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Immunoelectron microscopic analyses of Maillard reaction products in bovine anterior lens capsule and Descemet's membrane

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Abstract

It has been hypothesized that Maillard reaction products form in basement membranes during aging and may affect protein turnover. The purpose of this study was to localize Maillard reaction products in intact lens capsules and Descemet's membranes by immunoelectron microscopy to determine whether Maillard products accumulated with age and whether basement membrane thickness increased to a similar degree. The monoclonal antibodies antiglucitollysine and antipyrraline were employed to detect the products in native and glucose-treated bovine basement membranes. The content of basic amino acids, furosine, and fluorophores (370/440), as well as resistance to trypsin digestion showed that the basement membranes formed significant quantities of Maillard products when incubated with 200 mM glucose in vitro ($P < 0.05$). Likewise, incubation in 200 mM glucose resulted in at least a 4-fold increase in immunoreactivity ($P < 0.001$). Native basement membranes increased in thickness more than 2-fold with age ($P < 0.001$). Immunoreactivity varied similarly in that bound antiglucitollysine increased approx. 2-fold and antipyrraline approx. 3-fold in old vs. young basement membranes, but these differences were significant only in pyrraline immunoreactivity in the lens capsule ($P < 0.01$). Advanced products other than pyrraline may accumulate in Descemet's membrane since significant increases in fluorescence and resistance to trypsin were noted. These data suggest that the Maillard reaction may, to a small degree, contribute to basement membrane thickening.

Key words: Maillard reaction; Glycation; Aging; Immunoelectron microscopy; Basement membrane; Eye

1. Introduction

Basement membranes, such as those in the kidney [1,2] and eye [3–5], thicken in aging and undergo accelerated thickening in diabetes mellitus. The anterior lens capsule and Descemet's membrane are two ocular basement membranes which show age-related thickening [6–9]. The mechanism(s) underlying the thickening is unclear. One hypothesis suggests a slowing of the degradation process. In agreement with this, there is evidence that collagen becomes progressively less soluble and more resistant to proteolysis with age [10,11]. It also has been shown that incubation of basement membrane components with reducing sugars alters their conformation, spacing and binding properties, traps soluble proteins, and decreases solubility and digestibility [12,13]. Therefore, it is believed that exposure of basement membranes to normal glucose levels for extended periods of time or to elevated glucose levels for shorter periods affects protein turnover.

The mechanism by which sugars may alter protein degradation is not entirely elucidated but currently it is believed that the Maillard reaction may play an important role. This reaction is initiated by nonenzymatic condensation of an aldehyde or ketone of a reducing sugar with a free amino group to form a Schiff base which rearranges to form a more stable ketoamine, the Amadori product. Degradation of the Amadori product regenerates the intact amine and dehydrates the sugar to deoxyosones which can react further with free amino groups to form stable advanced glycosylation endproducts (AGEs). AGEs are often heterocyclic chromophores and fluorophores [14], some of which can be detected by immunological assays [15–17].

Age-related increases in collagen glycation have been identified [11,18,19], but recently it is believed

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that age-related increases in glycation are correlated with increases in glycemia [20,21]. AGEs, in the form of fluorescent chromophores (335/385, 370/440), consistently have been shown to accumulate with age in collagen [12–14]. The majority of these studies have used biochemical methods to detect early or advanced Maillard reaction products following solubilization of the tissue. Few studies, however, have analyzed intact basement membranes for Maillard products and none have localized both early products and AGEs in the same intact tissues.

The purpose of this study was to localize and quantitate, by immunoelectron microscopy, early and advanced Maillard reaction products in glucose-treated and untreated bovine anterior lens capsules and Descemet's membranes to investigate the relationships between density of Maillard reaction products and age of the animal as well as basement membrane thickness. These two basement membranes are exposed to identical sugars and concentrations in situ via the aqueous humor. The two early products, Schiff base adduct and Amadori product, were detected with a monoclonal antibody directed against glucitollysine [22], while the nonfluorescent AGE, 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde (pyrraline) [23], was detected with a monoclonal antipyrraline antibody [15,24]. These antibodies have been used previously in light microscopic studies of human diabetic tissue which identified increased glucitollysine immunoreactivity in skin [25], and increased pyrraline immunoreactivity in renal extracellular matrix [24]. Quantitation was limited since it was based on color intensity.

2. Materials and methods

Basement membrane preparation

Intact bovine eyes were obtained from old (8–12 years old) and young (3–18 months old) animals. Anterior lens capsules were removed from lenses and separated from posterior lens capsules. Descemet's membranes were isolated from all corneas, except those used for thickness measurements, by scraping from underlying stroma. Acellular lens capsules and Descemet's membranes were obtained by sequential detergent treatments [26].

Basement membrane thickness measurements

Thickness measurements of young and old lens capsules and Descemet's membranes were acquired from light micrographs. Basement membranes were prepared for light microscopy as described by Carlson and Hinds [27]. Transverse sections (1.0 μm thick, obtained from a Dupont-Sorvall MT-2B ultramicrotome) were stained with Toluidine blue (1% in 1% sodium borate), then viewed and photographed through an Olympus BH2 light microscope with the objective at 40× magnification.

Incubation of basement membranes with sugars

Lens capsules and Descemet's membranes were incubated in 100 mM phosphate-buffered saline (pH 7.4) containing either glucose (5, 50, or 200 mM), 3-deoxyglucosone (5 or 50 mM), or fructose (500 mM). Controls were incubated in 100 mM phosphate-buffered saline (pH 7.4) only. Sodium azide (0.5 mg/ml) was added to all sugar solutions and each solution was filtered through a 0.2 μm filter (Supor; Gelman Sciences, via Baxter Scientific Products, McGaw Park, IL) to prevent bacterial growth. Aliquots (5 ml) of these solutions were incubated for 10, 50 or 100 days at 37°C with approx. 10 mg of each acellular basement membrane.

Amino acid analysis and furosine determination

Native or incubated basement membranes were rinsed and, whenever indicated were reduced with 150 mM $NaBH_4$ in phosphate-buffered saline (pH 8–9). Reduction was terminated with 50 mM acetic acid. Dry weights of one mg per basement membrane (amino acid analysis) or wet weights of 20 mg per basement membrane (furosine assay) were hydrolyzed in 1.0 ml 6 M HCl at 100–110°C for 30 h. Amino acid composition analyses were performed by the Department of Biochemistry at North Dakota State University in Fargo, ND on a Beckman model 6300 amino acid analyzer.

Acid hydrolyzed basement membranes were analyzed for furosine by HPLC (Beckman model 332) [28]. The eluent was 4.0 mM H_3PO_4 (pH 5.0–6.0) in 7% methanol. Fructosyllysine standards were acquired from Dr. E. Schleicher, Munchen, Germany, and Dr. P.A. Finot, La Tour de Peilz, Switzerland. Predictable variations in furosine peak height as a result of glucose or $NaBH_4$ treatment of lens capsules and Descemet's membranes confirmed furosine elution time. Furosine concentrations, expressed in nmol/mg, were estimated from tyrosine concentrations.

Fluorescence

In order to determine collagen-linked fluorescence, lyophilized basement membranes were trypsinized in a ratio of 5 mg of basement membrane per ml of 0.3% trypsin (Type IX in 50 mM Trizma® (pH 7.6), Sigma, St. Louis, MO). Solutions were agitated at 37°C for 24 h. Solubilized material was analyzed for the presence and quantity of fluorescent products (370/440) [14] with a Shimadzu recording spectrofluorophotometer, model RF-540. Undigested portions were rinsed, lyophilized and weighed.

Immunoelectron microscopy

Embedding. Basement membranes were conventionally fixed in formaldehyde and embedded in Lowicryl

embedding medium (Polysciences) [29,30]. In brief, they were rinsed with 10 mM phosphate-buffered saline (pH 7.4), fixed in 2% formaldehyde (2 h, 0–4°C), washed with 500 mM NH_4Cl, dehydrated with graded aqueous dimethylformamide (DMF, Sigma; 25°C), infiltrated with Lowicryl (25°C) and embedded in fresh Lowicryl in gelatin capsules (size No. 3). These were suspended 10 cm above ultraviolet lamps (25 watts, 18 long, General Electric) at 4°C and polymerized 45–60 min. Hardened blocks were sectioned such that lens capsules or Descemet's membranes were cut transversely. Thin sections (0.11–0.14 μm) were picked up on uncoated 400 mesh nickel grids (Pelco, Ted Pella, Redding, CA).

Immunolabeling for glucitollysine and pyrraline. Treatments of section-laden grids were as described by Abrahamson [30] and occurred at 25°C, except treatments with primary antibodies which occurred at 0–4°C. The latter were monoclonal antibodies specific for glucitollysine (G6C9), pyrraline, and Sendai virus, diluted 1:5000, 1:300, and 1:300 respectively, with 0.05% egg albumin in 0.01 M phosphate-buffered saline (pH 7.4). A portion of antiglucitollysine was diluted with reduced glucosylated poly(Lys) hydrochloride (blocking antigen) in 0.05% egg albumin to result in final concentrations of 1:5000 antiglucitollysine and 90 μM antigen. A portion of antipyrraline was diluted with caproyl pyrraline (blocking antigen) giving final concentrations of 1:300 antipyrraline and 800 μM antigen. The antiglucitollysine was a gift from Dr. L.K. Curtiss, La Jolla, CA. The antipyrraline, caproyl pyrraline, and 3-deoxyglucosone were gifts of Dr. V. Monnier, Case Western Reserve University, Cleveland, OH.

Grids were immersed in 1 M NH_4Cl (60 min), rinsed with phosphate-buffered saline, immersed in 0.5% egg albumin (60 min) then in primary antibody (16 h, 4°C), rinsed with 10 mM phosphate-buffered saline, immersed in 0.05% egg albumin (15 min), and finally immersed in immunogold (30 nm gold-labeled goat anti-mouse IgG; Amersham Corporation, Arlington Heights, IL) diluted 1:6 with 10 mM phosphate-buffered saline (pH 7.4; 2–3 h). Grids were rinsed with water, stained with saturated uranyl acetate (8 min), then rinsed with water. Basement membranes were viewed and photographed with a JEOL 100S transmission electron microscope. Gold particles appeared as black dots on micrographs of lens capsules and Descemet's membranes, with negative magnifications of 5000 diameters. Number of particles within 100 cm^2 areas on electron micrographs (final magnification 13 000 diameters) of randomly selected sections of lens capsules and Descemet's membranes were marked and counted.

Statistical analyses

Results reported are the arithmetic means of values obtained from n samples. Analysis of variance (ANOVA) was used to calculate statistically significant differences between the mean values.

3. Results

Age-related changes in the basement membranes

Basement membrane thickness increased 2–3-fold in lens capsule and almost 4-fold in Descemet's membrane as a function of age ($P < 0.001$; Table 1). Concomitantly, fluorescence at 440 nm (excitation at 370 nm) increased by a factor of two in old vs. young basement membranes. The resistance to trypsin as a function of age in lens capsule was unchanged, but was significantly greater in old compared to young Descemet's membrane ($P < 0.05$). The amount of hydroxylysine, lysine, and arginine residues as potential sites of post-synthetic modification in aging were quantitated. There was a trend toward a loss of lysine and arginine residues in lens capsules, and a similar trend for hydroxylysine and arginine residues in Descemet's membranes, but in neither tissue did the observed changes reach statistical significance (Table 1).

Table 1
Age-related alterations in physical and biochemical properties of bovine lens capsules and Descemet's membranes

Alteration	n	Lens capsule		Descemet's membrane	
		young	old	young	old
Thickness (μm)	7	9.42 (±0.40)	24.83 (±0.73) ***	3.34 (±0.39)	12.50 (±1.01) ***
Fluorescence-370/440 (arbitrary units/mg)	5	0.71 (±0.10)	1.41 (±0.08) *	1.68 (±0.20)	3.03 (±0.22) **
Resistance to trypsin (percent undigested)	5	5.72 (±0.94)	5.24 (±1.03)	18.96 (±2.77)	27.60 (±2.10) *
Basic amino acids (residues/1000)					
Hyl	3	43.92 (±0.54)	45.43 (±1.02)	19.37 (±0.55)	18.58 (±0.47)
Lys	3	12.69 (±0.05)	11.48 (±1.07)	24.20 (±1.04)	25.70 (±0.62)
Arg	3	41.95 (±1.91)	38.78 (±1.07)	41.29 (±1.23)	39.35 (±1.40)

n, number of samples = number of eyes. Values presented are means (±S.E.). * $P < 0.05$: young vs. old. ** $P < 0.01$: young vs. old. *** $P < 0.001$: young vs. old.

Immunolocalization and quantitation of glucitollysine in vitro and in vivo

The effect of glucose incubation on the in vitro formation of glucitollysine in lens capsule and Descemet's membrane was investigated by immunoelectron microscopy. The basement membranes were incubated for 10 days in 200 mM glucose containing $NaCNBH_3$ in order to shift the equilibrium towards glucitollysine formation. Immunolabeling with antiglucitollysine showed a dispersed pattern of gold particles suggesting diffuse formation of glucitollysine in both basement membranes (Figs. 1B,D). Those immunolabeled with antiSendai virus as control contained little bound antibody (Fig. 1A). Considerable reduction of bound antibody was also observed when antiglucitollysine was preabsorbed with glycated poly(Lys) (Fig. 1C). Quantitative analysis (Table 2) revealed that 10 day glucose incubations, with reduction following glycation, resulted in a 4–13-fold increase in glucitollysine formation ($P < 0.001$) in lens capsule and Descemet's membrane, respectively, relative to controls. The presence of reducing agent during incubation with glucose led to an 80- and 40-fold increase in glucitollysine formation, respectively, over the amount formed in glucose incubations without reducing agent. Thus, the data in Fig. 1 and Table 2 confirm the validity of the method and the detectability of glucitollysine by immunoelectron microscopy. Quantitative determination of furosine by HPLC in lens capsule and Descemet's membrane, following 50 day incubations with glucose, confirmed the

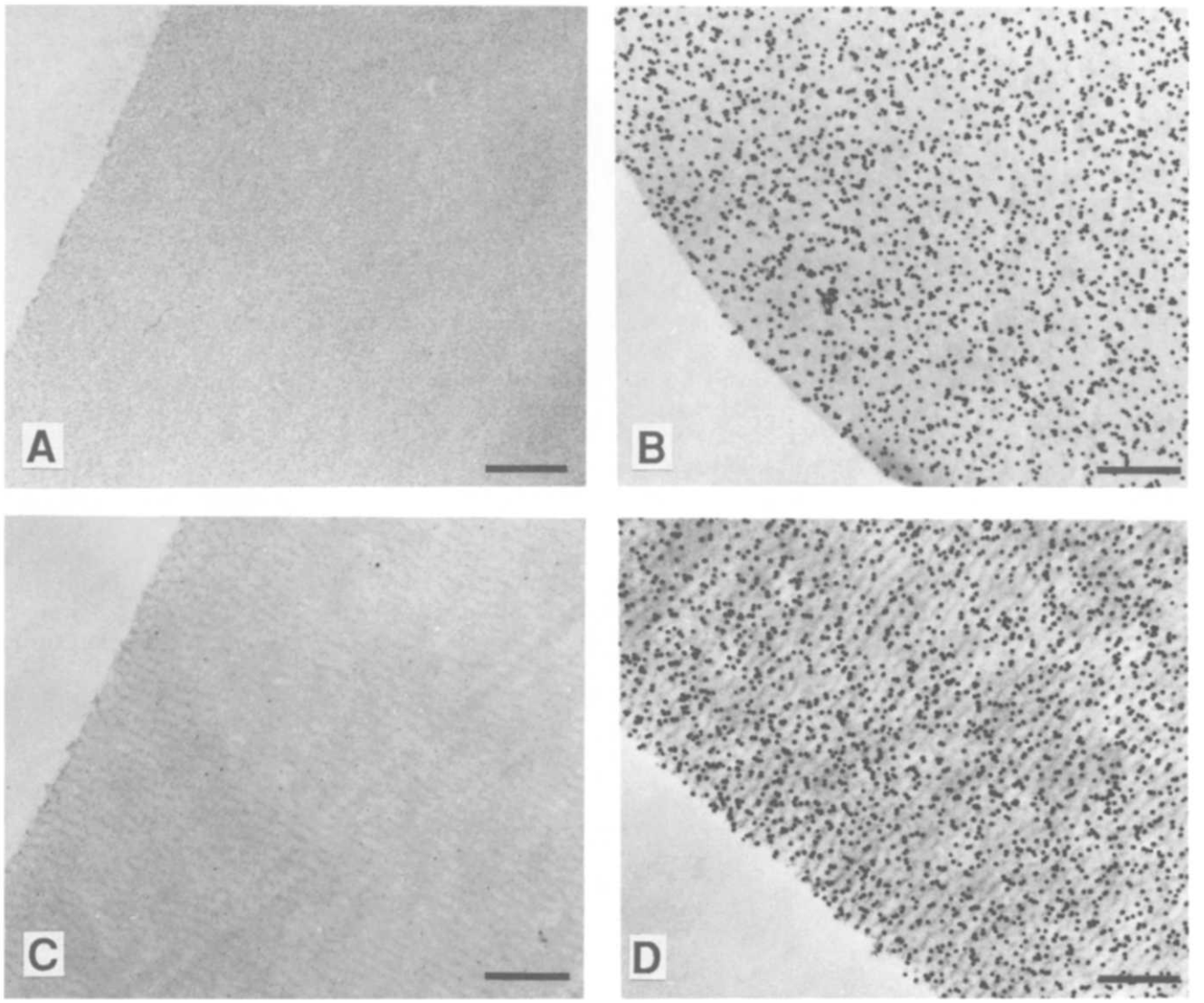

Fig. 1. Immunoelectron microscopic localization of glucitollysine on lens capsules and Descemet's membranes incubated 10 days in 200 mM glucose in the presence of 150 mM $NaCNBH_3$ (pH 7.8). (A) Lens capsule labeled with antiSendai virus (1 : 300) and immunogold (control). (B) Lens capsule labeled with antiglucitollysine (1 : 5000) and immunogold. (C) Descemet's membrane labeled with antigen-treated antiglucitollysine (1 : 5000) and immunogold (control). (D) Descemet's membrane labeled with antiglucitollysine (1 : 5000) and immunogold. Gold particles appear as black dots. Scale bar = 1.0 μm.

presence of early Maillard products in these basement membranes. Incubations with glucose resulted in 4- and 6-fold increases in furosine concentration over native lens capsules and Descemet's membranes, respectively ($P < 0.001$; data not shown).

The effect of age on glycation and on the susceptibility of basement membranes to undergo further glycation was investigated. Age comparisons of gold particle densities on native lens capsules and Descemet's membranes (Table 2) showed a 40–50% increase in basement membrane immunoreactivity toward antiglucitollysine as a function of age in both lens capsule and Descemet's membrane, but the increase was not significant ($P > 0.05$) due to large standard deviation. The susceptibility of young and old lens capsules and Descemet's membranes to undergo further glycation was investigated by incubation with 200 mM glucose for 10 and 50 days. The data in Table 2 indicate that glycation increases further as a function of time, and show that old basement membranes, especially Descemet's membrane, are less susceptible to glycation than young basement membranes. At 10 days of incubation with glucose no difference was noted between young and old lens capsules, but at 50 days old lens capsules were 25% less glycated than young lens capsules ($P < 0.05$). Old Descemet's membranes were markedly less

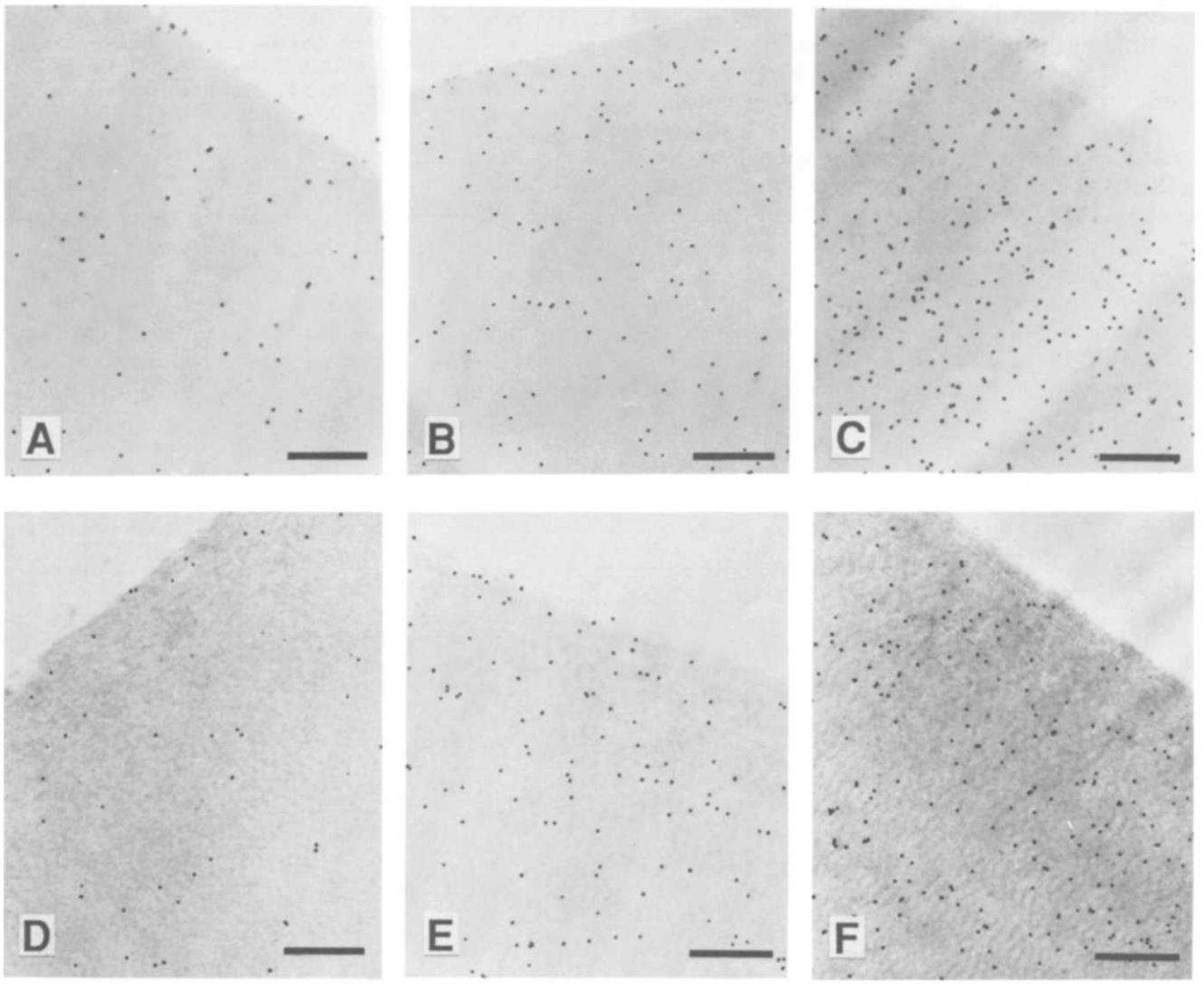

Fig. 2. Immunoelectron microscopic localization of pyrraline on sugar-treated lens capsules (A,B,C) and Descemet's membranes (D,E,F), and the effect of sugar type and concentration on immunoreactivity. Basement membranes were incubated 50 days with: (A,D) 50 mM glucose, (B,E) 200 mM glucose, or (C,F) 50 mM 3-deoxyglucosone (all pH 7.4). All were immunolabeled with antipyrraline (1:300). Gold particles are evenly distributed on each basement membrane, but densities increase from A to C and D to F. Scale bar = 1.0 μm.

susceptible to glycation both at 10 and 50 days ($P < 0.01$). In these experiments, reduction with $NaBH_4$ occurred only at the end of the incubation period.

Effect of sugar incubation and age on formation of pyrraline

The ability of intact lens capsules and Descemet's membranes to form the advanced Maillard reaction product pyrraline in vitro and the relative content of pyrraline in native basement membranes as a function of age was investigated by immunoelectron microscopy. Typically, gold particles were evenly distributed in both lens capsule (Figs. 2A–C) and Descemet's membrane (Figs. 2D–F) following incubation in glucose or 3-deoxyglucosone suggesting diffuse formation of pyrraline in basement membranes in vitro. Basement membranes incubated 50 days in 200 mM glucose (Figs. 2B,E) were more immunoreactive than those incubated in 50 mM glucose (Figs. 2A,D), but overall the most immunoreactivity was seen in basement membranes incubated in 50 mM 3-deoxyglucosone (Figs. 2C,F), an immediate precursor of pyrraline. Fig. 3 demonstrates quantitatively that lens capsules incubated in 200 mM glucose were significantly more immunoreactive than those incubated in lower glucose concentrations ($P < 0.001$) while lens capsules incubated in 50 mM 3-deoxyglucosone were significantly more immunoreactive than those incubated in 50 mM glucose ($P < 0.01$). Compared to controls, incubations with 200 mM glucose resulted in an 8-fold increase in gold particle densities, while incubations with 50 mM 3-deoxyglucosone resulted in a 14-fold increase in particle densities. Overall, similar results were obtained for Descemet's membrane incubated with glucose or 3-deoxyglucosone (data not shown).

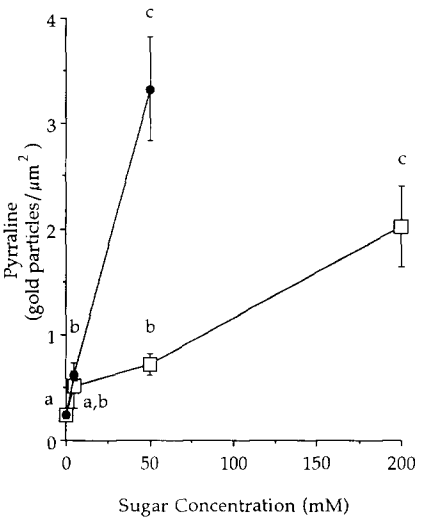

Fig. 3. Alterations in lens capsule immunoreactivity towards antipyrraline in relation to in vitro exposure to sugar, and to sugar type and concentration. Lens capsules were incubated 50 days in either glucose (0, 5, 50, or 200 mM (pH 7.4), □), or 3-deoxyglucosone (0, 5 or 50 mM (pH 7.4), •) then immunolabeled with antipyrraline (1:300). Vertical bars denote S.E. of the mean number of particles on approx. ten 56 μm^2 areas. For either sugar, means which do not share the same superscript are significantly different ($P < 0.01$).

The ability of fructose to form pyrraline in vitro was investigated. Significant densities of evenly distributed gold particles were observed on Descemet's membranes incubated for 100 days with 500 mM fructose

Table 2
Gold particle densities on reduced native, phosphate-buffered saline-treated and glucose-treated lens capsules and Descemet's membranes immunolabeled with antiglucitollysine

Treatment	Lens capsule				Descemet's membrane			
	young		old		young		old	
	n [a]	mean [b]	n [a]	mean [b]	n [a]	mean [b]	n [a]	mean [b]
In Vivo	12	0.029 (±0.005)	10	0.052 (±0.015)	9	0.019 (±0.006)	17	0.040 (±0.012)
Buffer - 10 d + $NaCNBH_3$ [c]	–	–	7	0.063 (±0.020)	–	–	10	0.042 (±0.007)
Glucose [d] - 10 d then $NaBH_4$ *** + $NaCNBH_3$	14	0.205 (±0.030)	7	0.248 (±0.026)	9	1.316 *** (±0.190)	14	0.551 (±0.089)
	–	–	18	20.398 (±1.122)	–	–	15	22.456 (±0.777)
Glucose - 50 d then $NaBH_4$ [c]	12	1.152 * (±0.139)	12	0.861 (±0.077)	16	3.011 ** (±0.274)	16	2.131 (±0.185)

[a] Number of 56 μm^2 areas.
[b] Number of gold particles per μm^2 (±S.E.).
[c] 150 mM.
[d] 200 mM.
* $P < 0.05$: young vs. old.
** $P < 0.01$: young vs. old.
*** $P < 0.001$: young vs. old, 10 day glucose then $NaBH_4$-treated vs. buffer-, glucose + $NaCNBH_3$-, or 50 day glucose-treated.

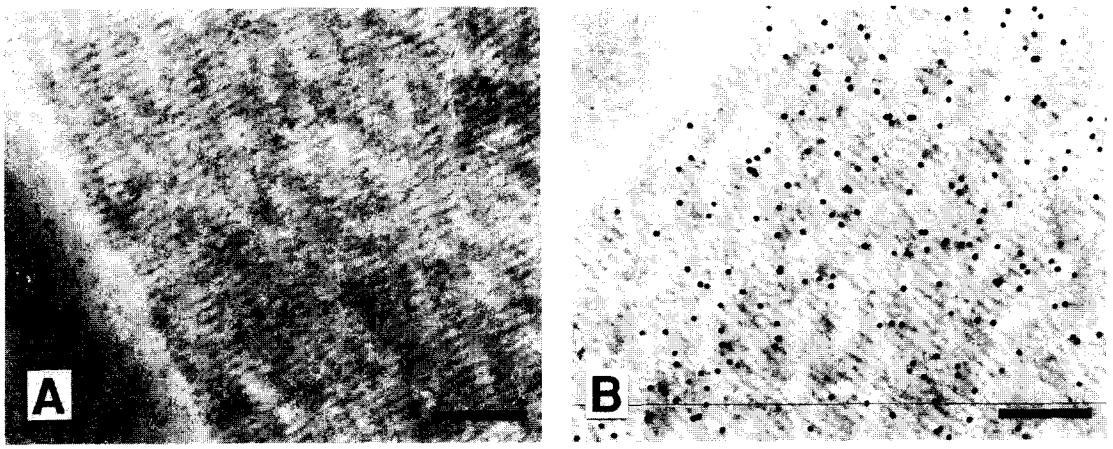

Fig. 4. Electron micrographs of antipyrraline labeled (1:300), fructose-treated Descemet's membranes showing specificity of antipyrraline. All were incubated 100 days in 500 mM fructose (pH 7.4) then labeled with either caproyl pyrraline-treated antipyrraline (A), or with unaltered antipyrraline (B). Scale bar = 1.0 μm.

(Fig. 4B). Preincubation of antipyrraline with the free hapten, ϵ-caproic pyrraline, abolished binding of antibody (Fig. 4A). Pyrraline formation increased by a factor of 11 in Descemet's membranes incubated with fructose compared to untreated Descemet's membranes ($P < 0.001$).

The effect of age on pyrraline immunoreactivity in lens capsule and Descemet's membrane was investigated (Table 3). A 3-fold increase in pyrraline immunoreactivity was observed in lens capsules ($P < 0.01$) as a function of age, and a 2–1/2-fold increase in pyrraline immunoreactivity was found in old vs. young Descemet's membrane, though this increase was not statistically significant.

Incubations with glucose induced biochemical changes in the basement membranes which confirmed the formation of advanced Maillard reaction products (Table 4). Fluorescence increased 6-fold in lens capsule and nearly 5-fold in Descemet's membrane following incubation in glucose. Descemet's membrane also

Table 3
Gold particle densities on native lens capsules and Descemet's membranes immunolabeled with antipyrraline

Treatment	Lens capsule				Descemet's membrane			
	young		old		young		old	
	n [a]	mean [b]	n [a]	mean [b]	n [a]	mean [b]	n [a]	mean [b]
In Vivo	7	0.180 (±0.053)	6	0.555 (±0.094) **	4	0.108 (±0.087)	7	0.292 (±0.042)

[a] Number of 56 μm^2 areas.
[b] Number of gold particles per μm^2 (±S.E.).
** $P < 0.01$: young vs. old.

Table 4
Glucose induced biochemical alterations in bovine lens capsules and Descemet's membranes

Alteration	n [a]	Lens capsule		Descemet's membrane	
		control	glucose [b]	control	glucose [b]
Fluorescence-370/440 (arbitrary units/mg)	5	1.41 (±0.08)	9.18 (±0.32) ***	3.03 (±0.22)	14.15 (±1.57) ***
Resistance to trypsin (percent undigested)	5	5.24 (±1.03)	7.85 (±1.43)	27.60 (±2.10)	59.70 (±3.55) ***
Basic amino acids (residues/1000)					
Hyl	3	45.43 (±1.02)	39.96 (±0.30) **	18.58 (±0.47)	17.59 (±0.77)
Lys	3	11.48 (±1.07)	8.17 (±0.73) *	25.70 (±0.62)	19.55 (±0.39) **
Arg	3	38.78 (±1.07)	32.29 (±2.17) *	39.35 (±1.40)	35.84 (±0.15)

Values presented are means (±S.E.).
[a] Number of samples = number of eyes.
[b] Fluorescence and trypsin = 100 d, 200 mM; amino acids = 50 d, 200 mM.
* $P < 0.05$: control vs. glucose-treated.
** $P < 0.01$: control vs. glucose-treated.
*** $P < 0.001$: control vs. glucose-treated.

showed a 2-fold increase in resistance to trypsinization. Amino acid analysis showed significant losses of hydroxylysine ($P < 0.01$), lysine ($P < 0.05$), and arginine ($P < 0.05$) residues in lens capsules. A similar trend was noted in Descemet's membranes, but only the loss of lysine residues was significant ($P < 0.01$). Losses of these basic amino acids suggested their incorporation into Maillard reaction products.

4. Discussion

The purpose of this study was to establish the feasibility of localizing and quantitating, by immunoelectron microscopy, early and advanced Maillard reaction products in bovine lens capsules and Descemet's membranes as a preamble for correlating the density of Maillard reaction products with animal age and basement membrane thickness. Both basement membranes showed age-related increases in thickness and fluorescence, while Descemet's membrane also showed an age-related increase in resistance to trypsin. Both basement membranes showed moderate age-related increases in glucitollysine and pyrraline immunoreactivity, 2-fold and 3-fold, respectively, but only the increase in pyrraline immunoreactivity in lens capsule was significant ($P < 0.01$).

The age-related thickness increases in bovine lens capsule and Descemet's membrane measured in this study were similar to those observed by others for the human lens capsule and Descemet's membrane [6–8]. The Maillard reaction could contribute to this thickening by increasing the number of intra- or intermolecular crosslinks present [31,32], and by blocking sites for degradative enzymes [33], as these changes would result in increased resistance to digestion. The age-related resistance of Descemet's membrane to trypsin is probably not due to heightened maturation of lysyl oxidase dependent crosslinks [34] because 3-hydroxypyridinium mature crosslinks are absent from both bovine Descemet's membrane and lens capsule [35]. Immature (reducible) enzymatic crosslinks have been identified in both basement membranes [36], but in many connective tissues, including bovine lens capsule, their concentration decreases with age [37,38]. Trypsin resistance differences between lens capsules and Descemet's membranes noted in this study, as well as other studies [39], may be related to differences in their constituent collagen types, since lens capsule is composed primarily of type IV collagen [40] while Descemet's membrane combines types IV and VIII collagens [41,42]. Moreover, differences in the organization of all proteins within the basement membranes could result in differential resistance to trypsin.

The age-related trend toward a loss of lysine and arginine residues in lens capsules, and hydroxylysine and arginine residues in Descemet's membranes was similar to that observed by others [36,39,41,43]. It was not surprising that this trend was not significant since many Maillard reaction products could be destroyed during acid hydrolysis [44]. The age-related doubling of fluorescence most directly suggested age-related increases in Maillard reaction products [12,13,45].

The diffuse formation of glucitollysine in intact basement membranes incubated with glucose indicated that if Maillard products were to accumulate with age and lead to decreased digestibility, the entire basement membrane would be affected. The very high density of glucitollysine in the basement membranes incubated with sodium cyanoborohydride – a 300–500-fold increase in glucitollysine over untreated basement membranes – suggested, as others have shown, that reduction shifts the equilibrium of the Maillard reaction towards glucitollysine formation through stabilization of early products such that formation of more advanced products as well as degradation of the Schiff base are prevented [46]. This in turn could alter protein conformation such that lysines not normally available to glucose molecules become exposed [47,48]. The 4–13-fold increase in glucitollysine formation in basement membranes reduced following glycation over untreated controls suggested that although the density of lysines capable of forming early products within the intact basement membranes are relatively high, only a small percentage of lysines (approx. 1–3%) will form early products at any given time under physiologic conditions. The presence of 4–6 fold increased concentrations of furosine following glucose incubations confirmed the formation of early Maillard products in lens capsule and Descemet's membrane in vitro.

The age-related decrease in glucitollysine immunoreactivity following incubation with glucose, suggested that the old basement membranes, especially Descemet's membranes, contain fewer available lysines than the young basement membranes. Some of the lysines in the old basement membranes may be incorporated into advanced Maillard reaction products, thereby suggesting age-related increases in advanced products. The lack of significant age-related differences in early products in native basement membranes reflect results from other laboratories which have shown age-related increases in some tissues and species but not others [18,20,21,45]. Several investigators have concluded that Amadori product concentrations reach a steady-state with respect to serum (or lens) glucose concentration [20]. In light of these investigations, results in the current study suggest that the slight age-related differences in immunoreactivity of native lens capsules and Descemet's membranes may be the result of differences in aqueous humor glucose concentration in each animal, rather than accumulation of early Maillard reaction products with age.

The diffuse pyrraline immunoreactivity following incubations in glucose and 3-deoxyglucosone, both known precursors of pyrraline [15], showed that pyrraline can form throughout the thickness of intact lens capsules and Descemet's membranes. Increasing immunoreactivity with increasing sugar concentrations and the greatest immunoreactivity with 3-deoxyglucosone, an immediate precursor, confirmed that pyrraline would form in the intact basement membranes if exposed to these sugars. The ability of fructose to form pyrraline in vitro was not unexpected since fructose has been shown to be more reactive than glucose in forming Maillard products [14].

The significant loss of lysine residues detected by amino acid analysis and the increase in fluorescence in both basement membranes as well as the increase in trypsin resistance in Descemet's membrane following incubation in glucose, confirmed that advanced Maillard products can form in both intact basement membranes. The additional losses of hydroxylysine and arginine residues, the lack of resistance to trypsin, and the formation of similar densities of pyrraline in lens capsules as compared to Descemet's membrane suggested that these two basement membranes may form different types of advanced Maillard reaction products.

Although only a small number of advanced products were localized in both native basement membranes, 4–10-fold less than when each was incubated with 200 mM glucose, native lens capsules showed a significant increase in pyrraline immunoreactivity as a function of age. In contrast, Descemet's membranes did not accumulate this advanced product with age. In situ, Descemet's membrane may be protected from the formation of pyrraline by the corneal endothelium, unlike anterior lens capsule which is in direct contact with the aqueous humor.

It should be noted that a recent report apparently failed to confirm pyrraline immunoreactivity in glucose-incubated proteins or proteins isolated from diabetic plasma [49]. The exact reason for the discrepancy is unclear. Two possibilities are that the polyclonal antibodies raised by Smith and co-workers had different specificities, and that the concentration of glucose used in the protein incubations, that is 0.23 mM, was too low. Clearly, our data support a strong relationship between glycation and pyrraline immunoreactivity.

In conclusion, early Maillard reaction products and the advanced product pyrraline were localized throughout the thickness of both basement membranes following incubation in glucose. Advanced products are capable of forming crosslinks, but the density of pyrraline that formed in situ was low and did not appear sufficient to affect basement membrane digestibility by trypsin. Therefore, pyrraline alone likely did not account for the observed large age-related increases in thickness. The significant decreases in susceptibility to glycation and increases in fluorescence with age suggested that perhaps a combination of different advanced Maillard products, oxidation-derived products and unidentified mature enzymatic crosslinks together contribute to decreased degradation. Decreased degradation, increased intermolecular spacing [48] and trapping of non-basement membrane proteins [50] would lead to thicker basement membranes. Future studies should concentrate on the characterization of additional Maillard products to permit direct connections to be made between the accumulation of Maillard products and changes in tissue morphology.

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6. References

[1] Ashworth, C.T., Erdmann, R.R. and Arnold, N.J. (1960) Am. J. Pathol. 36, 165–179.
[2] Osterby, R. (1983) in Diabetes Mellitus: Theory and Practice (Ellenberg, M. and Rifkin, H., eds.), pp. 323–341, Medical Examination Publishing, New York.
[3] Bloodworth, J.M.B., Jr. (1963) Diabetes 12, 99–114.
[4] Carlson, E.C. and Bjork, N.J. (1990) Anat. Rec. 226, 295–306.
[5] Leuenberger, P.M. (1973) Gerontology 19, 1–15.
[6] Hogan, M.J., Alvarado, J.A. and Weddell, J.E. (1971) Histology of the Human Eye, pp. 93–102, 652–659, W.B. Saunders, Philadelphia.
[7] Fisher, R.F. and Pettet, B.E. (1972) J. Anat. 112, 207–214.
[8] Johnson, D.H., Bourne, W.M. and Campbell, R.J. (1982) Arch. Ophthalmol. 100, 1942–1947.
[9] Engerman, R.L. and Colquhoun, P.J. (1982) Diabetologia 23, 521–524.
[10] Hamlin, C.R. and Kohn, R.R. (1971) Biochim. Biophys. Acta 236, 458–467.
[11] Schnider, S.L. and Kohn, R.R. (1981) J. Clin. Invest. 67, 1630–1635.
[12] Reiser, K.M. (1991) Proc. Soc. Exp. Biol. Med. 196, 17–29.
[13] Van Boekel, M.A.M. (1991) Mol. Biol. Rep. 15, 57–64.
[14] Baynes, J.W. and Monnier, V.M. (1989) The Maillard Reaction in Aging, Diabetes, and Nutrition, pp. 1–410, Alan R. Liss, New York.
[15] Hayase, F., Nagaraj, R.H., Miyata, S., Njoroge, F.G. and Monnier, V.M. (1989) J. Biol. Chem. 263, 3758–3764.

[16] Araki, N., Ueno, N., Chakrabarti, B., Morino, Y. and Horiuchi, S. (1992) J. Biol. Chem. 267, 10211–10214.
[17] Makita, Z., Vlassara, H., Cerami, A. and Bucala, R. (1992) J. Biol. Chem. 267, 5133–5138.
[18] Garlick, R.L., Bunn, H.F. and Spiro, R.G. (1988) Diabetes 37, 1144–1150.
[19] Cohen, M.P. and Yu-Wu, V. (1983) Exp. Gerontol. 18, 461–469.
[20] Patrick, J.S., Thorpe, S.R. and Baynes, J.W. (1990) J. Gerontol. Biol. Sci. 45, B18–B23.
[21] Dunn, J.A., McCance, D.R., Thorpe, S.R., Lyons, T.J. and Baynes, J.W. (1991) Biochemistry 30, 1205–1210.
[22] Curtiss, L.K. and Witzum, J.L. (1983) J. Clin. Invest. 72, 1427–1438.
[23] Njoroge, F.G., Sayre, L.M. and Monnier, V.M. (1987) Carbohydr. Res. 167, 211–220.
[24] Miyata, S. and Monnier, V. (1992) J. Clin. Invest. 89, 1102–1112.
[25] Kelly, S.B., Olerud, J.E., Witztum, J.L., Curtiss, L.K., Gown, A.M. and Odland, G.F. (1989) J. Invest. Dermatol. 93, 327–331.
[26] Carlson, E.C., Brendel, K., Hjelle, J.T. and Meezan, E. (1978) J. Ultrastruct. Res. 62, 26–53.
[27] Carlson, E.C. and Hinds, D. (1981) J. Ultrastruct. Res. 77, 241–247.
[28] Schleicher, E. and Wieland, O.H. (1981) J. Clin. Chem. Clin. Biochem. 19, 81–87.
[29] Altman, L.G., Schneider, B.G. and Papermaster, D.S. (1984) J. Histochem. Cytochem. 32, 1217–1223.
[30] Abrahamson, D.R. (1986) J. Histochem. Cytochem. 34, 847–853.
[31] Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P. and Cerami, A. (1986) Science 232, 1629–1632.
[32] Tanaka, S., Avigad, G., Eikenberry, E.F. and Brodsky, B. (1988) J. Biol. Chem. 263, 17650–17657.
[33] Bailey, A.J. and Kent, M.J.C. (1989) in The Maillard Reaction in Aging, Diabetes, and Nutrition (Baynes, J.W. and Monnier, V.M., eds.), Vol. 304, pp. 109–122, Alan R. Liss, New York.
[34] Brennan, M. (1989) J. Biol. Chem. 264, 20947–20952.
[35] Eyre, D.R., Koob, T.J. and Van Ness, K.P. (1984) Anal. Biochem. 137, 380–388.
[36] Heathcote, J.G., Eyre, D.R. and Gross, J. (1982) Biochem. Biophys. Res. Commun. 108, 1588–1594.
[37] Heathcote, J.G., Bailey, A.J. and Grant, M.E. (1980) Biochem. J. 190, 229–237.
[38] Barnard, K., Light, N.D., Sims, T.J. and Bailey, A.J. (1987) Biochem. J. 244, 303–309.
[39] Kefalides, N.A. (1971) Int. Rev. Exp. Pathol. 10, 1–39.
[40] Schwartz, D., Chin-Quee, T. and Veis, A. (1980) Eur. J. Biochem. 103, 21–27.
[41] Labermeier, U., Demlow, T.A. and Kenney, M.C. (1983) Exp. Eye Res. 37, 225–237.
[42] Kapoor, R., Sakai, L.Y., Funk, S., Roux, E., Bornstein, P. and Sage, E.H. (1988) J. Cell Biol. 107, 721–730.
[43] Fukushi, S. and Spiro, R.G. (1969) J. Biol. Chem. 244, 2041–2048.
[44] Njoroge, F.G. and Monnier, V.M. (1989) in The Maillard Reaction in Aging, Diabetes, and Nutrition (Baynes, J.W. and Monnier, V.M., eds.), Vol. 304, pp. 85–107, Alan R. Liss, New York.
[45] Miksik, I. and Deyl, Z. (1991) J. Gerontol. 46, B111-B116.
[46] Wolff, S.P., Jiang, Z.Y. and Hunt, J.V. (1991) Free Radic. Biol. Med. 10, 339–352.
[47] Beswick, H.T. and Harding, J.J. (1987) Biochem. J. 246, 761–769.
[48] Tanaka, S., Avigad, G., Brodsky, B. and Eikenberry, E.F. (1988) J. Mol. Biol. 203, 495–505.
[49] Smith, P.R., Somani, H.H., Thornalley, P.J., Benn, J. and Sonksen, P.H. (1993) Clin. Sci. 84, 87–93.
[50] Brownlee, M., Pongor, S. and Cerami, A. (1983) J. Exp. Med. 158, 1739–1744.