpha'$ ), and the aliphatic lysyl protons at 1.3–2.3 ppm. The  $^{13}\text{C}$  spectrum showed 2 peaks between 170 and 180 ppm (attributed to two carboxylic groups), 7 peaks (2 of which are superimposed) between 90 and 160 ppm (from the aromatic ring), 1 water side band (at 40.4 ppm), and 10 peaks (including  $4 \times 2$  superimposed peaks) between 20 and 60 ppm (from two aliphatic chains) (Fig. 3B). Taken together, these data suggested that the isolated compound was identical with vesperlysine A (Fig. 4) described by Nakamura (6). This was confirmed by both heterocorrelation analysis (Fig. 3C), which showed which protons are linked to which carbon, and ES mass spectrometry using ion-trap methodology (LCQ), which revealed  $m/z$  at 393.4 with MS/MS Fragments at 376 (loss of OH), 348 (loss of 1 COOH), 264 (loss of 1 norleucine), and 219 (the 264 fragment with additional loss of 1 COOH) (Fig. 5, A

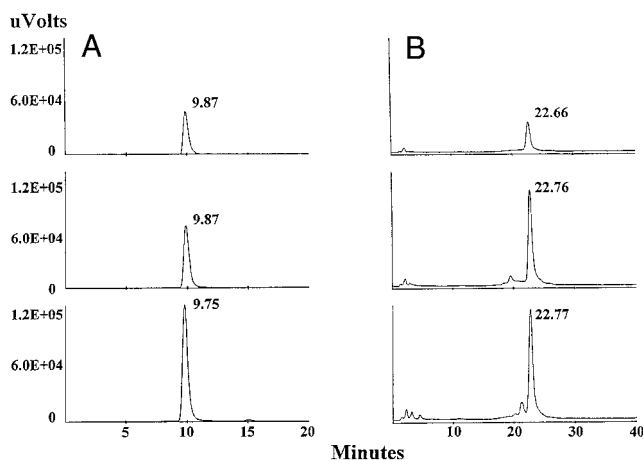


FIG. 2. Comparison of HPLC chromatograms of synthetic vesperlysine A and LM-1 from human lenses, and co-chromatography. Separations were performed on analytical C-18 reverse phase column (A) and cation exchange column (B). Vesperlysine A (upper) and LM-1 (middle) were also mixed before injection in both columns (lower). Fluorescence was monitored as in Fig. 1.

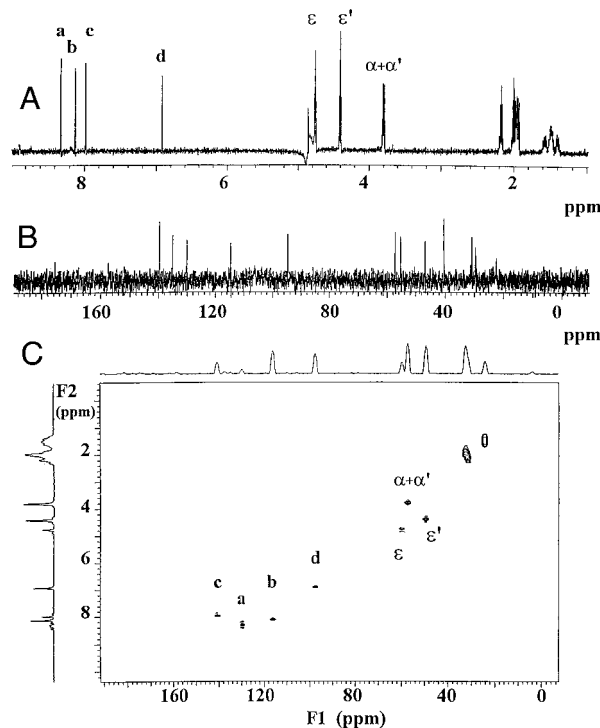


FIG. 3.  $^1\text{H}$  (A),  $^{13}\text{C}$  (B), and hetero-correlation (C) NMR spectra (500  $\mu$ g in 300  $\mu$ l of  $\text{D}_2\text{O}$ , 600 MHz, 25 °C) of vesperlysine A isolated from  $N^{\alpha}$ -(acetyl-L-lysine and ribose mixture).

and B). When fragment 264 was analyzed by MS/MS/MS, a new fragment at  $m/z$  135 attributed to the aromatic ring of vesperlysine A was obtained (data not shown).

#### Comparison of Synthetic Vesperlysine A with Native Fluorophore LM-1

For comparative studies, LM-1 was isolated through repetitive HPLC chromatography essentially as described for vesperlysine A. From a pool of 22 lenses (Pirie pigmentation stage II–IV and brunescent; age range: 44–86 years), 2.6 and 4.6  $\mu$ g (based on the extinction coefficient for vesperlysine A, see below) were obtained from the water-soluble and water-insoluble fractions, respectively. Fluorescence at various pHs and UV spectra are shown in Fig. 6. The fluorescence spectra of syn-



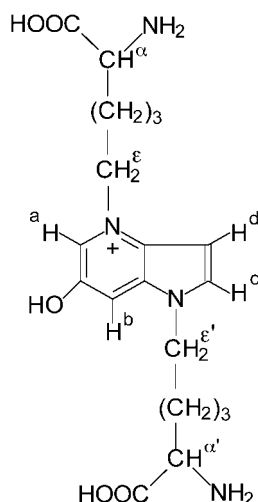


FIG. 4. Structure of vesperlysine A.

thetic and native LM-1 varied with the pH, confirming the presence of a titrable proton on the ring, and were identical for both the synthetic and native compound (Fig. 6, A and C). Vesperlysine A had two previously unreported UV maxima at 302 and 363 nm, which were identical with those of native LM-1 (Fig. 6, B and D) and those originally reported by Nagaraj and Monnier (1). Extinction maxima calculated for the synthetic vesperlysine A were 1841 and 1812 mol<sup>-1</sup>·liter·cm<sup>-1</sup>, respectively.

To further strengthen the possible homology between vesperlysine A and LM-1, the preparations were analyzed by ES mass spectrometry using direct injection into the LCQ mass spectrometer. The full spectrum for LM-1 shows a *m/z* signal at 393.3 (Fig. 5C). The MS/MS of *m/z* 393.3 shows the identical fragments for LM-1 (Fig. 5D) corresponding to those of vesperlysine A (Fig. 5B). It is important to note here that, out of concern for potential carryover of material from previous injections, no synthetic standard was injected into the LCQ instrument on the day the native LM-1 analysis was performed. Furthermore, the HPLC injector that was used for serial purification of LM-1 had never been exposed to synthetic vesperlysine A, thereby excluding the possibility of cross-contamination between the synthetic and native material. As a final proof of the identity of LM-1 with vesperlysine A, both synthetic and native compounds were acetylated with acetic anhydride and the reaction products were analyzed by C-18 reverse phase chromatography. In both cases the retention times of the fluorescent compounds were greatly retarded and eluted at 41.2 min, and in both cases a degradation compound was formed upon storage of the preparation, which eluted at 40.8 min (data not shown). The former peak likely corresponds to the fully acetylated vesperlysine A of which the unstable *O*-acetyl group on the ring is spontaneously cleaved off upon storage. Mass spectrometric analysis with the LCQ mass spectrometer showed a *m/z* signal at 477.7 for the acetylated vesperlysine A. The MS/MS of *m/z* 477.7 shows four major fragments with *m/z* signal at 459.2 (loss of 1 OH), 435.2 (loss of 1 acetyl), 389.3 (loss of 2 COOH), and 306.2 (loss of 1 *N*<sup>α</sup>-acetylnorleucine) (spectra not shown).

Based on the findings above indicating identity between LM-1 and vesperlysine A, LM-1 is referred to as vesperlysine A for all subsequent studies described below.

#### Vesperlysine A in Biological Tissues

**Preanalytical Studies**—For analysis of vesperlysine A in biological tissue, a number of pre-analytical variables were eval-

uated. They are summarized as follows. The yield of vesperlysine A from bovine serum albumin that was glycosylated for 80 days with 53 mM D-ribose in 200 mM phosphate buffer (pH 7.4) was unaffected by hydrolysis time (12 to 18 h) or presence of oxidizing conditions (O<sub>2</sub> and presence of 100 μM CuSO<sub>4</sub>) during acid hydrolysis of 3 mg/ml protein for 18 h at 110 °C. Furthermore, presence of added glucose or ribose (2 mM each) during hydrolysis had no effect on the yield. Finally, vesperlysine A was not an artifact of acid-hydrolyzed ribated protein because vesperlysine A could also be obtained from unprotected L-lysine and D-ribose reacted at 50 °C (pH 7.4) without acid hydrolysis.

**Detection of Vesperlysine A in the Human Lens**—Human lenses (age: 16–86 years; *n* = 46) were separated into WS and WI fractions and analyzed by a two-step HPLC system using fluorescence at 440 nm (excitation at 370 nm). An age-related increase was noted in both water-soluble and water-insoluble fractions (Fig. 7A). A shallow increase of the regression line (*r*<sup>2</sup> = 0.32, *p* < 0.05) was noted in the water-soluble fraction, which reached only 2.5 pmol/mg of protein at 90 years (individual data points not shown). In contrast, a sharp curvilinear increase (*r*<sup>2</sup> = 0.85, *p* < 0.001) was noted in the insoluble fraction, which reached 20 pmol/mg at 90 years, i.e. 6–8 fold higher than in the soluble fraction. Moreover, most of the values from diabetic lenses were increased, including in those the WS fraction in which the highest diabetic value reached 10 pmol/mg (data not shown).

**Comparison of Vesperlysine A with Other Markers of the Maillard Reaction**—A comparative analysis of Maillard reaction markers was carried out in the water-insoluble fractions from 46 lenses for vesperlysine A (Fig. 7A) and a subgroup of 36 lenses for the markers glycosylated lysine (furosine) (Fig. 7B), pentosidine (Fig. 7C), and CML (Fig. 7D). It is readily apparent that, with age, a curvilinear increase is observed for CML, pentosidine, and vesperlysine A, in contrast to furosine, the concentration of which remains unchanged, as previously reported by Patrick (7). Furthermore, only vesperlysine A and furosine are elevated beyond normal values in diabetic lenses. Finally, CML stands out as the major modification, followed by furosine, vesperlysine A, and pentosidine, the levels of which are 6, 150, and 500 times lower than CML at the end of life span, respectively.

Further correlation was sought with the degree of pigmentation of the lenses using the classification of Pirie (5) (Fig. 8). A strong relationship was noted with lens color for all advanced Maillard products, except for vesperlysine A, the concentration of which is decreased in the highly pigmented, so-called brunescient lenses. Differences between diabetic and non-diabetic lenses were significant for furosine in all lens types (*p* < 0.001), and for vesperlysine A in type III and IV lenses (*p* < 0.001).

**Correlation with Total Lenticular Fluorescence**—The markers described above were correlated with lens crystallin fluorescence at the emission/excitation maxima of insoluble native crystallins, which were detected at 310/430 nm. As shown in Table I, all markers were significantly correlated with total fluorescence except furosine. Interestingly, removing the values for the diabetic lenses ameliorated the correlation coefficients for all advanced glycation end products (AGEs), and removing the brunescient lenses ameliorated the correlation coefficient of vesperlysine A and pentosidine. Most impressively, CML had the highest correlation coefficient in normal subjects, suggesting that it is an excellent surrogate marker for the process responsible for total fluorescence at 310/430 nm. We determined that vesperlysine A accounted for at most 5% of the total fluorescence at 370/440 nm in the water-insoluble proteins of old lens crystallins.

**Mechanism of Formation of Vesperlysine A**—Studies on the

FIG. 5. Electron spray ionization-MS and MS/MS spectra of synthetic vesperlysine A (A and B, respectively) and LM-1 (C and D, respectively) isolated from human lenses. MS/MS was performed on the  $m/z$  393. Major fragments are 376 (loss of OH), 348 (loss of 1 COOH), 264 (loss of 1 norleucine), and 219 (a 264 fragment with additional loss of 1 COOH).

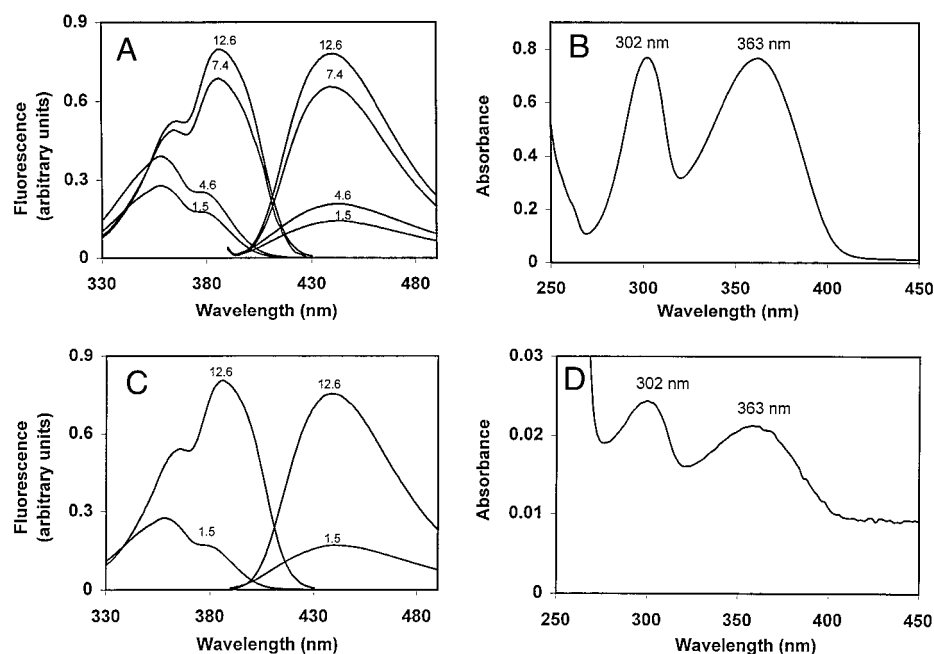
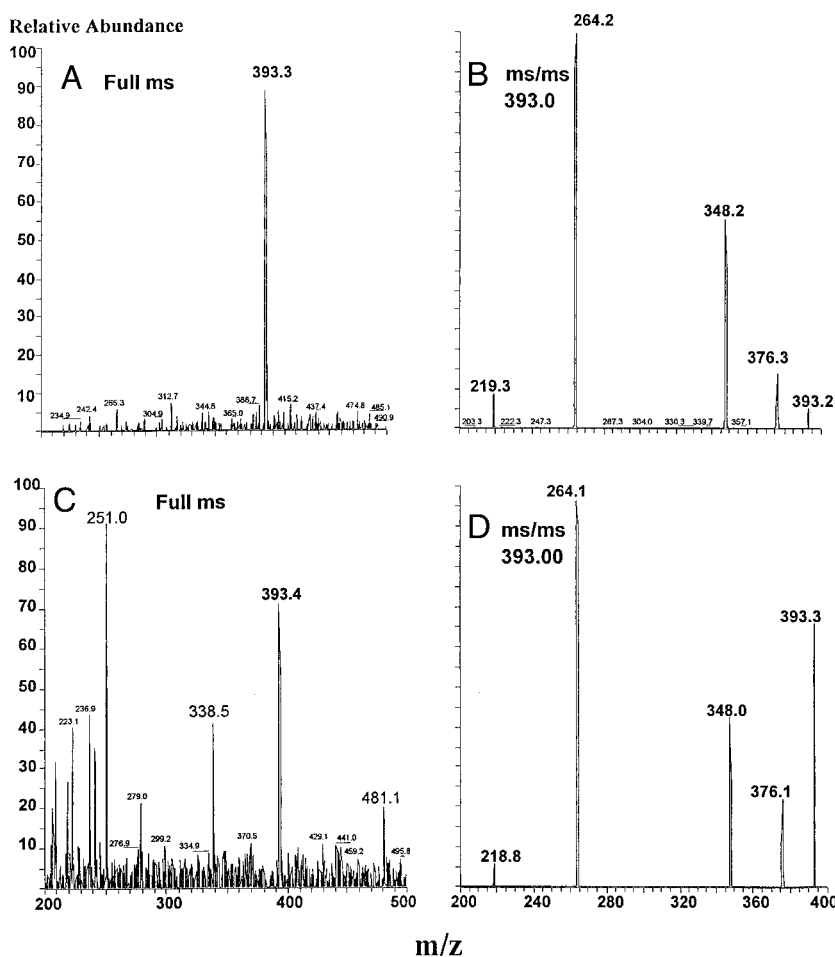


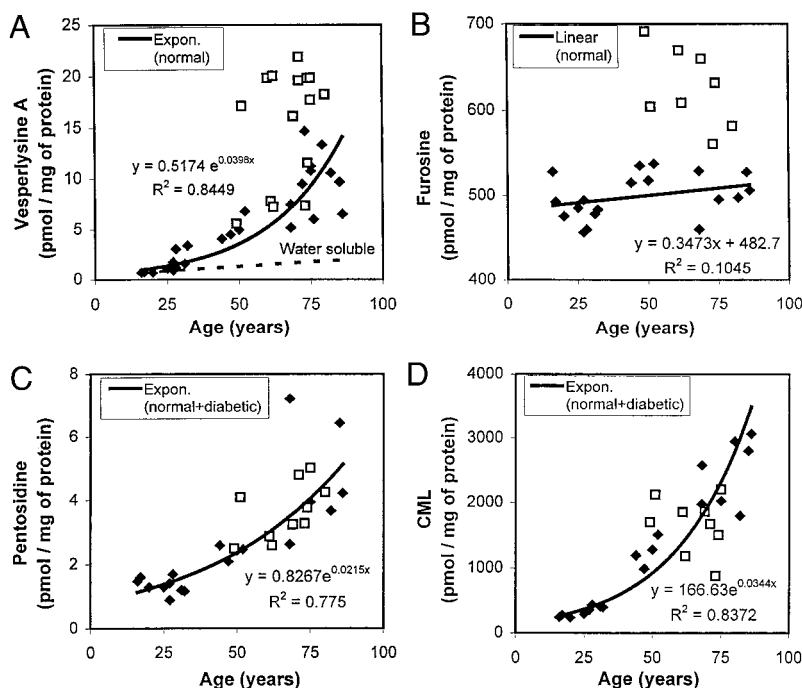
FIG. 6. Fluorescence (at pH 1.5, 4.6, 7.4, and 12.6) and absorption spectra of vesperlysine A (A and B) and LM-1 (C and D) isolated from the incubation of *N*-acetyl-L-lysine and D-ribose and from human lenses, respectively. Fluorescence excitation was set at 380 nm for the emission spectra, and emission was set at 440 nm for the excitation spectra.

mechanism of formation of vesperlysine A were performed. Poly-L-lysine (9 g/liter) was incubated in 200 mM phosphate buffer with 50 mM carbonyl compound (D-glucose, D-ribose, D-arabinose, D-threose, DL-glyceraldehyde, methylglyoxal, glyoxal, and ascorbic acid) for 7 days at 37 °C. The results (Table II) show that vesperlysine A could be efficiently synthesized from ribose, threose, and ascorbate, but not from glucose,

glyoxal, methylglyoxal, and glyceraldehyde. Arabinose and xylose were also precursors.

Because the heterocyclic ring of vesperlysine A requires the participation of 7 carbon atoms, we hypothesized addition of C-2 and C-3 carbonyl compounds should have a catalytic effect on vesperlysine A formation from C-5 sugars. A dramatic, dose-dependent increase in vesperlysine A was noted with

FIG. 7. Levels of vesperlysine A (A), furosine (B), pentosidine (C) and  $N^{\epsilon}$ -(carboxymethyl)lysine (D) in water-insoluble lens crystallins from diabetic ( $\square$ ) and non-diabetic ( $\blacklozenge$ ) subjects as a function of age. The dashed line in panel A represents the regression line of data for the water-soluble fraction.



glyoxal and glycolaldehyde (Fig. 9, A and B). Although some effect was noted with DL-glyceraldehyde (Fig. 9D), methylglyoxal had not catalytic effect (Fig. 9C, see value of the y scale). We tentatively attribute the very small increase observed to the presence of potential contaminant(s) reported present in commercial batch of methylglyoxal (8).

In light of the fact that glyoxal and arabinose are autooxidation products of glucose (9), repeated attempts were made to obtain vesperlysine A from glucose under aerobic conditions and high phosphate buffer (200 mM) to enhance glucose autooxidation (9). Surprisingly, none succeeded.

**Vesperlysine A in Other Tissues**—Vesperlysine A was quantitated in the collagen-rich acid-insoluble fraction from heart, kidney, lung, and skin, as well as in the trichloroacetic acid precipitate from plasma, red blood cell, liver, and lens from streptozotocin diabetic and non-diabetic rats after 8 weeks of diabetes. Levels were almost nondetectable ( $<1$  pmol/mg of protein). Similarly, very low levels and no increase were found in highly insoluble skin collagen from a 90- versus 15-year-old human donor. Vesperlysine A was also quantitated in the trichloroacetic acid-precipitated fraction from 4 healthy subjects, 4 diabetic, and 4 uremic subjects. Levels were  $4.72 \pm 0.86$ ,  $3.5 \pm 0.58$ , and  $5.33 \pm 0.66$  pmol/mg of protein, respectively.

#### DISCUSSION

A chromatographic peculiarity appears to underlie former failures to synthesize LM-1 from  $N^{\alpha}$ -acetyl-L-lysine and D-ribose, in that no fluorescent peak was observed at the expected retention time for LM-1 using the C-18 reverse phase column.<sup>2</sup> This led to the erroneous conclusion LM-1 could not possibly be a lysine dimer. Similar observations were made in the initial phase of the current study. Subsequently, we found the retention time of vesperlysine A was significantly affected by the concomitant presence of amino acids. Thus, identical retention times between LM-1 and vesperlysine A were observed only when vesperlysine A was either added to lens protein hydrolysate, or injected mixed together with, e.g., acid-hydrolyzed bovine serum albumin. The awareness of this chromatographic idiosyncrasy eventually allowed us to demonstrate full identity

between LM-1 and vesperlysine A in terms of chromatographic behavior in two systems, with and without derivatization with acetic anhydride, as well as spectral analysis.

Vesperlysines were first described *in vitro* by Ienaga and colleagues (2) as a family of fluorescent sugar-derived cross-links of L-lysine. In our studies, the single major fluorescent peak at LM-1 retention time corresponded to vesperlysine A. However, a second minor peak that was also elevated in old lenses co-chromatographed at 18 min with another synthetic lysine-ribose advanced product (data not shown), suggesting other vesperlysines might also be present in the human lens.

In depth studies on the mechanism of LM-1/vesperlysine A formation were carried out, and the results were compared with our former study (1) and that of Nakamura (2). First, we were able to reproduce our previous findings (1) that LM-1 could be most efficiently made from D-ribose and ascorbate, but also D-threose. In our previous study, neither fructose nor glucose were precursors. In contrast to Nakamura *et al.*, numerous attempts to make vesperlysine A from glucose, even in presence of 200 mM non-Chelex-treated phosphate buffer, failed. This was rather unexpected in view of the fact that both D-arabinose and glyoxal are autooxidation products of glucose (9), which should act in concert as vesperlysine A precursors, as suggested by the kinetic experiments in Fig. 9. This discrepancy suggests presence of competing reactions, possibly by glucose itself and other products for sites necessary for vesperlysine A formation. Further possible differences include the utilization by Nakamura *et al.* of pentylamine instead of lysine as a reactant. Finally, we have utilized a highly specific two-column system in all our analytical studies, which may have helped achieve better separation of vesperlysine isomers.

The inability of C-2 and C-3 sugar fragments and oxoaldehydes *per se* to make vesperlysine A is in agreement with its structure. However, based on its structure, we hypothesized and confirmed that both glyoxal and glycolaldehyde are vesperlysine A precursors when added to D-ribose. The presence of these oxoaldehydes in the lens is anticipated based on the documented accumulation of the glyoxal lysine dimer (GOLD) (11). Similarly, glyceraldehyde, which by itself was not a vesperlysine A precursor, could moderately catalyze its formation from D-ribose. Most importantly, however, methylglyoxal was

<sup>2</sup> F. Tessier, M. Obrenovich, and V. M. Monnier, unpublished data.

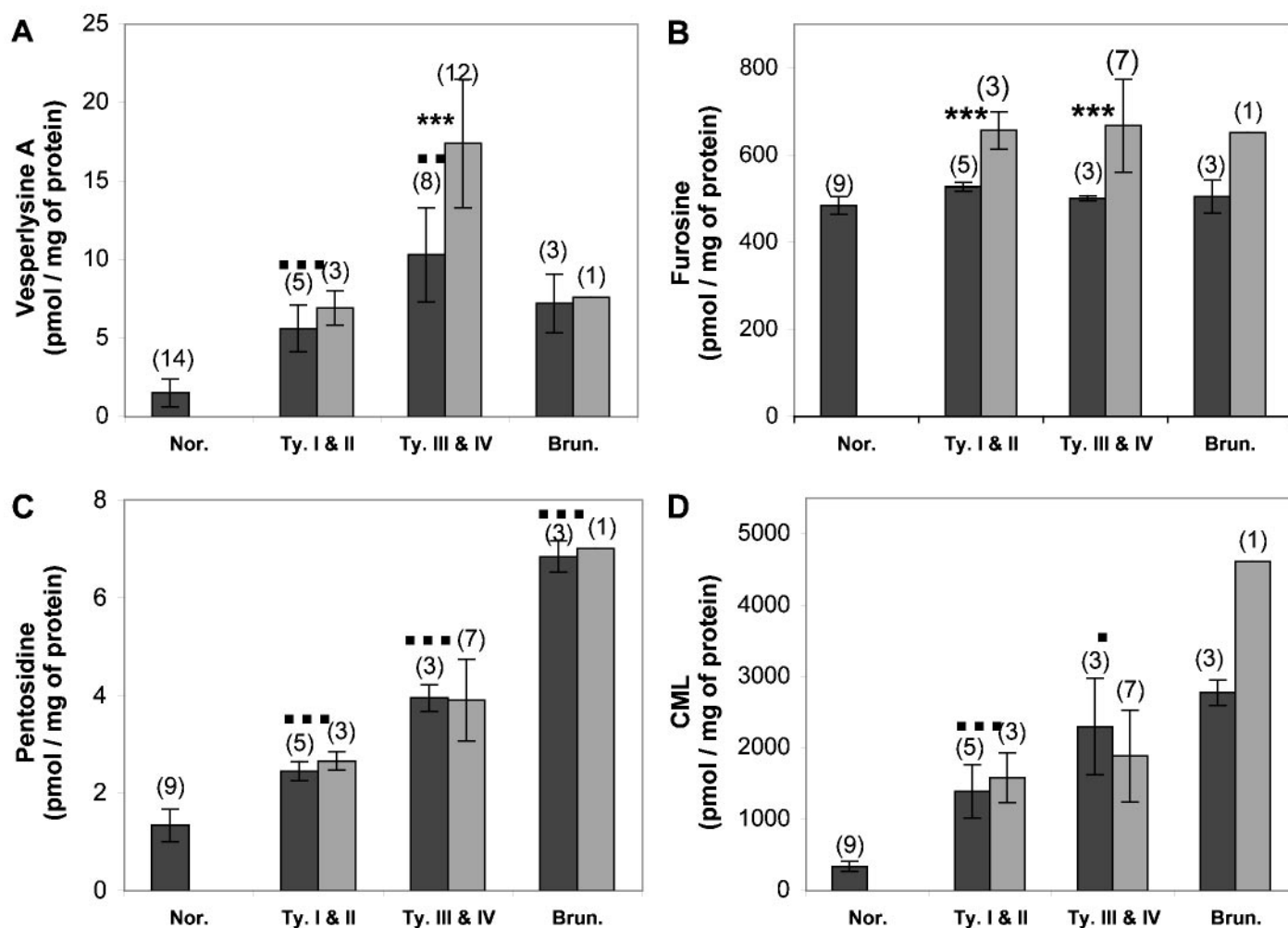


FIG. 8. Levels of vesperlysine A (A), furosine (B), pentosidine (C), and  $N^{\epsilon}$ -(carboxymethyl)lysine (D) in water-insoluble crystallins from lenses classified on the basis of pigmentation. Statistical significance between diabetic (gray bars) and non-diabetic (black bars) was calculated using Student's nonpaired  $t$  test (\*\*\*,  $p < 0.001$ ). Nor., normal; Ty., type; Brun., brunescient. The means were also compared between each consecutive non-diabetic groups (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , Student's nonpaired  $t$  test).

TABLE I

Correlation coefficients ( $r^2$ ) between total lenticular fluorescence (310/430 nm) and AGEs or furosine in water-insoluble crystallins  $p < 0.001$  except for furosine ( $p > 0.05$ ).

Glycation product	Normal and diabetic ( $n = 31$ )	Normal ( $n = 21$ )	Normal <sup>a</sup> and diabetic ( $n = 27$ )	Normal <sup>a</sup> ( $n = 17$ )
	$r^2$	$r^2$	$r^2$	$r^2$
Vesperlysine A	0.676	0.725	0.854	0.829
Pentosidine	0.784	0.825	0.923	0.894
CML	0.852	0.917	0.757	0.861
Furosine	0.163	0.102	0.201	0.005

<sup>a</sup> Brunescient lenses excluded.

neither a precursor nor a catalyst of vesperlysine A formation. We tentatively attribute the minute increase in vesperlysine A from ribose in presence of 10 mM methylglyoxal (Fig. 9C) to contaminants, which have been reported in the commercial batch of methylglyoxal.

The inability of methylglyoxal and glucose to serve as vesperlysine A precursor in the lens is an important finding as vesperlysine A thus becomes a marker for metabolic pathways different from those represented by existing markers of the advanced Maillard reaction *in vivo*, i.e. the glycoxidation markers and those derived from methylglyoxal, i.e. methylglyoxal lysine dimer (MOLD)/imidazolylysine (11, 12), argpyrimidine (13), and  $N^{\epsilon}$ -(carboxymethyl)lysine (14).

TABLE II

The effects of incubation of various sugars, oxoaldehydes and ascorbic acid with poly-L-lysine (10 g/liter) for 7 days at 37 °C on the formation of vesperlysine A

Incubation was in 200 mM sodium phosphate buffer (pH 7.4).

Sugar (50 mM)	Vesperlysine A
	nmol / mmol L-lysine
Glucose	ND <sup>a</sup>
Ribose	20.4
Arabinose	3.3
Xylose	4.5
Threose	22.9
Glyceraldehyde	ND
Glyoxal	ND
Methylglyoxal	ND
Ascorbic acid	8

<sup>a</sup> ND, not detected.

The fact that threose, a known ascorbate degradation product (15, 16), is a potent precursor of vesperlysine A, together with our previous data showing that LM-1 could form from ascorbate oxidation products under anaerobic conditions (1), strongly suggests ascorbate is a likely precursor of vesperlysine A in the aging and diabetic lens. In support, we previously reported exposure of rat lenses to high galactose *in vitro* and *in vivo* favored the uptake of ascorbate oxidation products, which are otherwise excluded by the healthy lens, such as 2,3-dike-



FIG. 9. Catalytic effect of C-2 and C-3 carbonyl compounds on vesperlysine A formation. Increasing concentration (0, 1, 5, and 10 mM) of glyoxal (A), glycolaldehyde (B), methylglyoxal (C), or glyceraldehyde (D) were initially added to the incubation mixture of poly-L-lysine (9 g/liter) and D-ribose (10 mM) in 200 mM phosphate buffer (pH 7.4). Vesperlysine A was detected and quantified using a two-column HPLC system.

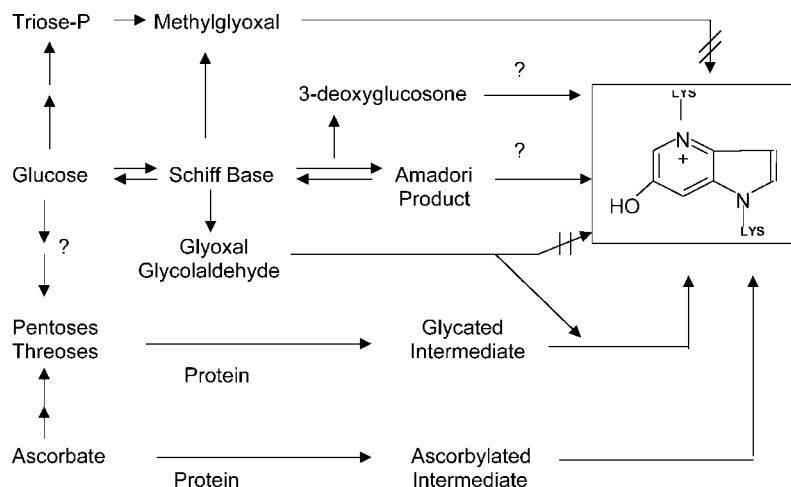
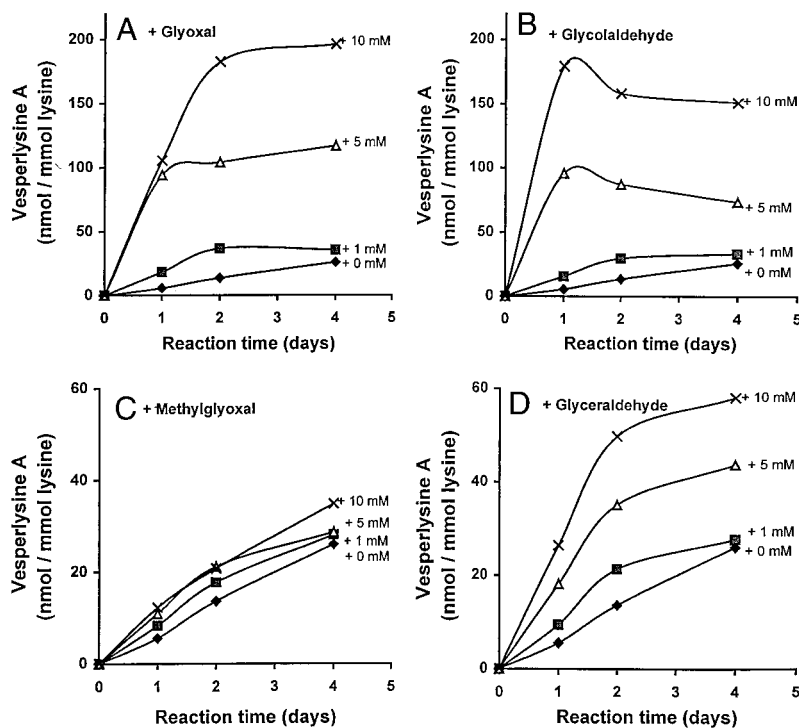


FIG. 10. Proposed mechanism for the formation of vesperlysine A in the human lens.

togulonic acid. (17). The latter was previously found to be a LM-1 precursor (1).

The proposed mechanism of non-oxidative formation of vesperlysine A is strongly supported by the data in the lens whereby the comparison with the glycoxidation markers pentosidine and CML is quite revealing (Fig. 7). First, a simple metal-catalyzed mechanism, as *e.g.* observed during glucose autooxidation, can be excluded on the basis that neither CML nor pentosidine are elevated in the diabetic lenses examined. Second, we formerly showed pentosidine was elevated only in lenses from poorly controlled diabetic dogs, and the sharp increase corresponded to a concomitant increase in Amadori product of glucose (4). This led to the conclusion that most pentosidine formed in diabetic human and dog lens is likely linked to a permeability breakdown of the lens. Thus, the dichotomy between the pentosidine and the vesperlysine A data in the diabetic lens leads to the conclusion that pentosidine in the diabetic lens unlikely originates from ascorbate, as originally proposed (18). In contrast, vesperlysine A/LM-1 formed readily from ascorbate oxidation products even under

anaerobic conditions (1). Thus, when structural constraints and the data above are taken into consideration, ascorbate emerges as one of the most likely precursors of vesperlysine A, whereby a catalytic effect from trioses or glycolaldehyde/glyoxal cannot be excluded. The absence of elevated CML excludes lipoxidation as a vesperlysine A precursor in the diabetic lens. Similar absence of increased CML in the diabetic lens was reported previously by Lyons *et al.* (19). Proposed mechanistic pathways are summarized in Fig. 10.

As expected, all Maillard reaction markers strongly correlated with age and the age-related pigmentation of the lens. Again, we confirmed the previous findings that LM-1/vesperlysine A levels were comparatively lower in the brunescient lenses, *i.e.* a rare form of advanced cataract that is characterized by extreme pigmentation, covalent cross-linking, and insolubilization of the crystallins. Several explanations for this finding are possible, among which are the blocking of vesperlysine A formation sites by kinetically more favored reactions, and possibly also the increased photo-oxidative destruction of LM-1/vesperlysine A, which has been found to act as photosen-



sitizer in UVA-irradiated lens crystallins (10).

This study is to our knowledge the first to document the existence of vesperlysine A *in vivo*. In view of the fact that it was either non-detectable, or present in minute quantities in other human and rat tissues, and only mildly increased in plasma proteins from patients with end stage renal disease, a condition associated with dramatic increase in many advanced glycation end products (AGEs), vesperlysine A emerges as novel Maillard reaction marker of human lenticular aging, which is most likely specific for carbonyl stress by ascorbate in the diabetic lens.

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