2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) Is a Newly Identified Advanced Glycation End Product in Cataractous and Aged Human Lenses*

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Post-translational modifications of proteins take place during the aging of human lens. The present study describes a newly isolated glycation product of lysine, which was found in the human lens. Cataractous and aged human lenses were hydrolyzed and fractionated using reverse-phase and ion-exchange high-performance liquid chromatography (HPLC). One of the non-proteinogenic amino acid components of the hydrolysates was identified as a 3-hydroxypyridinium derivative of lysine, 2-ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine). The compound was synthesized independently from 3-hydroxypyridine and methyl 2-[(tert-butoxycarbonyl)amino]-6-iodohexanoate. The spectral and chromatographic properties of the synthetic OP-lysine and the substance isolated from hydrolyzed lenses were identical. HPLC analysis showed that the amounts of OP-lysine were higher in water-insoluble compared with water-soluble proteins and was higher in a pool of cataractous lenses compared with normal aged lenses, reaching 500 pmol/mg protein. The model incubations showed that an anaerobic reaction mixture of N'-(tert-butoxycarbonyl)lysine, glycolaldehyde, and glyceraldehyde could produce the N'-4-butoxycarboxybenzyl derivative of OP-lysine. The irradiation of OP-lysine with UVA under anaerobic conditions in the presence of ascorbate led to a photochemical bleaching of this compound. Our results argue that OP-lysine is a newly identified glycation product of lysine in the lens. It is a marker of aging and pathology of the lens, and its formation could be considered as a potential cataract risk-factor based on its concentration and its photochemical properties.

Glycation is a natural process observed in living systems, consisting in post-translational modifications of the side chains of amino acids in proteins by reactive carbonyl components. Glycation is a multistage and multidirection process. Initially, so-called “early glycation products” are formed. At this stage the modifications of amino acids are reversible. Further reactions lead to the formation of advanced glycation end products (AGEs), which are relatively stable and tend to accumulate in biological or model systems with time. AGEs are important from a medical point of view because their concentration increases during aging (1) or in different medical complications such as cataract formation (2), retinopathy (3), and nephropathy (4).

A natural protective mechanism against glycation and other harmful post-translational modifications is protein turnover. The vast majority of protein molecules has a limited life span and is periodically degraded and rebuilt. A remarkable exception are the proteins in lens fiber cells, especially those in the lens nucleus, being as old as the individual to whom they belong. Thus, the lens is a very appropriate tissue to study the accumulation of AGEs in vivo.

The characterization and quantification of AGEs are based on chemical, spectral, and immunological techniques. Two major types of experiments are used for this purpose: either the properties of the biological sample are compared with those of in vitro glycated protein standard(s) or the tissue specimens are characterized and quantified with respect to a specific AGE with known structure. The first approach is popular probably because the preparation of in vitro glycated standards is rapid, inexpensive, and requires little labor. A serious drawback of such an approach, however, is that the conditions for preparation of in vitro glycated proteins are very different from in vivo glycated proteins. Usage of enormous amounts of glycating agent and/or elevated reaction temperatures is more than the exception. There is no guarantee that the structures, and the relative amounts of products built in vitro, match those of AGEs formed in vivo. In the contrary, model glycation reaction mixtures can contain major glycation products, which do not exist in vivo (5). Another problem is that the first approach refers to an abstract or imaginary value, “total AGEs,” whereas it is clear that at least the known AGEs belong to completely different structural classes having little in common. In contrast, the approach based on characterization and quantification of known AGEs gives specific information about the progress and extent of glycation in vivo. The only limitation of this approach is that it could be applied only to AGEs whose structure has already been identified.

In this article we report for the first time that the amino acid

1 The abbreviations and trivial names used are: AGEs, advanced glycation end products; Asc, L-ascorbic acid; Boc-Lys, tert-butoxycarbonyl-L-lysine; CL, cataractous lenses; DHA, L-dehydroascorbic acid; ESI-MS, electrospray ionization mass-spectrometry; FCL, fetal calf lenses; HFB, heptfluorobutyric acid; IE, ion-exchange; NAL, normal aged lenses; ODS, octadecylsulfate; OP-lysine, 2-ammonio-6-3-oxidopyridinium-1-yl)hexanoate; PDA, photo diode array; RP, reverse-phase; HPLC, high performance liquid chromatography; WI, water-insoluble; WS, water-soluble; DTPA, diethylentriaminepentaacetic acid; HOP-lysine, 4-hydroxymethyl derivative of OP-lysine.
2-ammonio-6-(3-oxidopyridinium-1-yl)hexanoate I (Fig. 1) is a component of the proteins in aged and cataractous lenses. Being a 3-oxidopyridinium derivative of lysine, we suggest the trivial name OP-lysine for compound I.

We also present a possible reaction pathway for its formation as a result of post-translational modification of lysine residues on proteins.

In our experiments OP-lysine appeared in RP-HPLC profiles of processed cataractous lens acid hydrolases as one of the major peaks absorbing at 290 nm and the strongest fluorescence peak at excitation/emission at 290/390 nm. The spectral and RP-HPLC properties of this compound were very similar to those of another glycation product, HOP-lysine 2. In a previous study (6) we reported that HOP-lysine (a 4-hydroxymethyl derivative of OP-lysine) can be formed from dehydroascorbic acid, l-erythrose, l-threose, and glycolaldehyde in vitro when reacted with lysine-containing material. The same compound, referred to as GA-pyridine, was reported as a component of proteins in human atherosclerotic lesions based on immunohistochemical staining pictures (7). This encouraged us to gather reliable chemical evidence about the structure of the lens protein component with absorbance at 290 nm and fluorescence at excitation/emission at 290/390 nm. As a result, the presence of OP-lysine in pooled cataractous and aged lenses was discovered. This compound is interesting also because the structure of OP-lysine is generic for other glycation products: HOP-lysine (6, 7), structure 2; GLAP (8), structure 3; the blue fluorophore LM-1 (9) (also referred to as vespex-lysine A), structure 4; and the post-translational collagen modification, lysylpyridinoline (10), structure 5 (Fig. 1).

**EXPERIMENTAL PROCEDURES**

**Materials**—Boc-Lys, chelating resin Chelex, heptafluorobutyric acid (HFBA), 1,4-dioxane, and all the carbohydrates were purchased from Sigma. Celite 521, 1,4-dioxane, and glycolaldehyde dimer were purchased from Aldrich. Methanol-d4 and DMSO-d6 from Cambridge Isotope Laboratories, Inc. (Andover, MA). Trifluoroacetic acid, acetonitrile, and methanol were from Fisher.

Oxacetylsulfate (ODS) cartridges were purchased from Supelco (Belleville, PA). The cartridges were activated with methanol and washed with 0.1% trifluoroacetic acid in water prior to use. Solutions of diazomethane in ethyl ether (see under “Materials”) was added dropwise with stirring until the appearance of a stable yellow color of diazomethane. The initial solid was dissolved completely at the end of the reaction. The solvent was evaporated under reduced pressure to give 3.3 g of the crude product 7. The latter was dissolved in 150 ml of tetrahydrofuran, and triphenylphosphine (5.0 g, 19 mmol), imidazole (1.4 g, 20 mmol), and iodine (4.8 g, 19 mmol) were subsequently added. The mixture was stirred at room temperature under argon for 2 h. The solvent was evaporated under reduced pressure, and 15 ml of ethyl acetate was added to the product. The solid residue was eliminated by filtration, and the filtrate was separated on a 120-g Redi-Sep column using hexanes/ethyl acetate 4:1 (v/v). Fractions of 7 ml were collected and analyzed by TLC using hexanes/ethyl acetate 4:1 (v/v). The fractions with 

**Preparative RP isolations and purifications** were carried out using a 250 × 21.2 mm Prodigy ODS (3) column. The eluants used are as follows: A, 0.1% HFBA in water; B, 50% acetonitrile containing 0.1% HFBA. The gradients used are as follows: 0–10 min, isocratic 10% B; 10–50 min, linear gradient 10–100% B; 50–100 min, isocratic 100% B at a flow rate of 1.0 ml/min.

**Synthesis of OP-lysine**—OP-lysine was prepared according to a modified method published by (11). The reaction pathway is presented in Fig. 2.

Briefly, Boc-Lys (4.92 g, 20 mmol) was dissolved in 50 ml of water; pH of the solution was brought to 9.5 with 4 M sodium hydroxide, and the temperature was maintained at 60 °C for 6 h. pH of the reaction mixture was corrected to 9.5 periodically during incubation. The sample was cooled and filtered through Celite 521. The filtrate was saturated with sodium chloride, pH was brought to 3.5 and the solution was extracted with ethyl acetate (4 × 40 ml). The combined organic layers were washed with 40 ml of 30% NaCl and dried overnight over anhydrous sodium sulfate. The solution was concentrated under reduced pressure. The yield was 4.1 g of the crude compound 6. Part of this product (3.5 g) was stirred with 10 ml of ether, which caused partial crystallization of the compound. A solution of diazomethane in diethyl ether (see under “Materials”) was added dropwise with stirring until the appearance of a stable yellow color of diazomethane. The initial solid was dissolved completely at the end of the reaction. The solvent was evaporated under reduced pressure to give 3.3 g of the crude product 7. The latter was dissolved in 150 ml of tetrahydrofuran, and triphenylphosphine (5.0 g, 19 mmol), imidazole (1.4 g, 20 mmol), and iodine (4.8 g, 19 mmol) were subsequently added. The mixture was stirred at room temperature under argon for 2 h. The solvent was evaporated under reduced pressure, and 15 ml of ethyl acetate was added to the product. The solid residue was eliminated by filtration, and the filtrate was separated on a 120-g Redi-Sep column using hexanes/ethyl acetate 4:1 (v/v). Fractions of 7 ml were collected and analyzed by TLC using hexanes/ethyl acetate 4:1 (v/v). The fractions with 

**Fig. 1. Structures of OP-lysine and its analogs.**

**Fig. 2. Synthesis of OP-lysine.**—OP-lysine was prepared according to a modified method published by (11). The reaction pathway is presented in Fig. 2.
at 290/390 nm, was collected and dried under reduced pressure. The yield of the HFBA adduct of OP-lysine (1) was 39.7 mg. In a separate experiment compound 9 was deprotected as described above. The crude product 1 was purified according to the procedure described previously (11) in order to obtain trifluoroacetic acid adduct of OP-lysine, 1-2 trifluoroacetic acid. This product (10.4 mg, 23 μmol) was dissolved in 2 ml of water and was used as a stock solution for fluorescence and UV measurements, including preparation of calibration curves for quantification of OP-lysine based on the peak area in RP-HPLC. The chromatographic profiles were recorded by absorbance at 289 nm and fluorescence at excitation/emission at 290/390 nm.

A sample, containing 5.0 nmol OP-lysine in 100 μl of water, was hydrolyzed in 5 ml of 6 N HCl for 22 h at 110 °C. The solvent was evaporated under reduced pressure; 5 ml of water was added, re-evaporated, and finally, the residue was dissolved in 100 μl of water. Both the starting solution and the hydrolysate were analyzed by analytical RP-HPLC. The chromatogram of the hydrolysate showed a single major peak with the UV spectrum, retention time, and fluorescence spectrum corresponding to those of the original OP-lysine sample. The sample was analyzed also by ESI-MS spectrometry. The recovery of the peak following acid hydrolysis was 90 (according to a fluorescence calibration curve) and 85% (according to the A289 calibration curve) compared with the peak area of the initial OP-lysine standard.

**Preparation and Analysis of Lens Protein Hydrolysates—Fifty cataractous lenses (52–75 years old, average age 64 years, mostly type II–III (19)), 50 human lenses (60–75 years old, average age 65 years), and 10 fetal calf lenses were processed in the following way. Each type of lens was pooled, decapsulated, homogenized in 25 ml of water, and separated by centrifugation at 30,000 × g. The pellet was washed twice with 20 ml of water followed by centrifugation. The water-soluble fractions were extensively dialyzed against water for 3 days and freeze-dried. The water-insoluble fractions of cataractous lenses and aged normal human lenses were dried under reduced pressure. The slight water-insoluble fraction of calf lenses was discarded.

Samples containing 100 mg of lyophilized protein were hydrolyzed with 10 ml of 6 N HCl under argon for 22 h at 110 °C in Teflon-sealed glass tubes. The samples were dried under reduced pressure; 5 ml of water was added, re-evaporated, and finally, the residue was dissolved in 1 ml of water. The samples from cataractous, normal human aged, and fetal calf lenses were processed in the following way. Each type of lens was pooled, decapsulated, homogenized in 25 ml of water, and separated by centrifugation at 30,000 × g. The pellet was washed twice with 20 ml of water followed by centrifugation. The water-soluble fractions were extensively dialyzed against water for 3 days and freeze-dried. The water-insoluble fractions of cataractous lenses and aged normal human lenses were dried under reduced pressure. The slight water-insoluble fraction of calf lenses was discarded.

Isolation of OP-lysine from Hydrolyzed Cataractous Lenses—Part of the hydrolyzed water-insoluble cataractous lens protein (see above), consisting of 200 mg of normal human aged lenses or 500 mg of fetal calf protein, was separated using a 60-ml (10 g solid phase) ODS cartridge using eluant water/methanol/trifluoroacetic acid in water. Fractions of 1.0 ml were collected and analyzed by analytical RP-HPLC. Chromatograms were recorded using ESI-MS/MS and PDA detectors as well as fluorescence and PDA detectors. The amounts of OP-lysine in the hydrolysates were calculated using the fluorescence peak areas at excitation/emission at 290/390 nm and the UV peak areas at 289 nm.

Detection of OP-lysine in Digested Cataractous Lenses—Enzymatic digest of water-insoluble sonicated supernatant from cataractous lenses was prepared as described previously (2). The final solution was concentrated 10-fold and analyzed by liquid chromatography ESI-MS/MS. Analyses of in Vitro Reaction Mixtures for the Presence of OP-lysine—Topa-DNA, DHA, n-glycolfructose, t-xylene, glyceraldehyde, methylglyoxal, and glycolaldehyde, were reacted with Boc-Lys. Reaction mixtures containing 100 mM carbonyl compound, 50 mM Boc-Lys, 1 mM DTPA, and 100 mM potassium phosphate, pH 7, were incubated at 37 °C for 7 days under argon. The mixtures were analyzed by preparative RP-HPLC. The chromatograms were monitored for UV absorbance in the range of 200–400 nm and the fluorescence at excitation/emission at 290/390 nm. None of the analyzed reaction mixtures showed a peak with spectral features and retention times close to those of the synthetic OP-lysine except the reaction mixture Boc-Lys + glyceraldehyde. The peak from this reaction system was collected, concentrated under reduced pressure, and deprotected using 200 μl of trifluoroacetic acid at 0 °C for 15 min. Trifluoroacetic acid was eluted by a stream of argon, and the residue was analyzed by ion-exchange chromatography in both the absence and presence of the synthetic OP-lysine standard using analytical ion-exchange chromatography (gradient B).

This study of OP-lysine involved isolation of small amounts of the compound from cataractous lenses, estimation of its structure based on UV, H NMR, and MS spectra, unequivocal synthesis, and characterization of larger amounts of standard OP-lysine, experiments that proved the identity between the biological material and the standard quantification of OP-lysine in acid hydrolysates of lens proteins and liquid chromatography-MS/MS experiments showing that OP-lysine exists in lens enzymatic digests. Our initial interest was provoked by the similarity of the UV and fluorescence spectral properties between this specific and unknown component of acid-hydrolyzed lenses and another known AG, HOP-lysine. The final result of the study was that OP-lysine is one of the most...
abundant known UVA-absorbing AGEs in cataractous and aged lenses.

*Synthesis and Characterization of OP-lysine*—OP-lysine was prepared using the synthetic path presented in Fig. 2. The preparation is a modified version of a synthesis published by Adamczyk et al. (11). The major change was the usage of methyl ester protection of the carboxyl group of Boc-Lys instead of tert-butyl. The esterification of compound 6 with diazo-methane was quantitative, and the only major side product was nitrogen gas, which allowed us to omit one preparative chromatographic separation. In addition, the use of methyl instead of tert-butyl protection allowed for the selective deprotection of both the carboxyl and amino groups of condensation product 9 when necessary. Otherwise, the NMR spectral characteristics of compound 1 obtained by us perfectly matched the data published by Adamczyk et al. (11), namely 1H NMR (CD3OD): δ 8.51 (m, 1H), 8.4 (m, 1H), 7.94 (m, 1H), 7.87 (m, 1H), 4.57 (t, 2H, J = 7.5 Hz), 3.97 (t, 1H, J = 6.4 Hz), 2.07–1.94 (m, 4H), 1.60–1.50 (m, 2H) ppm. For 13C NMR (CD3OD): δ 171.6, 159.4, 136.6, 134.1, 132.7, 129.8, 62.4, 53.5, 32.6, 31.6, 28.3, 26.1, 6.1 ppm. The important intermediate 8 was also purified and its NMR spectral characteristics are as follows: 1H NMR (CD3OD): δ 5.06 (d, 1H, J = 7.7), 4.31 (m, 1H), 3.75 (s, 3H), 3.18 (s, 2H, J = 6.9), 1.86 - 1.40 (m, 6H), 1.45 (s, 9H) ppm. 13C NMR (CD3OD): δ 173.0, 155.3, 79.9, 53.1, 52.3, 32.6, 31.6, 28.3, 26.1, 6.1 ppm.

ESI-MS spectrometry of OP-lysine revealed a [M + 1]+ peak at m/z = 225, which was in agreement with the calculated molecular mass. The ESI-MS/MS spectrum (Fig. 3) showed three major fragmentation peaks at m/z = 130, 96, and 84. A possible fragmentation scheme of OP-lysine is shown in Fig. 4.

As we described under “Materials,” OP-lysine was purified by RP-HPLC using gradients of acetonitrile in water in the presence of HFBA or trifluoroacetic acid. We found that the usage of HFBA results in a longer retention time of OP-lysine compared with trifluoroacetic acid-based eluants. Improved chromatographic separation due to HFBA was reported for other AGEs (13). This makes HFBA more appropriate for isolation of OP-lysine from complex mixtures or its chromatographic quantification. However, HFBA is much less volatile than trifluoroacetic acid, and we experienced difficulties with elimination of HFBA and moisture from preparative fractions containing OP-lysine. On the other hand the concentration of fractions containing OP-lysine obtained after RP-HPLC separation in water/acetonitrile/trifluoroacetic acid systems resulted in the production of the trifluoroacetic acid adduct of compound 1, OP-lysine-2 trifluoroacetic acid as described previously (11). We used trifluoroacetic acid-based separation for preparation of a standard for quantification of OP-lysine by UV-spectroscopy and for preparation of standard curves for OP-lysine content based upon the absorbance at 289 nm and fluorescence at excitation/emission at 290/390 nm. The UV-spectrum of OP-lysine depends on pH as can be seen in Fig. 5 due to the existence of two different forms.

At pH 2, which is typical for RP-HPLC separations, OP-lysine showed absorption maxima at 203, 223, and 289 nm. At pH 7, which corresponds to the physiological conditions, the absorption maxima were at 214, 249, and 320 nm. These values match the absorption maxima published for 3-hydroxy-1-methylpyridinium chloride: pH 2, 224 and 288 nm, and pH 7, 249 and 320 nm (14). The fluorescence spectrum of OP-lysine was also pH-dependent: pH 2, excitation/emission at 287/392 nm; pH 7, excitation/emission at 320/393 nm.

Detection and Quantification of OP-lysine in Lens Proteins—Proteins from cataractous and aged human lenses as well as fetal calf lenses were separated to water-soluble and water-insoluble fractions. The insignificant water-insoluble fraction of calf lenses was discarded. The proteins obtained were acid-hydrolyzed and passed through ODS cartridges in order to eliminate most of the brown-colored components of the hydrolysates. Model experiments with synthetic OP-lysine standard confirmed that it is not retained effectively under these conditions and elutes close to the dead volume of the cartridges used. At the same time most of the brown coloration produced by hydrolysis was retained on the top of the cartridge. The separation on ODS cartridges was used as a first step in purification of OP-lysine from hydrolyzed proteins for both preparative and analytical purposes as described below.

A fraction of hydrolyzed water-insoluble protein, correspond-
ing to 25 pooled cataractous lenses, was separated consequently on 60 ml of ODS cartridge, IE-HPLC and RP-HPLC by multiple injections. The peak corresponding to the spectral and chromatographic properties of OP-lysine was collected at each separation step. Finally, a purified fraction was obtained and analyzed by 1H NMR spectroscopy. An expanded part of the spectrum of the product isolated from the lenses is shown in Fig. 6A.

The chemical shifts and the fine structure of the signals of the aromatic protons of this sample perfectly matched those of the synthetic standard (Fig. 6B). Furthermore, co-elution experiments using analytical RP-HPLC and analytical IE-HPLC (gradient B) confirmed the identity between the sample isolated from hydrolyzed cataractous lenses and the synthetic standard of OP-lysine.

In order to evaluate the amounts of OP-lysine in lens proteins, pooled samples of WS and WI fractions of cataractous and normal aged lenses were acid-hydrolyzed as described above. The WS fraction of calf lens proteins was also acid-hydrolyzed as a negative control. The hydrolysates were passed through ODS cartridges, and the fractions collected were analyzed by analytical RP-HPLC in triplicate. Typical UV and fluorescence profiles, used for measurement of OP-lysine in WI cataractous lenses, are shown in Fig. 7. The amounts measured for different types of lens proteins are shown in Fig. 8.

In order to confirm that the absorbance and fluorescence peaks used are due to OP-lysine, we analyzed the lens hydrolysates by HPLC-ESI-MS/MS. In all cases the human lens hydrolysates produced the all three daughter peaks of OP-lysine. Representative chromatograms of hydrolyzed WI cataractous human lens protein and hydrolyzed calf lens proteins are shown in Fig. 9.

The figure represents the profiles for all the three daughter ions at m/z = 130, 96, and 84 obtained from a parent ion [M + 1]+ at m/z = 225, as well as the absorbance at 289 nm. Interestingly, the results showed a minimal amount of OP-lysine even in hydrolyzed calf lens protein because of the extremely high sensitivity of selected ion monitoring ESI-MS/MS HPLC analysis. The abundance of the peaks, however, were ~10^4 lower than those of hydrolyzed WI cataractous proteins. Once the traces for hydrolyzed WI cataractous lens protein and calf lens protein were plotted at the same scale, the latter produced flat lines, suggesting that the peaks observed in human lenses are not artifacts of the protein hydrolysis by itself. Furthermore, we analyzed an enzymatic digest (2) of the same sample of WI cataractous lens proteins by selected ion monitoring ESI-MS/MS HPLC. The appearance of all three daughter ions of OP-lysine at appropriate retention time (Fig. 10) is also solid evidence that this post-translational modification of lysine exists in cataractous lenses.

**Formation of OP-lysine in Model Systems Containing Boc-Lys and Carbonyl Compounds**—We studied the ability of 10 potential carbonyl precursors to produce OP-lysine with Boc-Lys under conditions that do not favor glycoxidation, i.e. in absence of metals and anaerobic environment. DHA, glucose,
Fructose, xylose, erythulose, glyceraldehyde, glyceraldehyde 3-phosphate, methylglyoxal, 1,3-dihydroxyacetone, and glycolaldehyde are reactive carbonyl compounds known to be able to modify the amino group of lysine residues in lens proteins (6, 15–21). The RP-HPLC analyses showed that none of the above carbonyl compounds was able to produce measurable amounts of OP-lysine when reacted with Boc-Lys at pH 7 for 5 days based on UV and fluorescence HPLC profiles of the reaction mixtures. In the case of glyceraldehyde, there was a peak having a retention time and UV spectrum close to those of OP-lysine. The product was collected using preparative RP-HPLC, dried, and deprotected with trifluoroacetic acid. After evaporation of trifluoroacetic acid, the residue was further analyzed by 1H NMR spectroscopy and analytical IE-HPLC (gradient B). The 1H NMR spectrum, recorded in CD3OD, revealed the presence of only three protons in the aromatic region having chemical shifts at δ: 8.41 (s, 1H), 8.38 (s, 1H), and 7.88 (s, 1H) ppm. These values as well as the fine structures of the peaks were completely different compared with those of the synthetic sample of OP-lysine. It is quite possible that the product we isolated is compound 3, which has singlet signals for the aromatic protons at δ: 8.38, 8.34, and 7.94 ppm (in D2O) according to Ref. 8. The analytical IE-HPLC co-elution experiment of the product obtained from the model mixture Boc-Lys + glyceraldehyde and the synthetic OP-lysine showed two differ-
The absorbance of OP-lysine at 320 nm practically did not change in the presence of Asc without irradiation (Fig. 12C). The results presented in Fig. 12 argue that Asc is an active participant in the photochemical bleaching of 3-oxidopyridinium heterocycle by UVA.

**DISCUSSION**

The major purpose of this article is to provide evidence for the existence of OP-lysine *in vivo* as a newly identified AGE of lysine residues on proteins.

Similarly to other known AGEs, the amount of OP-lysine in the biological material was not sufficient for the recording of its $^{13}$C NMR spectrum, which is one of the key confirmations of the structure of an analyte. We were able, however, to isolate enough material for recording the $^1$H NMR spectrum due to the relatively high content of this AGE in cataractous human lenses. We suggested a possible structure (1) of the new AGE based on its NMR, UV, and fluorescence spectral data as well as its similarities with another AGE, HOP-lysine 2, which we isolated earlier from a DHA-containing model reaction system (6). We performed independent synthesis of OP-lysine (Fig. 2), which was a modified version of a procedure published previously (11). This allowed us to produce a sufficient amount of synthetic standard to permit characterization by chemical and spectral methods. The usage of known reactions during the independent synthesis, the chemical and spectral properties of the synthetic product, and the identity of $^1$H NMR, $^{13}$C NMR, and ESI-MS spectral data obtained by us and those published by another laboratory (11) were solid evidence that the synthetic standard of OP-lysine we prepared had the structure 1, shown in Fig. 2. The relative high stability of OP-lysine toward acid hydrolysis encouraged us to use this method for its release from proteins. Furthermore, we used the synthetic standard in order to prove the existence of OP-lysine *in vivo*.

First, we compared the properties of the synthetic standard of OP-lysine with the substance isolated from hydrolyzed cataractous lenses mentioned in the previous paragraph. The data given in Fig. 6 showed that the chemical shifts and the fine structures of $^1$H NMR peaks in the spectrum of the substance isolated from hydrolyzed lenses were identical to those of the synthetic standard of OP-lysine. Similarly, the UV and fluorescence spectra of the two samples were identical (data not shown). Two chromatographic experiments using analytical
exists this 3-oxidopyridinium derivative of lysine digested lens proteins (Fig. 10) clearly confirms the existence of these ions of OP-lysine in the chromatogram of enzymatically digested proteins. Also, the presence of peaks for all three daughter ions of OP-lysine argues that OP-lysine cannot be generated during acid hydrolysis of water-insoluble and water-soluble calf lens proteins shown in Fig. 9. Traces of daughter ions of OP-lysine for hydrolyzed WI cataracts and dehydroascorbic acid could be OP-lysine precursors under oxidative conditions.

We estimated the amount of OP-lysine in pooled samples of water-insoluble and water-soluble fractions, obtained from cataractous and aged human lenses. The data shown in Fig. 8 argue that increased amounts of OP-lysine can be related to cataractogenesis and transformation of water-soluble to water-insoluble lens proteins. Trying to answer the concerns presented in the previous paragraph, which are not completely resolved in this study, we are working on development of an HPLC-ESI-MS/MS quantification method based on usage of isotopically labeled internal standard of OP-lysine. Still, in the current study we have an excellent agreement between the values obtained from UV and fluorescence HPLC peak areas (Fig. 8), which supports our confidence that the numbers presented in Fig. 8 are correct. In general, the values we report for OP-lysine are lower than those published for fructose lysine (21), N-oxy-1-(1-carboxyethyl)lysine (23, 24), and N-carboxymethyllysine (24) and are higher, although of the same magnitude as these published for argpyrimidine (25).

We studied the ability of 10 model reaction systems based on Boc-Lys and 2-, 3-, 4-, 5-, and 6-carbon-containing AGE precursors to generate OP-lysine under anaerobic conditions. Interestingly, the UV and fluorescence chromatograms of the resulting mixtures did not reveal measurable amounts of this compound. At the same time there was an evident peak for OP-lysine in the system Boc-Lys + glycolaldehyde + glyceraldehyde (Fig. 11). The fractions, corresponding to OP-lysine, were further purified, and the product showed a \(^{1}H\) NMR spectrum identical to those of the synthetic standard and the substance isolated from hydrolyzed lenses (Fig. 6). The molecular mass of the product of the model system was 224 as expected, and there were successful co-elution experiments with the synthetic standard using RP- and IE-HPLC. The data obtained argue that the combination of the two carbonyl precursors used, glycolaldehyde plus glyceraldehyde, is a better source for generation of OP-lysine from Boc-Lys than any of the single carbonyl compounds we tested \textit{in vitro}. In our model experiments we used incubation conditions that suppress glycoxidation. Therefore, we cannot exclude that other carbohydrates and dehydroascorbic acid could be OP-lysine precursors under oxidative conditions.

A possible reaction pathway for formation of OP-lysine includes a di-Amadori adduct between lysine residue, glycolaldehyde and glyceraldehyde, as an intermediate and further aldol-like cyclization, followed by elimination of water (Fig. 13).

Although there are no data that di-Amadori products like OP-lysine are involved in glycation \textit{in vivo}, it is well known that similar compounds are highly reactive in Maillard reaction even at 37 °C (26). Our results argue that some of the AGEs existing \textit{in vivo} could be products of co-operative interactions between reactive carbonyl compounds and side chains of amino acids. In the real biological systems, lysine and arginine side chains are immersed in a “mixture” of glycation agents, and it should be not a surprise that in some cases the most favorable reaction path would include participation of more than one carbonyl compound. This hypothesis is in agreement with results published previously (9) showing that the addition of glyceraldehyde to a reaction mixture of poly-L-lysine and ribose results in approximately a 7-fold increase of the yield of vepser lysine A.

The formation of OP-lysine on protein molecules could affect their properties. Compared with the original lysine residue, OP-lysine bears an additional negative charge because the 3-oxidopyridinium ring is deprotonated at physiological pH values (Fig. 5B). This could affect the tertiary and quaternary structure of proteins. For example, the loss of a single positive charge in αA-crystalline due to a mutation of arginine to cysteine results in significant change of the tertiary structure of this protein (27), significant diminishment of its chaperon-like activity, and has been suggested as a probable cause for development of congenital cataract (28).

**Fig. 13.** A hypothetical reaction path for formation of OP-lysine.
By taking into account that UVA can penetrate in the human lens (29) and that the latter contains high concentrations of ascorbate (30), we can expect that OP-lysine could play an important role in photochemical processes in the lens, based on the data for its photo-bleaching shown in Fig. 12.

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2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) Is a Newly Identified Advanced Glycation End Product in Cataractous and Aged Human Lenses
Ognyan K. Argirov, Bin Lin and Beryl J. Ortwerth

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The coiled-coil domain of EspA is essential for the assembly of the type III secretion translocon on the surface of enteropathogenic Escherichia coli.

Robin M. Delahay, Stuart Knutton, Robert K. Shaw, Elizabeth L. Hartland, Mark J. Pallen, and Gad Frankel

A typographical error occurred during the preparation of the manuscript whereby heptad repeat “d” position residues in the putative coiled-coil domain of EspA were mistakenly notated as “a” position residues. Where reference is made to the heptad position of residues selected for substitution, “a” should be “d” in all cases. The number designation of amino acids as they occur in the EspA sequence and the identity of residues mutated are unchanged and as reported. This error is purely notational and has no bearing on any of the results either presented or discussed.

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2-Ammonio-6-(3-oxidopyridinium-1-yl)hexanoate (OP-lysine) is a newly identified advanced glycation end product in cataractous and aged human lenses.

Ognyan K. Argirov, Bin Lin, and Beryl J. Ortwerth

Page 6488, “Experimental Procedures”: Under the subheading “Synthesis of OP-lysine,” the beginning of the second paragraph was incomplete. The sentence should read: Briefly, Boc-Lys (4.92 g, 20 mmol) was dissolved in 50 ml of water, pH of the solution was brought to 9.5 with 4 M sodium hydroxide, sodium nitroprusside (9.43 g, 32 mmol) was added within 30 min, and the temperature was maintained at 60 °C for 6 h.

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The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Krüppel-like factor 5 through direct interaction.

Takayoshi Matsumura, Toru Suzuki, Kenichi Aizawa, Yoshiko Munemasa, Shinsuke Muto, Masami Horikoshi, and Ryozo Nagai

Page 12123: There was an error in the e-mail address for Dr. Nagai. The correct e-mail address is: nagai-tky@umin.ac.jp.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.