FEBS Letters 382 (1996) 234–238 FEBS 16756

The presence of a glucose-derived Maillard reaction product in the human lens

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Received 27 November 1995; revised version received 30 January 1996

Abstract Pyrraline is an advanced Maillard reaction product formed by the non-enzymatic reaction initiated by glucose with lysine residues on proteins. This reaction involves an intermediate, 3-deoxyglucosone, concentration of which is shown to be elevated in plasma and lenses during diabetes. Bovine lens alpha crystallins incubated with 3-deoxyglucosone showed that pyrraline formation was a major modification and its quantification by two different methods revealed time-dependent accumulation. Pyrraline was quantified in normal, senile cataractous and diabetic lenses. Although a wide variation was observed, the mean value in cataractous lenses (mean ± S.E.: 48.4 ± 12.67 pmol/mg protein) was higher than in age-matched normal lenses (30.9 ± 10.26 pmol). Surprisingly, in diabetic lenses, the mean value was lower than normal lenses (28.4 ± 15.3 pmol). These results suggest that glucose-specific advanced Maillard products occur in the human lens and such modifications may play a role in lens aging and cataract formation.

Key words: Glycation; Protein aging; Lens crystallin; Diabetes

1. Introduction

Advanced Maillard reaction products derived from non-enzymatic glycosylation of proteins by reducing sugars have been implicated in protein aging and pathogenesis of diabetic complications. The human lens was one of the first tissues to be studied for the role of Maillard reaction in protein aging, since proteins here hardly turn over and thus are expected to accumulate advanced Maillard products. The detection of the initial product of glycation (Amadori product), and its increase in aging and cataractous lenses suggested that glycation may in part be responsible for protein aggregation, crosslinking and pigmentation. Evidence for the presence of advanced Maillard products was mostly indirect, measured as protein-bound fluorescence. However, the protein-bound fluorescence can originate not only from the Maillard reaction, but also from the reaction of lipid peroxidation products. Thus, the specific effects of advanced Maillard products could not be appreciated. Recently, evidence for the advanced Maillard reaction in the human lens has been provided by the detection of specific products, LM-1, pentosidine and carboxymethyl lysine (CML) [1–3]. Their progressive accumulation in aging, and higher levels in cataractous lenses compared to age-matched normal lenses provided further evidence for a role for advanced Maillard reaction in lens protein aging and cataractogenesis.

Studies on the synthetic mechanisms for the advanced Maillard products described above have shown that they can be synthesized by a variety of sugars other than glucose, including ascorbate oxidation products [2,4,5]. In fact, previous studies have implicated ascorbate oxidation products as the major precursors for LM-1 and pentosidine in the lens [1,2,5,6]. The only exception to this is pyrraline, an advanced Maillard reaction product also shown to be present in vivo (Fig. 3).

Pyrraline is synthesized by glucose through an intermediate, 3-deoxyglucosone. The presence of 3-deoxyglucosone in plasma and its elevation in diabetes have been demonstrated in recent reports [7,8]. Its increase in diabetic lenses has been established in experimental animals [9], and has been suggested to occur through polyol pathway intermediates [10]. It can also form from the Amadori product directly [11]. Although pyrraline formation in vivo is disputed in a recent report [12], several studies using immunochemical and chromatographic methods have demonstrated its presence in vivo [13–15]. Our recent study has shown that pyrraline can form pyrraline-pyrraline and pyrraline-cysteine crosslinks, which may explain in part protein crosslinking in aging and diabetes [16]. Since protein glycation by glucose is enhanced and the polyol pathway is activated in diabetic lenses, we hypothesized that these events may lead to increased formation of pyrraline. In this study, we report the synthesis of pyrraline in lens crystallins incubated with 3-DG and present quantitative data on pyrraline in normal, cataractous and diabetic lenses.

2. Materials and methods

Human lenses were obtained from the Cleveland Eye Bank and the National Disease Research Interchange (NDRI), Philadelphia, PA. Cataractous lenses were obtained from the Department of Ophthalmology, University Hospitals of Cleveland, Cleveland, OH. Bovine lens α-crystallins were a kind gift from Dr. Krishna Sharma, University of Missouri, Columbia, MO. Pronase E (from *Streptomyces griseus*), peptidase (from porcine intestinal mucosa) and barium hydroxide octahydrate were from Sigma Chemical Co. All other reagents were of analytical grade. Pyrraline was purified from glucose-lysine incubation mixture as described before [13]. 3-Deoxyglucosone was synthesized by the method of Madson and Feather [17].

2.1. Incubation of α-crystallins with 3-deoxyglucosone

α-Crystallin at a concentration of 100 mg/ml was incubated with 100 mM 3-DG in phosphate-buffered saline (PBS) at 37°C. Aliquots of 0.4 ml were withdrawn on day 0, 4, 8, 12 and 19 and dialyzed against 2×2 l PBS at 4°C for 48 h. The dialyzed fractions were stored at −80°C until use. Proteins incubated without the sugar served as control.

2.2. Enzymatic digestion

5 mg proteins (lens α-crystallins incubated with or without 3-DG) were taken in 400 μl phosphate-buffered saline (PBS). To this 1% peptidase (w/w) was added and incubated at 37°C for 16 h. The

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SSDI S0014-5793(96)00142-1

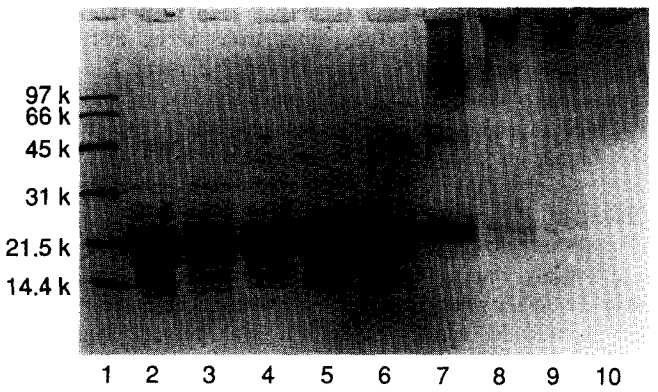

Fig. 1. SDS-polyacrylamide gel electrophoresis of bovine α-crystallins incubated with 3-deoxyglucosone. Lane 1, protein standards; lanes 2, 3, 4 and 5 are crystallins incubated without 3-DG for 4, 8, 12 and 19 days, respectively, and lanes 6, 7, 8, 9, 10 are crystallins incubated with 3-DG for 0, 4, 8, 12 and 19 days, respectively.

digestion was continued with the addition of pronase E (1%, w/w) for 16 h. After digestion, the samples were clarified by centrifugation at 3500×*g* for 15 min and filtered through 0.45 μm centrifugal filters (Rainin Instruments Co. Inc., Woburn, MA).

2.3. Barium hydroxide hydrolysis

5 mg lens proteins (incubated with or without 3-DG) were taken in a screw capped tube and 1.5 ml water and 800 mg barium hydroxide octahydrate were added. Hydrolysis was carried out at 120°C for 24 h. The pH of the hydrolyzate was adjusted between 7 and 8 with 4 N H_2SO_4. The solution was then centrifuged at 14000×*g* for 30 min. The supernatant was taken out and dried in a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY). In the case of human lenses, tissue corresponding to 100 mg wet weight was minced into small pieces, and digested with barium hydroxide (2.5 g) in 4.8 ml water. Other procedures were as described above. In the protein digests obtained by the above methods, amino acid estimation was done by the method of Moore and Stein using L-leucine as standard [18].

2.4. HPLC assay of pyrraline

Pyrraline estimation was accomplished by HPLC using a C_{18} reversed phase column (10 μm, Vydac, The Separations Group, Hesperia, CA). The mobile phase consisted of water (A) and 50% acetonitrile in water (B) and with 0.1% trifluoroacetic acid (TFA). The gradient program was as follows 0–10 min: 0–15% B; 10–40 min: 15–25% B; 42–49 min: 100% B and 50–57 min: 0% B at a flow rate of 1.2 ml per min. The column eluate was monitored for absorbance at 298 nm. Pyrraline standard eluted at 16.8 min. In the case of enzyme digested samples, the mobile phase B was 60% acetonitrile in water with 0.01 M heptafluorobutyric acid (HFBA). The column was eluted with a linear gradient of B from 0 to 35 min at a flow rate of 1.0 ml per min. Pyrraline standard eluted at about 15.0 min under these conditions.

In the case of human lenses, the mobile phase was water/acetonitrile with 0.1% TFA a described above. The gradient program was as follows: 0–10 min: 0–29% B, 20–40 min: 29–57% B; 41–47 min: 100% B and 48–58 min: 0% B. Pyrraline standard eluted at 19 min. Since pyrraline was not well resolved pyrraline containing eluate (from 17 to 22 min) was collected, pH was adjusted to 7.0 by 1 N NaOH and dried. The dried material was reconstituted in 200 μl water (+0.01 M HFBA) and subjected again to HPLC on a reversed phase column using the following program (solvent A: water + 0.1 M HFBA, solvent B: 60% acetonitrile in water+0.01 M HFBA; 0–15 min: 0–20% B; 15–20 min: 20–25% B; 20–40 min: 25–42% B; 41–48 min: 100% B; 49–56 min: 100% A. Pyrraline eluted at 24.5 min. Pyrraline was quantified in the lenses by comparing with the peak area of the standard and expressed as pmoles/mg protein by considering the molecular weight of lens cystallins as 20000 kDa.

2.5. Purification of pyrraline from brunescent lenses

Human brunescent lenses (pool of five) were extracted into water soluble and insoluble fractions as described previously [1]. The water insoluble fraction (50 mg) was digested with barium hydroxide and subjected to HPLC using a C_{18} reversed phase semi-preparative column (10 μm, Vydac). The HPLC eluate corresponding to the retention time of pyrraline standard was collected and reinjected for further purification as described above for the human lenses, except that the flow rate was set at 2.0 ml per min. The absorption spectrum of pyrraline was recorded by an on-line UV/vis absorbance detector (model 975, Jasco Corp., Japan).

SDS-PAGE was performed on a 12.5% gel in the presence of mercaptoethanol. Briefly, 25 μg of proteins was applied to each lane with 4% stacking gel. The gel was run at 40 mA and stained with Coomassie brilliant blue.

3. Results

Bovine lens α-crystallins incubated with 3-deoxyglucosone progressively turned yellow and underwent crosslinking. SDS-PAGE (Fig. 1) showed the formation of dimers and some polymers after only 4 days of incubation. After 8 days, there was a significant reduction in monomers and formation of protein polymers. Most of the protein turned polymeric after 12 days of incubation. The absorption spectra showed that pyrraline may be a major modification in these incubations, since the region where pyrraline has maximum absorbance (between 285 and 320 nm) increased as a function of time (Fig. 2).

Pyrraline was quantified in the incubated samples by two methods. The barium hydroxide hydrolysis followed by HPLC showed a distinct peak corresponding to the retention time of purified pyrraline (not shown). This peak had the absorption spectra (scanned by the on-line detector) identical to that of purified pyrraline (not shown). Pyrraline level increased as a function of time and reached 419 pmol/mg protein after 19 days of incubation (Fig. 3). We also tested whether pyrraline could be released from protein by enzyme digestion and whether this method could be used for its quantification in proteins. Sequential addition of peptidase and pronase was efficient in releasing pyrraline. HPLC of the digested material showed a well separated peak at 15.3 min, which was identical to the retention of purified pyrraline (Fig. 4). The samples which were spiked with purified pyrra-

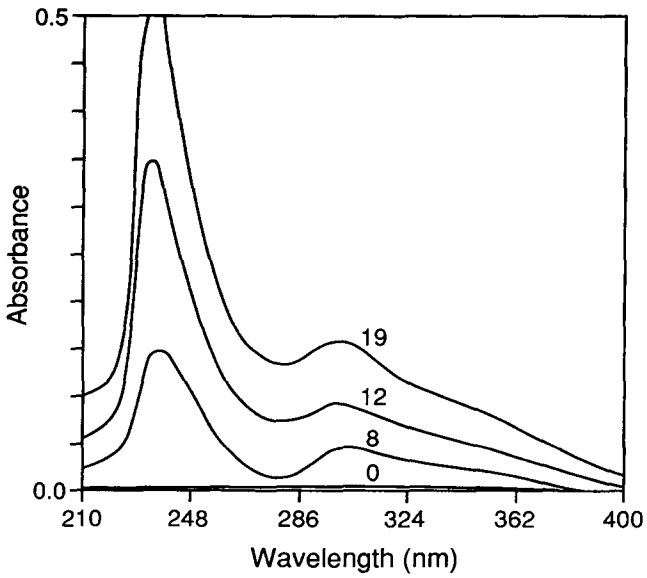

Fig. 2. Absorption spectra of bovine α-crystallins incubated with 3-deoxyglucosone.

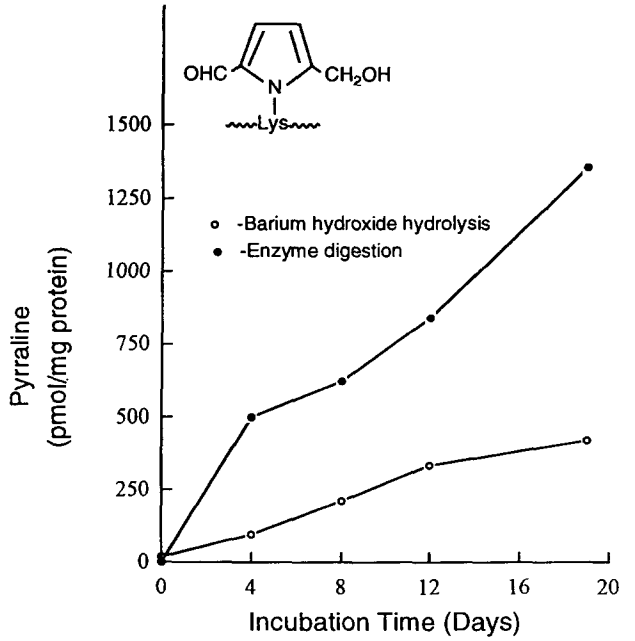

Fig. 3. Formation of pyrraline in bovine α-crystallins incubated with 3-deoxyglucosone. Pyrraline was estimated by HPLC either after enzyme digestion (●) or after barium hydroxide hydrolysis (○). Inset shows the structure of pyrraline.

line showed one single peak with a corresponding increase in the peak area. Pyrraline level measured by this method was about 3 times higher than the barium hydroxide method. After 19 days of incubation 1354 pmol/mg protein had formed (Fig. 3). The difference between the two methods could be due to partial destruction of pyrraline by alkaline hydrolysis or due to overestimation of pyrraline due to contamination of the peak in HPLC in the method with enzyme digestion. However, for quantification in lenses barium hydroxide hydrolysis was employed for the reason described below. Proteins incubated without 3-DG did not show pyrraline in these methods.

To establish the presence of pyrraline in the human lens, an attempt was made to purify from protein of highly pigmented brunescent lenses. Our attempts to employ enzyme digestion were unsuccessful possibly due to insufficient digestion of the highly crosslinked proteins. The barium hydroxide hydrolysis method, on the other hand, showed the presence of pyrraline. Since the pyrraline peak was not well resolved during the first HPLC, we collected the effluent containing pyrraline, dried and reinjected onto HPLC under a different condition. A well resolved peak was observed at 27.4 min, which was similar to the retention time of purified pyrraline (Fig. 5). The absorption spectrum of this peak was fully compatible with that of purified pyrraline. This preparation of pyrraline when mixed with pyrraline prepared from lysine-glucose incubation and subjected to HPLC eluted as a single peak. These observations confirm the presence of pyrraline in the human lens. However, mass spectral data is required as additional confirmatory evidence. The amount of purified material was not enough to perform this analysis.

Our attempt to quantify pyrraline in the water soluble and insoluble fractions of the lens separately was not successful by either enzymatic or barium hydroxide hydrolysis method. It was then decided to use lenses without protein extraction (about 100 mg wet weight). Barium hydroxide hydrolysis, followed by two-step HPLC was employed. Pyrraline standard was processed similarly for comparison. Seven normal, 10 cataractous lenses (senile, nuclear) and 8 diabetic lenses (not cataractous) were used. A wide variation of pyrraline was observed in these lenses (Fig. 6). The mean level however was higher in cataractous lenses (mean ± S.E., 48.4 ± 12.67 pmol/mg protein) when compared with normal lenses (30.93 ± 10.26 pmol). Surprisingly in diabetic lenses, levels in general were lower than normal lenses (28.4 ± 15.38 pmol). Interestingly, one normal, 2 cataractous and one lens in diabetic group showed extremely high levels. The pyrraline content in normal, senile cataractous and diabetic lenses accounted for 666, 968 and 570 μmol/mol crystallins, respectively. This corresponded to 0.095, 0.13 and 0.081% lysine modification in α-crystallins, respectively. Due to large differences in the range of pyrraline within groups, means were compared by a nonparametric statistic (Mann-Whitney U-test). No significant difference ($P > 0.05$) in the scores between any two groups was observed.

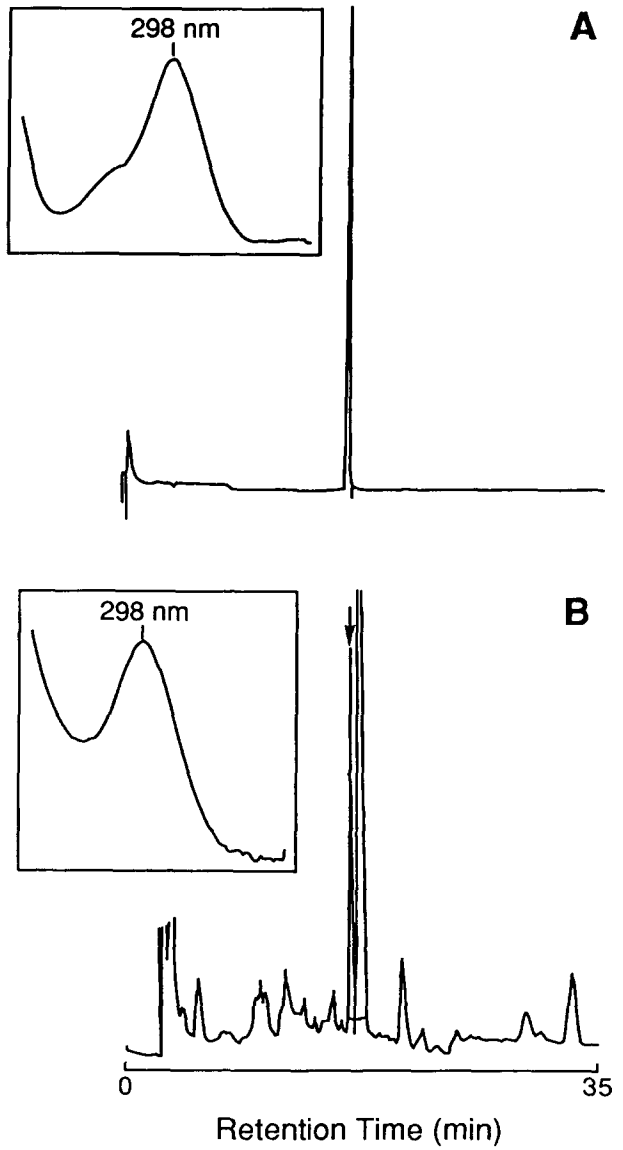

Fig. 4. Representative chromatograms showing pyrraline peak in α-crystallins incubated with 3-DG. A, pyrraline standard and B, crystallins incubated with 3-DG for 12 days.

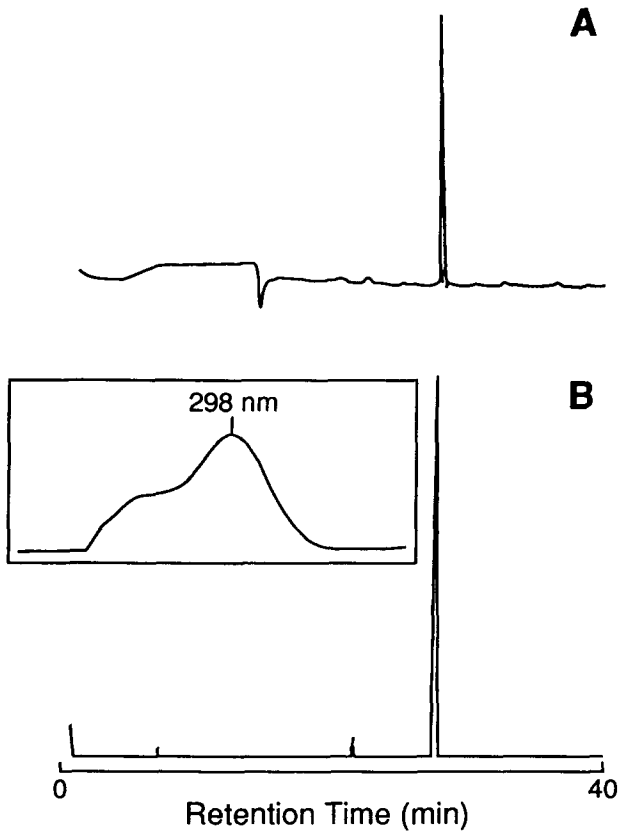

Fig. 5. Purification of pyrraline from human brunescent lenses. The pyrraline peak (in panel B) was obtained during the final step of purification. The chromatogram for pyrraline is shown in panel A. The inset shows the absorption spectrum of pyrraline purified from brunescent lenses.

4. Discussion

The purpose of this study was to investigate the presence of pyrraline in the human lens and its relation to cataract formation. In vitro experiments with α-crystallins showed that pyrraline can rapidly form when incubated with 3-deoxyglucosone. The absorption spectra of crystallins incubated with 3-DG clearly showed that pyrraline was a major modification. However, formation of pyrraline can not account for the extensive crosslinking of proteins observed in SDS-PAGE, since it by itself is a non-crosslinking product. It is possible however, that pyrraline formed on proteins may induce covalent crosslinking through oxidation by forming dipyrraline (pyrraline-pyrraline ether crosslinking), and thioether crosslinking with cysteine residues, as reported recently [16]. Under the incubation conditions used, other advanced Maillard reaction products are also likely to form. This is substantiated by the data on protein-fluorescence (excitation/emission 370/440 nm), which increased progressively in crystallins incubated with 3-DG (not shown). The formation of pyrrolopyridinium crosslink by the reaction of 3-DG with butylamine has been documented [19]. 3-DG is also a potent modifier of arginine residues on proteins. The structure of arginine modification has been recently reported [20].

The presence of pyrraline in normal non-cataractous lenses suggests that 3-deoxyglucosone is formed in these lenses. This is in agreement with the observation of Lal et al. [9] that poyol pathway metabolites, which are precursors of 3-DG are present in normal lenses. 3-Deoxyglucosone can also form from the Amadori product of the reaction between glucose with proteins [11]. The slightly acidic pH of the lens may promote the formation of 3-DG from Amadori products. The elevated levels in cataractous lenses reflects higher levels of Amadori products in these lenses. The wide range of distribution of pyrraline suggest that it may be oxidized or modified to different degrees in individual lenses due to differences in oxidative stress or cellular constituents reactive with pyrraline, e.g. glutathione [16].

Surprisingly, in the majority of diabetic lenses, the levels were lower than normal lenses. Considering the fact that polyol pathway and glycation are enhanced in diabetic lenses, it was expected that pyrraline levels would be higher in these lenses. One likely explanation for the lower levels is, increased oxidation of pyrraline in diabetes because of enhanced oxidative stress. Several studies have shown an enhancement in oxidative stress in diabetic lenses [21]. This may result in pyrraline ether crosslinking [16], leading to decreased levels of unmodified pyrraline. Alternatively, metabolism of 3-DG by activated aldose reductase [22] in diabetic lens may limit the formation of pyrraline. It should be pointed out here that the diabetic lenses used were not cataractous, but were only highly pigmented. It is interesting to note that in some lenses pyrraline levels were extremely high, reasons for this are unknown. It is noteworthy here that the presence of nephropathy in diabetic patients greatly enhances serum 3-DG level [8]. Whether this will have any effect on lens pyrraline remains to be investigated.

In summary, the demonstration of pyrraline in the human lens offers a probe to assess glucose-specific modification in lens aging and cataract formation. Although pyrraline may not itself contribute significantly to protein aggregation, its formation may compromise the ability of α-crystallins to function as a molecular chaperone [23] in analogy to the observation of Cherian and Abraham [24].

Acknowledgements: This work was supported by National Eye Institute Grant EY 09912, and a Grant-in-Aid from Johannsen Research Fund of Fight for Sight Research Division of Prevent Blindness America and Greater Cleveland Diabetes Association. We thank Drs. E. Kean and J. Plantner for extending us instrument facilities.

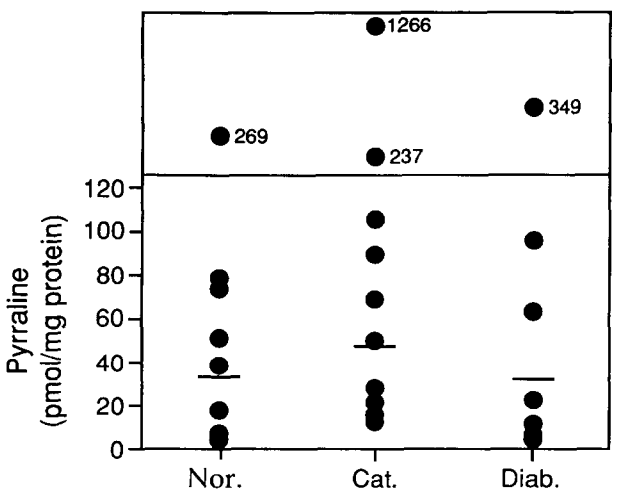

Fig. 6. Quantification of pyrraline in human lens crystallins. Nor, normal; Cat, senile cataractous and Diab, diabetic.

References

[1] Nagaraj, R.H. and Monnier, V.M. (1992) Biochim. Biophys. Acta 1116, 34–42.
[2] Nagaraj, R.H., Prabhakaram, M., Sell, D.R., Ortwerth, B.J. and Monnier, V.M. (1991) Proc. Natl. Acad. Sci. USA 88, 10257–10261.
[3] Lyons, T.J., Silvestri, G., Dunn, J., Dyer, D.G. and Baynes, J.W. (1991) Diabetes 40, 1010–1015.
[4] Dunn, J.A., Ahmed, A.U., Murtiashaw, M.H., Richardson, J.M., Walla, M.D., Thorpe, S.R. and Baynes, J.W. (1990) Biochemistry 29, 10964–10970.
[5] Grandhee, S.K. and Monnier, V.M. (1991) J. Biol. Chem. 266, 11654–11660.
[6] Nagaraj, R.H., Prabhakaram, M., Ortwerth, B.J. and Monnier, V.M. (1994) Diabetes 43, 580–586.
[7] Yamada, H., Miyata, S., Igaki, N., Yatabe, H., Miyauchi, Y., Ohara, T., Sakai, M., Shoda, H., Oimomi, M. and Kasuga, M. (1994) J. Biol. Chem. 269, 20275–20280.
[8] Niwa, T., Takeda, N., Yoshizumi, H., Tatematsu, A., Ohara, M., Tomiyama, S. and Niimura, K. (1993) Biochem. Biophys. Res. Commun. 196, 837–846.
[9] Lal, S., Szwergold, B., Taylor, A.H., Randall, W.C., Kappler, F., Well-Knecht, K., Baynes, J.W. and Brown, T.R. (1995) Arch. Biochem. Biophys. 318, 191–199.
[10] Szwergold, B.S., Kappler, F. and Brown, T.R. (1990) Science 247, 451–454.
[11] Hayase, F., Nagaraj, R.H., Miyata, S., Njoroge, F.G. and Monnier, V.M. (1989) J. Biol. Chem. 264, 3758–3764.
[12] Smith, P.R., Somani, H.H., Thornalley, P.J., Benn, J. and Sonksen, P.H. (1993) Life Sci. 84, 87–93.
[13] Portero-Otin, M., Nagaraj, R.H. and Monnier, V.M. (1995) Biochim. Biophys. Acta 1247, 74–80.
[14] Miyata, M. and Monnier, V.M. (1992) J. Clin. Invest. 89, 1102–1112.
[15] Marion, M.S. and Carlson, E.C. (1994) Biochim. Biophys. Acta 1191, 31–42.
[16] Nagaraj, R.H., Porterop-Otin, M. and Monnier, V.M. (1996) Arch. Biochem. Biophys. 325, 152–158.
[17] Madson, M.A. and Feather, M.S. (1981) Carbohydr. Res. 94, 183–191.
[18] Moore, S. and Stein, W.H. (1954) J. Biol. Chem. 211, 907–913.
[19] Hayase, F., Hinuma, H., Asano, M., Kato, H. and Arai, S. (1994) Biosci. Biotech. Biochem. 58, 1936–1937.
[20] Konishi, Y., Hayase, F. and Kato, H. (1994) Biosci. Biotech. Biochem. 58, 1953–1955.
[21] Ann Yeh, L. and Ashton, M.A. (1990) Metabolism 39, 619–622.
[22] Sato, K., Inazu, A., Yamaguchi, S., Nakayama, T., Deyashiki, Y., Sawada, H. and Hara, A. (1993) Arch. Biochem. Biophys. 307, 286–294.
[23] Horowitz, J. (1992) Proc. Natl. Acad. Sci. USA 89, 1449–1453.
[24] Cherian, M. and Abraham, E.C. (1995) Biochem. Biophys. Res. Commun. 212, 184–189.