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Accelerated age-related browning of human collagen in diabetes mellitus

(nonenzymatic glycosylation/crosslinking/diabetic sequelae)

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ABSTRACT **The nonenzymatic glycosylation reaction that is accelerated in diabetes is the first step of the Maillard or nonenzymatic browning reaction that occurs in stored food. The glucose-protein adduct rearranges and dehydrates to form brown and fluorescent pigments, which can act as crosslinks, resulting in decreased protein solubility and altered mechanical properties. Evidence suggesting that this process occurs *in vivo* has been found in lens crystallins. The observation that nonenzymatic glycosylation and insolubility increases in collagen with age and diabetes led us to investigate the possible browning of human collagen. Insoluble human dura mater collagen was digested with collagenase. Absorbance at 350 nm and fluorescence at 440 nm (excitation at 370 nm) of the solubilized material was measured. A linear increase in the amounts of yellow and fluorescent material was observed with age. Samples obtained at autopsy from three type I diabetics and a young type II diabetic showed increased fluorescence and had absorbance values that corresponded to the amount of chromophore found in nondiabetics twice their age ($P < 0.025$). The collagen adducts from aged and diabetic individuals had absorption and fluorescence spectra identical to those of collagen samples that underwent nonenzymatic browning with glucose *in vitro*. The structure of these collagen adducts is unknown. However, their likely occurrence throughout the body could explain the correlation between arterial stiffening, decreased joint mobility, and the severity of microvascular complications in type I diabetics.**

Several complications of diabetes mellitus occur in collagen-rich tissues and resemble processes and diseases characteristic of aging. These include earlier onset and greater severity of atherosclerosis (1), stiffening of lungs (2) and large arteries (3, 4), thickening of capillary and glomerular basement membranes (5, 6), periarticular rigidity (7), and osteoarthritis (8). With age, collagen becomes less soluble (9, 10), more crosslinked (11), and more glycosylated (10, 12), and it accumulates yellow and fluorescent pigments (13, 14). In diabetes, several of these changes occur at an earlier age, suggesting an apparent acceleration of the aging process. Compared with age-matched nondiabetics, collagen from diabetics is less soluble (10), is more resistant to digestion by collagenase (15) and cyanogen bromide (16), has more pepsin-releasable high molecular weight peptides (10), and is more nonenzymatically glycosylated (10, 15, 17).

Glycosylation and subsequent crosslinking occur in food proteins that are stored or heated in the presence of reducing sugars and are due to the Maillard or nonenzymatic browning reaction. Glucose, for example, first reacts nonenzymatically with free amino groups on proteins to form a stable amino 1-deoxyketosyl adduct, also called the Amadori product. Nonenzymatic glycosylation has been shown to occur with hemoglobin to form hemoglobin A_{1c} and with a variety of other body proteins—e.g., lens crystallins, collagen, and nerve proteins (18). The relatively stable Amadori product can then undergo a series of dehydrations and rearrangements to form highly reactive carbonyl compounds that react with other amino groups to crosslink proteins and decrease protein solubility (19). These protein adducts and crosslinks are characteristically yellow and fluorescent.

Nonenzymatic browning has been hypothesized, by us, to occur in long-lived proteins such as crystallins and collagen and, in diabetes, to accelerate the aging process in tissues rich in such proteins (20). The recent demonstration that insolubility and resistance to enzymatic digestion of human collagen increase with age and diabetes in parallel with nonenzymatic glycosylation (10) led to the present investigation of the browning process in the same subject population. Insoluble dura mater collagen from nondiabetics and subjects with type I and type II diabetes was examined for the presence of yellow and fluorescent pigments similar to those that form in the nonenzymatic browning reaction with glucose.

MATERIAL AND METHODS

The samples of dura mater used in this study were obtained at autopsy from the *same* subjects in whom age- and diabetes-related changes in skin collagen solubility and glycosylation were reported by Schnider and Kohn (10). These include 17 samples of dura mater from subjects without clinical or pathological evidence of connective tissue disease or diabetes mellitus and similar samples from 3 subjects with type I diabetes and 3 subjects with type II diabetes. The data on the diabetic subjects are summarized in Table 1. The diagnosis of diabetes was based on criteria established by the National Diabetes Data Group (21).

Extraction Procedure. Insoluble collagen was prepared as described previously in detail (10). Briefly, neutral salt-soluble and acid-soluble fractions were extracted and the remaining material was digested with pepsin. The collagen remaining after these extraction procedures was considered insoluble; it represented about 85–95% of the original dura collagen.

Preparation of Collagen Digests. The insoluble fraction of dura collagen was washed twice with distilled water and centrifuged at 75,000 × *g* for 20 min at 4°C. The pellet was suspended in 30 ml of 1.0 M NaCl and sonicated until a fine suspension was obtained. Five hundred microliters was taken to determine the total amount of collagen present in the sample. A volume equivalent to 10 mg of collagen was then centrifuged at 20,000 × *g* for 20 min at 4°C. The pellet was resuspended in 1.0 ml of 0.1 M $CaCl_2$/0.02 M Tris·HCl, pH 7.55, containing 0.05% toluene to prevent bacterial growth (buffer A). To each sample was added 0.5 ml of a solution containing 1 mg of purified collagenase (type CLSPA, Wor-

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Table 1. Summary of patient data

Age, years	Race/sex	Duration of diabetes, years	Treatment	Complications
			Type I diabetics	
36	Black female	13	Insulin	Diffuse severe atherosclerosis, retinopathy, nephropathy, peripheral neuropathy, charcot joint
43	White male	22	Insulin	Diffuse severe atherosclerosis, retinopathy, nephropathy, peripheral neuropathy
42	White male	25	Insulin	Diffuse severe atherosclerosis, retinopathy, nephropathy, peripheral neuropathy, diffuse microangiopathy with gangrene
			Type II diabetics	
44	Black female	Unknown	Insulin	End-stage renal failure of undetermined etiology, hypertensive retinopathy
76	Black male	20	Insulin	Generalized atherosclerosis, nephropathy, peripheral neuropathy, peripheral microangiopathy with gangrene
79	Black female	24	Insulin/ chlorpropamide	Diffuse severe atherosclerosis, retinopathy, nephropathy, joint contractures

thington, Millipore) per ml of buffer A. This corresponds to a final concentration of 5% (wt/wt) collagenase. The samples were incubated for 24 hr at 37°C with shaking. An insoluble pellet accounting for 0–5% of total collagen was removed by centrifugation. Aliquots (50 μl) of the clear supernatant were used to determine the amount of digested collagen. The remaining supernatant was used for spectroscopic investigation. The soluble material, in having resisted pepsin digestion, while being hydrolyzed by a highly specific collagenase, represents quite pure collagen.

Measurement of Collagen. Collagen was estimated by measuring the amount of hydroxyproline. Hydroxyproline was assumed to make up 14% of the collagen by weight (22). Samples were hydrolyzed in 6.0 M HCl for 18 hr in evacuated sealed tubes. The HCl was removed under reduced pressure and the amount of hydroxyproline was determined by a modification of the method of Stegeman and Stalder (23).

Effect of Organic Solvent Extraction on Fluorescence and Absorbance. Insoluble dura collagen (10 mg) from three nondiabetics (19, 58, and 87 years old) and two type I diabetics (18 and 43 years old) was subjected to extraction with 10 ml of chloroform/methanol (2:1, vol/vol) for 5 hr under shaking at room temperature. Insoluble material was collected by centrifugation for 15 min at 800 × *g* and washed twice with 5 ml of chloroform/methanol (1:1), 5 ml of chloroform/methanol (1:5), 5 ml of methanol only, and 10 ml of distilled water. The residue was suspended in buffer A, centrifuged, and digested for 24 hr with 1 ml of collagenase (1 mg/ml) in buffer A. Hydroxyproline content, absorbance, and fluorescence were measured as described elsewhere in this paper.

Spectroscopical Measurements. Absorption at 350 nm and 420 nm was measured with a Zeiss PM 6 spectrophotometer. Absorption spectra were recorded with a double-beam Beckman Acta C III spectrophotometer. Fluorescence and fluorescence-excitation spectra were recorded with a Perkin-Elmer fluorimeter, model 204. The measurements were made against a blank containing collagenase in buffer A.

Nonenzymatic Browning *in Vitro*. Rat (200 g) tail tendon fibers were incubated at 37°C for 19 days in phosphate-buffered saline with or without 100 mM glucose. A small drop of chloroform was added to the solutions and corks were moistened with toluene to inhibit bacterial growth. Sterility was confirmed with conventional blood agar culture plates. The tendon fibers were minced, washed three times with phosphate-buffered saline, and digested in buffer A containing bacterial collagenase (type CLSPA, Worthington) at an enzyme-to-substrate ratio of approximately 1:10. A small amount of undigested material was removed by centrifugation. Collagen in the supernatant was adjusted to a final concentration of 0.16 mg/ml. This solution was used for fluorescence spectroscopy.

Statistical Methods. All assays were performed without knowledge of the age or clinical status of individual subjects. Linear equations, regression coefficients, confidence limits, and plot diagrams were generated with a linear regression program available on the Rockefeller University computer (Unix, Bell Systems). Formulae for determining confidence limits were obtained from Armitage (24).

RESULTS

Fig. 1 shows that there is, with age, a linear accumulation ($r = 0.87$) of collagen-linked pigments that absorb at 350 nm. The amount of pigment associated with the collagen of type I diabetics, however, does not fall within the 95% confidence limits of the regression line for the nondiabetics ($r = 0.87$, $P < 0.001$). The samples from type I diabetics had an amount of chromophore equal to that of nondiabetics about twice their age. Samples obtained from older type II diabetics had values that fell within the normal range. Similar results were

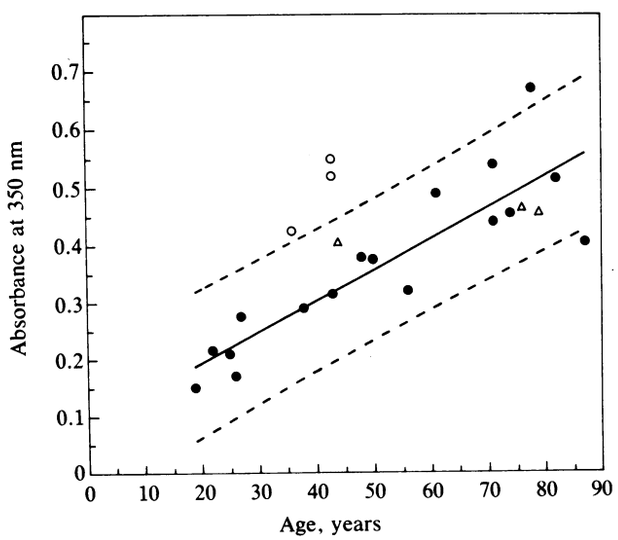

FIG. 1. Absorbance at 350 nm per mg of hydroxyproline in insoluble dura collagen solubilized by collagenase digestion. The solid line represents the regression equation $y = 0.0055x + 0.083$ ($r = 0.87$, $P < 0.001$) and is derived from the data of nondiabetics. The broken lines indicate the 95% confidence limits. ●, Nondiabetic subjects ($n = 17$); ○, type I diabetic ($n = 3$); △, type II diabetic ($n = 3$).

obtained when the amount of browning was detected by measuring the absorbance at 420 nm (data not shown). Since the formation of fluorescent compounds is typically observed during nonenzymatic browning, the supernatants of the digested collagen were also assayed for fluorescence at 440 nm upon excitation at 370 nm. Again, a linear increase of fluorescence with collagen age was observed ($r = 0.90$, $P < 0.001$). The fluorescence values for the three type I diabetics and the 44-year-old type II diabetic fell beyond the 95% confidence limits of the regression line for nondiabetic subjects, whereas those of the two older type II diabetics were within the normal range (Fig. 2). The number of diabetic samples is small, but the increase in pigmented and fluorescent material in diabetes is clearly significant. For the differences between the four diabetics between 35 and 57 years of age, and the five nondiabetics in the same age range, the P value was less than 0.025 by the Wilcoxon rank sum test.

The possibility that the increase in fluorescence and absorbance was due to lipids trapped in the insoluble collagen fraction was investigated. Exhaustive chloroform/methanol extraction was performed on samples from three nondiabetics (19, 58, and 87 years old) and two type I diabetics (18 and 43 years old). As shown in Table 2, extraction with organic solvent did not abolish the age-related increase in absorbance at 350 nm and fluorescence at 440 nm. Again, type I diabetics had values that corresponded to those of nondiabetics twice their chronological age.

These results suggest that the browning process that occurs in collagen with age is accelerated in type I diabetes. This view, however, implies that the chromophores and fluorogens formed in diabetes are identical with those found in older normal subjects. To gain information on this point, the absorption and fluorescence properties of typical samples from diabetic and nondiabetic subjects were investigated in more detail. Fig. 3 shows the absorption spectra of chromophores associated with dura collagen from the 43-year-old and 74-year-old nondiabetics and of the 43-year-old diabetic, using a sample from a 27-year-old nondiabetic as a reference. The spectra of the type I diabetic and the 74-year-old nondiabetic are identical, with a new absorption maximum at about 340 nm. Fluorescence-excitation spectra of these samples were also investigated at 370 nm (excitation) and 440 nm (emission) as previously described for nonenzymatic browning products (18). The spectra in Fig. 4 show that both excitation and fluorescence intensities increase with age and diabetes. No qualitative differences can be seen between the spectra from type I diabetic and nondiabetic subjects. Similar observations were made when these samples were systematically analyzed by exciting every 10 nm from 340 to 400 nm or monitored for fluorescence from 400 to 460 nm. That the observed excitation–emission maxima at 370 nm and 440 nm, respectively, result from nonenzymatic browning of glycosylated collagen is suggested by the spectra shown in Fig. 5. Collagen from rat tail tendons incubated with excess glucose had a specific increase in fluorogen that corresponds to that found in old and diabetic collagen.

Table 2. Absorbance and relative fluorescence per mg of hydroxyproline in insoluble collagen digested after extraction with chloroform/methanol

Age, years	Absorbance at 350 nm	Relative fluorescence at 440 nm (excitation at 370 nm)
	Nondiabetics	
19	0.140	53
58	0.204	115
87	0.289	234
	Diabetics	
18	0.182	112
43	0.287	200

Since collagen samples from type I diabetics had previously been shown to have more nonenzymatically glycosylated lysine residues, we have tested the possibility that the observed increased absorbance at 350 nm was an artifact due to browning of glycosylated lysine residues during digestion with collagenase. Two 5-mg samples of dura collagen from a 25-year-old nondiabetic were digested as described above, with and without 0.1 mmol ε-fructosyllysine, which is in 30 times excess over the amount of glycosylated residues found associated with tendon collagen (15). The absorbance at 350 nm of the digested collagen in the presence of glycosylated lysine was only 0.03 unit higher than without it. This small change rules out the possibility of a browning artifact.

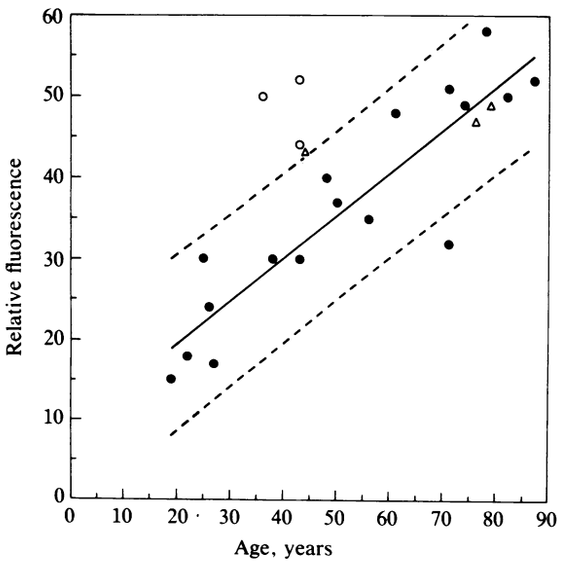

FIG. 2. Fluorescence at 440 nm upon excitation at 370 nm per mg of hydroxyproline in the samples shown in Fig. 1. The solid line represents the regression equation $y = 0.53x + 8.9$ ($r = 0.90$, $P < 0.001$). The confidence limits and symbols are the same as in Fig. 1.

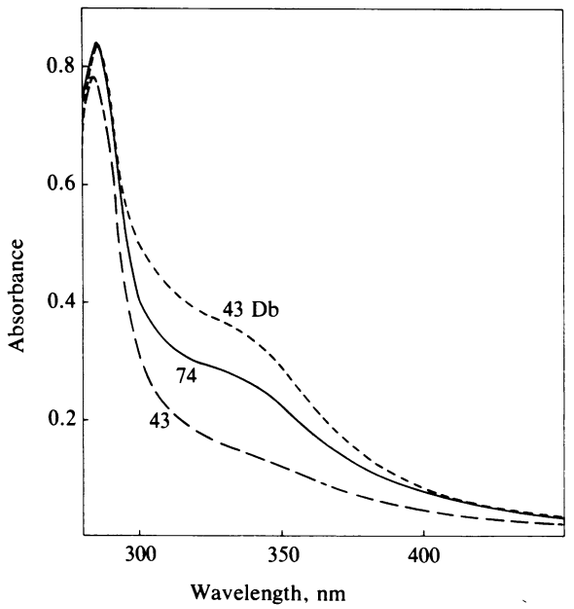

FIG. 3. Difference absorption spectra of three typical samples of insoluble dura collagen. The spectra were recorded against the collagen sample from the 27-year-old nondiabetic in the reference beam. The concentration was 1.1 mg of hydroxyproline per ml. – –, Nondiabetic 43-year-old; —, nondiabetic 74-year-old; - - -, type I diabetic (Db) 43-year-old.

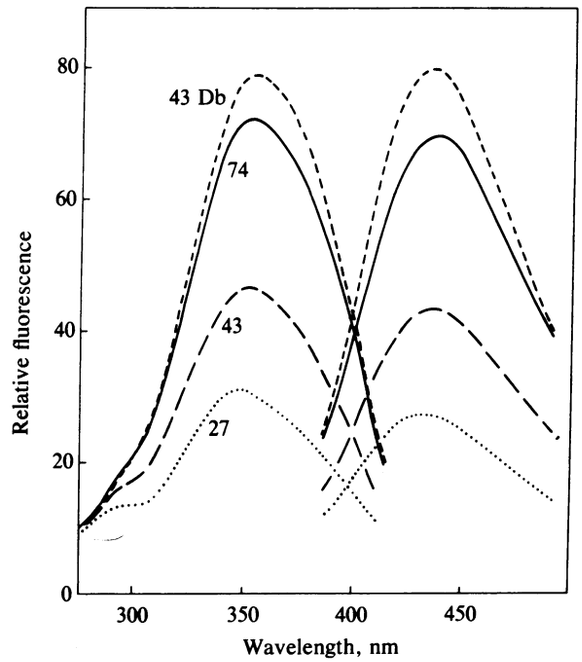

FIG. 4. Fluorescence–excitation spectra of the samples of dura collagen shown in Fig. 3. The spectra are differentiated as in Fig. 3, and ···· indicates the nondiabetic 27-year-old. For the emission spectra on the right, excitation was at 370 nm and, for the excitation spectra on the left, emission was monitored at 440 nm. Concentration was 0.38 mg of hydroxyproline per ml.

DISCUSSION

In previous studies on the effects of age and diabetes, it was shown that aging is associated with a decrease in the amount of soluble and pepsin-releasable collagen and an increase in nonenzymatic glycosylation of human skin collagen (10). These changes were generally increased by a factor of 2–3 in type I diabetes, indicating an apparent accelerated aging of collagen in diabetics.

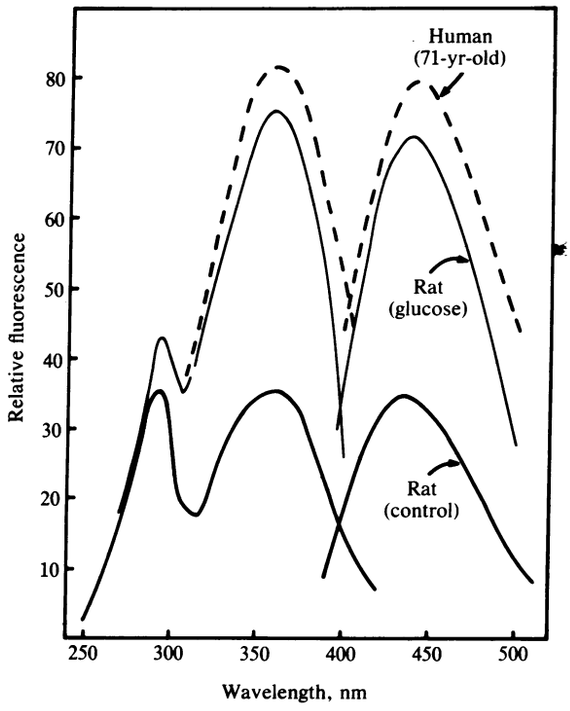

FIG. 5. Fluorescence–excitation spectra of collagenase digests of rat tail tendons incubated for 19 days with or without 100 mM glucose. Concentration was 0.22 mg of hydroxyproline per ml. The 71-year-old human sample was obtained from the series shown in Fig. 1 and the spectra were recorded as described for Fig. 4. Sensitivity was 1/10th for the human sample.

Comparison of these previous data with those reported in this work on dura collagen obtained from the same subjects shows a number of relevant similarities. First, the age-dependent accumulation of fluorescent and yellow pigments occurs linearly and parallels the changes reported in collagen solubility, digestibility, and glycosylation. Second, the amount of yellow color that is increased in dura collagen of type I diabetics parallels changes in the solubility and digestibility of skin collagen. Third, the two older type II diabetics in which collagen solubility and glycosylation were within normal limits also had browning values within the normal range. There are several possible explanations for the apparent absence of increased glycosylation and browning in these patients. The most likely is that nonenzymatic glycosylation may affect particularly recently synthesized collagen or collagen that is not densely crosslinked. Collagen in these studies makes up around 98% of the total collagen and was resistant to acid and pepsin solubilization, indicating a high degree of crosslinkage. In contrast, collagen from younger individuals, prior to onset of diabetes, would be expected to be less crosslinked than in older subjects. In addition, one could argue that, for the same duration of diabetes, a type I diabetic would have synthesized and glycosylated a larger proportion of total tissue collagen than a type II diabetic, thus making the relative increase in glycosylation and browning technically easier to detect. Finally, glycemia being more difficult to control in type I diabetes, browning rate would be accordingly much greater. This, however, does not imply that all type I diabetics would necessarily have increased browning. It may be anticipated that diabetics in good control and therefore not seen at autopsy might have browning values within the 95% interval of nondiabetics.

Browning does not seem to be due to uremia since the 76- and 79-year-old type II diabetics did not show increased browning although they had been uremic, like type I diabetics, in the last 2 years of their lives. Other factors such as atherosclerosis can be excluded as well, since atheromata do not occur in the dura.

Previous observations of age-related increases in pigmented and fluorescent compounds in human tendon collagen (13) and rat skin collagen (14) were tentatively explained on the basis of crosslinks involving tyrosine. Fluorescence was measured at 405 nm in the human tendon study (13) rather than at 440 nm as determined in the present study. Rat skin collagen (14), however, yielded fluorescence maxima similar to those for the human dura collagen reported reported here. More recently, pyridinoline, a fluorescent crosslink isolated from acid-hydrolyzed collagen, has been found in a variety of tissues from several species (25, 26). It is unlikely that the fluorescence properties of diabetic and old collagen are due to pyridinoline because its excitation-fluorescence maxima are found at much shorter wavelengths—i.e., at 295 nm and 395 nm, respectively (25). In addition, in human tissue, pyridinoline was found to increase up to the age of 20 years and decrease linearly up to the age of 90 years (26). A major finding in the present study is that the age-related increase in pigmented and fluorescent material was accelerated in diabetes. These changes are best explained by a direct effect of glucose on collagen as shown in Fig. 5. Moreover, model studies by food chemists of nonenzymatic browning of proteins *in vitro* have revealed the accumulation of nonenzymatic browning pigments with time in parallel with an increased amount of crosslinking and a decreased susceptibility to enzymatic digestion (27).

The recent observations of Rosenbloom *et al.* (28) that dia-

betics with joint limitation are at 83% risk of developing microvascular complications and of McMillan and Cook (4) that diabetics with increased stiffening of large arteries have a higher incidence of microangiopathy suggest that collagen crosslinking is implicated in the pathogenesis of various diabetic complications. Increased amounts of lysyl oxidase-dependent crosslinks have been reported in newly synthesized collagen from diabetic rats (29). Since, however, this observation was made in granulomatous tissue, its significance for diabetic lesions is unknown.

The nonenzymatic browning reaction yields an attractive hypothesis to explain the irreversibility of diabetic lesions based on extensive crosslinking and poor digestibility of browned collagen. Such changes appear to occur in diabetic kidneys, which have been found to be more autofluorescent than those of age-matched nondiabetic subjects (30). Other tissues that contain long-lived proteins, such as lens and nerve, might be affected by nonenzymatic browning as well. Isolation and structural analyses of protein pigments are required to further substantiate the browning concept and indicate more specifically how these modified proteins might play a role in the pathogenesis of the complications of diabetes and aging (31–33). In addition, further understanding of nonenzymatic browning *in vivo* is needed so that strategies for interfering with the process on long-lived proteins can be devised.

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