Collagen Browning and Cross-Linking Are Increased in Chronic Experimental Hyperglycemia

Relevance to Diabetes and Aging

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Diabetes and aging are associated with an increase in collagen-linked fluorescence and cross-linking that can be duplicated by incubating collagen with glucose. We have tested the hypothesis that browning and cross-linking can occur in vivo in rats by feeding them a diet containing 33% galactose. No significant increase in tail tendon browning or cross-linking, measured by tail tendon breaking time in urea, was observed after 3 mo of galactosemia. After 12 mo, however, twofold increases in tendon breaking time and collagen-linked chromophores absorbing >300 nm and fluorophores at 430 nm (excitation 355 nm, $P < .001$) analogous to those of diabetic and aging individuals were observed. The observed changes in collagen are attributed to the advanced Maillard (nonenzymatic glycosylation) reaction based on circumstantial evidence. With this premise, our data suggest that chronic galactosemia should be a powerful tool for investigating the role of the advanced Maillard reaction in complications of diabetes and aging. *Diabetes* 37:867–72, 1988

Human collagen undergoes progressive browning with aging characterized by yellowing, fluorescence, insolubilization, and decreased digestibility by proteolytic enzymes (1–6). These changes appear to be accelerated in diabetes (1–6). Kohn and associates (1,7) first noted that the age-related rate of collagen cross-linking was increased in diabetes and pointed out that several complications of aging that occur in collagen-rich tissues developed at an earlier age in diabetes. In a recent study, we demonstrated a twofold increase in the rate of collagen browning in skin from subjects with type I (insulin-dependent) diabetes (8,9). There was an overall correlation between the extent of browning, as indicated by the presence of collagen-linked fluorophores at 440 nm (excitation at 370 nm), and the severity of retinopathy and arterial and joint stiffness as well as blood pressure (8). The intercept between the age-related browning rates of diabetic and control subjects was found to correspond precisely to the mean age of diabetes onset, suggesting that the browning process has the attributes of a biological clock when it affects long-lived proteins such as skin collagen (9).

These postsynthetic modifications of diabetic and aging collagen are attributed to the Maillard or nonenzymatic browning reaction, which occurs as a consequence of nonenzymatic glycosylation (10,11). Collagen that is incubated with glucose becomes progressively less soluble and more cross-linked and acquires chromophores and fluorophores with spectroscopic properties similar to those of aging collagen (12). Based on evidence obtained from experiments in model systems, it is presumed that the glycosylated protein undergoes a sequence of dehydration and rearrangement reactions to form deoxyglucosones, which are responsible for the observed fluorescence and cross-linking (13). However, the precise mechanism that leads to browning of glycosylated proteins in vitro and in vivo remains to be elucidated.

Although browning and cross-linking of collagen with reducing sugars are readily demonstrable in vitro, progress delineating the contribution of the Maillard reaction in the pathogenesis of diabetic and age-related complications has been retarded by lack of an animal model of nonenzymatic browning without diabetes. In this study we tested the hypothesis that browning and cross-linking of collagen occur in nondiabetic animals exposed to high concentrations of a rapidly glycosylating sugar such as galactose (14). If confirmed, experimental galactosemia in animals with pharmacologically suppressed polyol pathways might be useful for the investigation of the pathology attributable to nonenzymatic glycosylation and browning.

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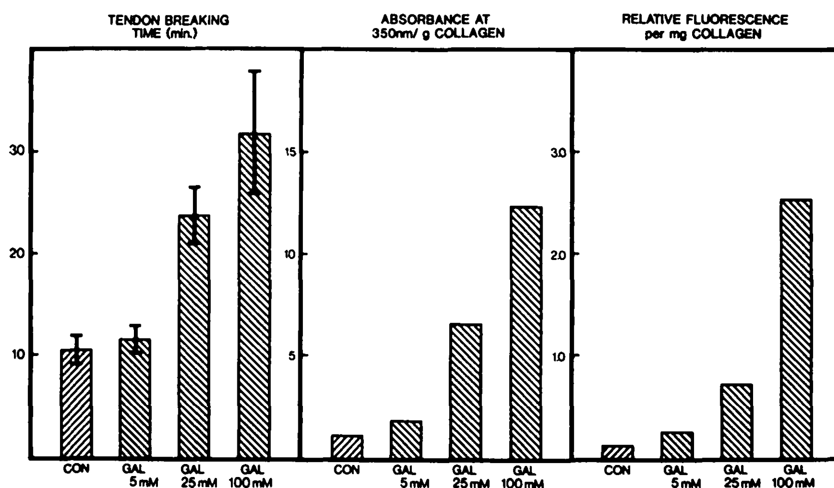

FIG. 1. In vitro effect of galactose (GAL) on tendon collagen (CON) cross-linking absorbance at 350 nm and fluorescence at 440 nm (excitation at 370 nm). Rat tail tendons were incubated for 2 wk with and without 5, 25, and 100 mM D-galactose. Cross-linking was assayed by method of tendon breaking time in urea. *Bars*, ± SD of mean of triplicate determinations. Tendon breaking time increase in 25 and 100 mM galactose was significantly elevated compared with that for 0 and 5 mM galactose ($P < .001$). Absorbance and fluorescence values were measured in pooled tendons and therefore have no SD values.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (190 g body wt) were fed a standard rodent powder-chow diet (Purina) that was 33% D-galactose (United States Biochemical, Cleveland, OH). Age-matched control rats received regular laboratory chow. After 3 and 12 mo, respectively, the animals were weighed and bled by cardiac puncture while under ether anesthesia. Blood was used for glycosylated hemoglobin assay. Autopsy and histopathological analysis were performed at a light-microscopic level for heart, lung, liver, kidney, intestine, pancreas, spleen, and aorta. Tails were used for preparation of tendons and measurements of collagen cross-linking, gylcosylation, absorbance at 350 nm, and fluorescence at 440 nm (excitation at 370 nm), as described earlier (12).

Incubation of tail tendons with galactose. The ability of galactose to induce browning and cross-linking in vitro was examined by incubating 10 freshly prepared tendons from a 150-g rat at 37°C with 0, 5, 25, and 100 mM D-galactose in phosphate-buffered saline (PBS) containing 2 μl/ml each of chloroform and toluene to prevent bacterial growth. After 15 days, tendons were washed with PBS and used for measurement of collagen cross-linking and collagen-linked fluorescence and absorbance as described below.

Tendon cross-linking assay. Tendon collagen cross-linking was assayed by the sensitive method of breaking-time measurement described by Harrison and Archer (15) and used by us previously (12). Briefly, tendons were attached on one end with a 4-0 surgical nylon string to an electric switch connected to a timer. A 275-g (±2 g) lead weight was attached to the lower end. Tendon and weight were immersed into a fresh 7-M urea solution at pH 7.5, as described previously (12). Temperature of the urea solution was kept constant at 40 ± 0.5°C by a surrounding bath. Tendons were assayed in triplicate. Interassay coefficient of variation varied from 1 to 12%.

Absorbance and fluorescence. Tendons were washed three times in PBS and once in distilled water and were delipidated with chloroform and methanol (2:1). Ten milligrams of delipidated tendons were minced and solubilized by digestion with 5% (wt/wt) clostridial collagenase (type VII, Sigma, St. Louis, MO) as described earlier (12). Absorbance at 350 nm, fluorescence at 440 nm (excitation at 370 nm), and absorbance and fluorescence spectra of the clear digest were determined and expressed per milligram of collagen, with the assumption of a hydroxyproline content of 14% (12).

Nonenzymatic glycosylation. Collagen-linked Amadori products were assayed with boronate affinity chromatography in the collagenase digest after borohydride reduction and acid hydrolysis as described previously (16). Approximately 0.5 mg of digested collagen was reduced with 200-fold molar excess of [^{3}H]NaBH$_4$ (50 mCi/mmol). The reaction was interrupted with 50 μl of 6 N HCl, and excess ^{3}H was evaporated exhaustively under the hood. Standards of α-formyl-ε-1-deoxyfructosyl lysine (glycosylated lysine) prepared as described by Finot and Mauron (17) were treated similarly. Glycosylated amino acids were released from the peptides by acid hydrolysis with 6 N HCl for 12 h at 110°C and separated by boronate affinity chromatography with the Glycogel B kit (Pierce, Rockford, IL). Radioactivity was counted in 500 μl eluate dissolved in 6 ml Hydrofluor (National Diagnostics, Sommerville, NJ).

Pyridinoline assay. Pyridinoline was quantified by high-performance liquid chromatography according to the method of Eyre et al. (18). Tendons from control and galactosemic rats were acid-hydrolyzed at 110°C in 6 N HCl for 20 h, followed by equalization of collagen content among samples based on the hydroxyproline assay of Stegeman and Stadler

TABLE 1
Nonenzymatic glycosylation of hemoglobin and tendon collagen after 1 yr of chronic galactosemia

	Control rats ($n=6$)	Galactosemic rats ($n=5$)
Body weight (g)	302 ± 28	269 ± 7
Glycosylated hemoglobin (%)	3.6 ± 0.6	10.2 ± 0.7*
Glycosylated collagen (nmol/mg)	31.7 ± 6.0	43.7 ± 8.2†

Values are means ± SE. Glycosylated hemoglobin was measured by boronate affinity chromatography. Interassay coefficient of variation was 16 and 7% in control and galactosemic rats, respectively. Glycosylation of collagen was measured by boronate affinity chromatography as described earlier (22).
*$P < .001$, †$P < .025$; galactosemic vs. control rats.

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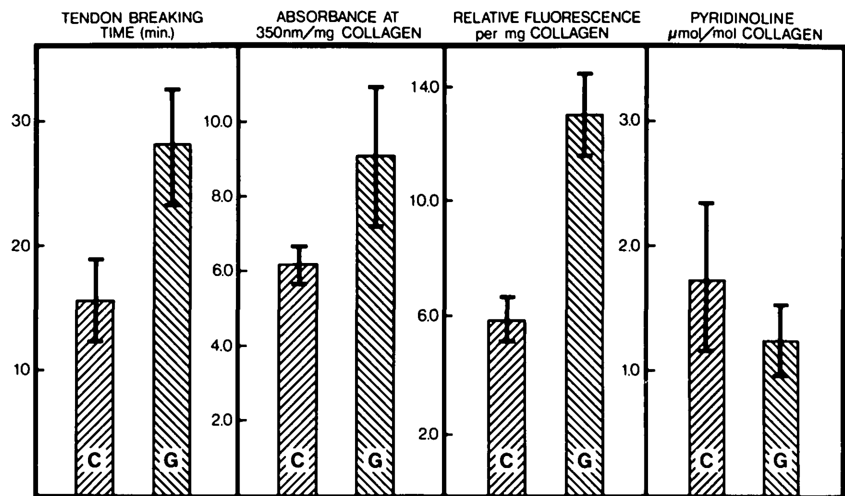

FIG. 2. Effect of 1 yr of galactosemia on rat tail tendon breaking time in urea, collagen-linked chromophores absorbing at 350 nm, fluorophores assayed at 440 nm (excitation 370 nm), and level of pyridinoline cross-links. Values are means ± SD of 5 Sprague-Dawley rats fed 33% galactose diet (G) and 6 control animals (C). With exception of pyridinoline levels ($P > .05$), values were significantly elevated in galactosemic vs. control rats at $P < .001$ level for breaking time and fluorescence and $P < .01$ for absorbance at 350 nm.

(19). Acid hydrolysate equivalent to 6 mg collagen contained in 90 µl water was injected into a high-performance liquid chromatograph (Waters Chromatography Division, Millipore, Milford, MA) equipped with 0.46 × 25 cm Vydac 218 TP 10-µm reverse-phase C_{18} column and an on-line fluorescence flow detector (Aminco-Bowman spectrofluorometer with flow cell, SLM Instruments, Urbana, IL). Pyridinoline was eluted at 54 min based on a standard provided as a gift from D. Fujimoto by application of a linear gradient from 0–17% acetonitrile in 0.01 M *n*-heptafluorobutyric acid from 10 to 97 min. Molarity was calculated with the same molar absorption coefficient at 295 nm as used by Fujimoto et al. (20).

Glycosylated hemoglobin. Glycosylated hemoglobin was assayed by boronate affinity chromatography with the Glycogel test kit and the manufacturer's instructions. Interassay coefficient of variation was 16 and 7% in control and galactosemic rats, respectively.

Light microscopy. Samples of heart, lung, liver, kidney, intestine, pancreas, spleen, and aorta were fixed in 10% formalin buffered to pH 7.6 with 0.01 M phosphate and embedded in paraffin. Coded sections cut at 4 µm were examined with a light microscope (American Optical, Rochester, NY).

Immunofluorescence. Deposition of plasma proteins (albumin, IgG, transferrin, and C-3) in tissues was evaluated by direct immunofluorescence. Samples of lung, aorta, tendon, and renal cortex from each rat were snap-frozen in liquid nitrogen and sectioned at 2 µm in a Cryostat (American Optical, Rochester, NY). After being washed in PBS, parallel sections were fixed for 1 min in acetone, washed in PBS, and overlaid with goat antisera specific for rat IgG (rhodamine conjugated), rat albumin, rat C-3, and rat transferrin (all fluorescein conjugated), all diluted at 1:20 in PBS and obtained commercially (United States Biochemical). Sections were incubated with antisera at 25°C in a moist chamber, washed with PBS, and mounted in 50% glycerol in PBS. Coded slides were examined in an epifluorescence microscope (Leitz, Rockleigh, NJ) to prevent observer bias. The distribution of deposits was recorded, and the intensity was scored semiquantitatively from 0 to 3+.

Statistical analysis. Means ± SD and significance of comparison were analyzed by the Microstat statistical program with paired Student's *t* test and analysis of variance available on the laboratory's IBM AT computer. Immunofluorescence data were compared between galactosemic and control rats by χ^2 tests, with Fisher's correction for small numbers.

RESULTS

The effect of D-galactose on collagen cross-linking in vitro was tested by incubating whole-rat tail tendons with increasing concentrations of galactose. After 15 days at 37°C, breaking time in 7 M urea had doubled ($P < .01$) and tripled ($P < .001$) in the presence of 25 and 100 mM galactose, respectively (Fig. 1). Concomitant increases in collagen-linked chromophores absorbing at 350 nm and fluorophores assayed at 440 nm (excitation 370 nm) analogous to those previously reported for rat tail tendons incubated with glucose (12) were also observed (Fig. 1).

Detection of browning and cross-linking of tendon collagen in vivo was initially attempted after 3 mo of galactosemia. At that time, the galactosemic animals had mature cataracts and glycosylated hemoglobin levels that were two- to threefold higher than in control animals ($P < .01$). No significant increases in tendon breaking time, collagen-linked fluorophores, or chromophores were noted (data not shown).

Cognizant of the kinetic relationship between nonenzymatic glycosylation [i.e., formation of Amadori products and nonenzymatic browning (21,22)], we reasoned that a period of 3 mo was too short for galactose-mediated cross-links to form from glycosylated collagen. Therefore, we repeated and extended the experiment over a period of 1 yr. Assays were performed with five galactosemic and six control animals.

Chronic galactosemia was well tolerated overall by the rats whose weight was only 11% less than that of the control rats (Table 1). Glycosylation of hemoglobin and collagen were increased 2.9 times ($P < .001$) in galactosemic animals and 1.4 times ($P < .025$) over control animals, respectively (Table 1). The larger increase in glycosylation of hemoglobin compared with collagen may be due to the fact that glycosylated collagen underwent nonenzymatic browning to a larger degree than did hemoglobin, because tail tendons are not turned over.

Evidence for increased browning and cross-linking of col-

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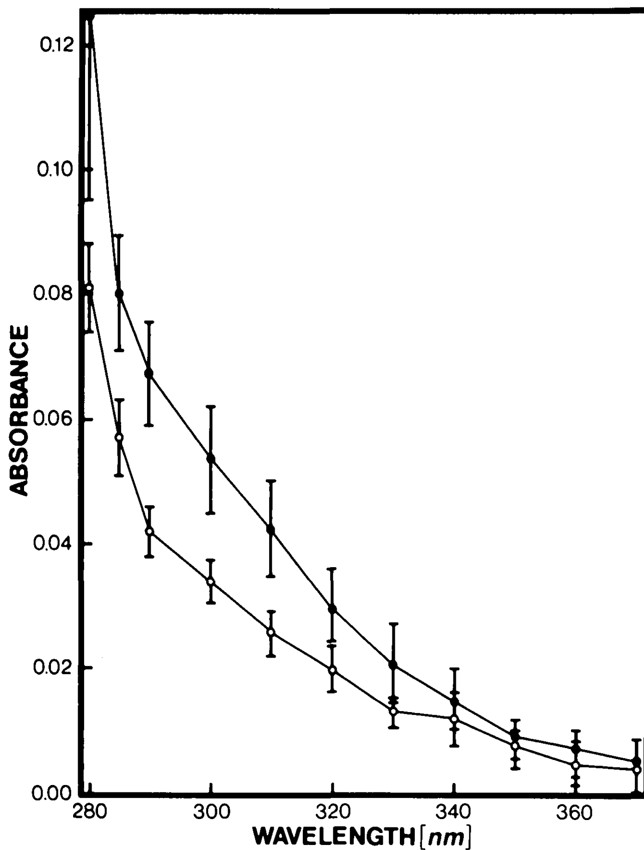

FIG. 3. Absorption spectra of tendon collagen digest from galactosemic (●) and control (○) rats. Samples were prepared as described in Fig. 1. Absorption spectrum was constructed by averaging absorption values at given wavelength (±SD) from individual spectra of 5 galactosemic and 6 control rats. Collagen concentration was adjusted to 4.64 mg/ml for each sample before individual spectra were recorded. Values between 280 and 330 nm were significantly different at *P* < .001 level.

lagen after 1 yr of galactosemia is presented in Fig. 2. Tendon breaking time and collagen-linked products fluorescent at 440 nm (excitation at 370 nm) increased approximately two-fold in galactosemic versus control animals ($P < .001$). The possibility that fluorescence and cross-linking were due to pyridinoline, a collagen cross-link mediated by the action of lysyl oxidase (20), was excluded because galactosemic rats had decreased rather than increased quantities of this cross-link ($P > .05$, Fig. 2). Collagen-linked chromophores absorbing at 350 nm were also significantly increased ($P < .01$). The most significant increase in absorbance, however, was observed between 280 and 320 nm (Fig. 3). A typical fluorescence-excitation spectrum of a collagenase digest of tendon from galactosemic rat showed the presence of excitation-emission maxima at 355 and 430 nm, respectively (Fig. 4), analogous to those generally obtained on incubating proteins with reducing sugars. Compared with those of the control animal, the maxima from the galactosemic rat are found at slightly longer wavelengths.

There were no differences between galactosemic and control animals in the morphologic features of heart, lung, liver, kidney, intestine, pancreas, spleen, or aorta at the light-microscopic level. Kidneys from four of the five galactosemic animals had linear immunofluorescence staining for albumin and IgG in glomerular and tubular basement membranes similar to the changes observed in diabetic nephropathy. In contrast, none of the control animals had this staining pattern ($P < .005$). This suggests that some of these serum proteins may have been trapped into tubular and glomerular basement membranes, possibly by means of galactose-derived cross-links (23). No differences in immunofluorescence for albumin, fibrinogen, IgG, or transferrin were observed between galactosemic and control rat arteries, lungs, or tendons.

DISCUSSION

This study demonstrates that chronic experimental hyperglycemia due to a reducing blood sugar analogous to glucose leads to browning and cross-linking of mature collagen in the absence of diabetes. Both browning and cross-linking properties resemble those seen in diabetic and aging individuals (5). The lag between onset of galactosemia and development of cross-links strongly favors a Maillard-mediated mechanism of cross-linking whereby, e.g., two Amadori products would be needed to generate the cross-link as described for furoyl furanyl imidazole (24). Preliminary experiments in our laboratory led to the identification of glucose-derived pyrrolic structures in a model system in which physiologic conditions of pH and temperature were used (25). Such pyrrolic compounds have been shown by others

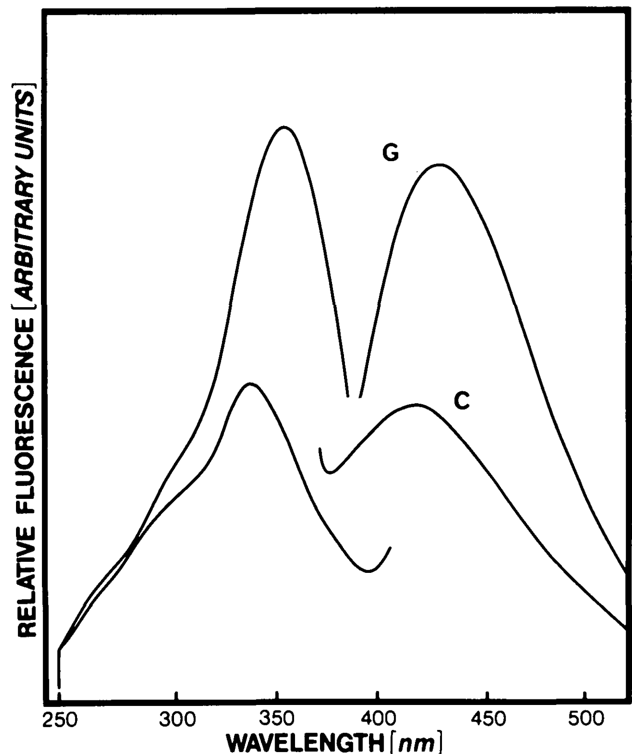

FIG. 4. Representative fluorescence-excitation spectrum of tendon collagen digest from galactosemic (G) and control (C) rats. Collagen concentration was 5.3 mg/ml in control animal and 4.6 mg/ml in galactosemic rat, respectively. Spectra were recorded at excitation-emission maxima detected at 335/400 nm and 355/430 nm for C and G rats, respectively. Aminco-Bowman spectrafluorometer was used (SLM Instruments, Urbana, IL). Sensitivity scale was ×10, blank adjust "low," photomultiplier scale 2.0, and entry and exit slits 4 mm.

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to condense together, thus mimicking a protein cross-link (26).

The cross-links described above should not be confused with cross-links developing in immature collagen or in young rats soon after the onset of diabetes (27–31). The fact that the latter cross-links can be prevented by lathyrogens (28), castration (31), or inhibitors of arachidonic acid metabolism (29) suggests that they are mediated by lysyl oxidase. Interestingly, arterial wall collagen cross-links that develop in diabetic animals might be mediated by both advanced glycosylation products and lysyl oxidase–dependent cross-links because aminoguanidine prevents glucose-mediated cross-links in vitro (32) but also has structure homology with the lathyrogen semicarbazide (33).

It remains to be demonstrated that the browning and cross-linking reported here are indeed derived from Amadori products of galactose and collagen. This has not been possible so far because the actual structure of the sugar-derived collagen cross-links is unknown. The absence of an increase in pyridinoline and the slow development of these cross-links, however, makes it unlikely that they are mediated by that enzyme or indirectly by aldose reductase, although this remains to be demonstrated. The results of this study contrast with the recent report by Chang et al. (34) that acute galactosemia decreases collagen cross-linking in granulation tissue. The comparison of the two studies clearly demonstrates that galactosemia does not exert the same effect on mature and immature collagen. Although these authors reject the possibility of a lathyrogenic effect due to glyosylation of collagen, the paradoxical effect of impaired maturation and accelerated aging of collagen (35) could be explained by the presence of Maillard reaction products that are not detectable by the thiobarbituric acid (TBA) assay (25). However, these are speculations that need to be addressed through experiments.

From the experiments described above, it appears that chronic galactosemia should be useful for testing the hypothesis that some of the complications of the diabetic and aging syndrome may relate to the nonenzymatic browning reaction. However, a number of observations should be taken into consideration when one attempts to incriminate the Maillard reaction in the pathogenesis of diabetic and age-related complications described above. First, cataract- (36) decreased motor nerve conduction velocity (37) and thickening of capillary basement membranes (38) have been demonstrated in experimental galactosemia but were preventable by inhibitors of aldose reductase. Similarly, limited joint mobility in the diabetic human was reversed after 2 mo of treatment with an aldose reductase inhibitor (39). Second, chronic galactosemia in dogs was shown to produce diabetes-like retinopathy (40) and glomerular basement membrane lesions (41), but mesangial matrix expansion and glomerular sclerosis that are typical of advanced diabetic nephropathy were not observed (42). Third, some age-related changes such as stiffening of arteries or decreased interleukin 2 production do not uniformly affect diabetic subjects (8,42). Thus, if chronic hyperglycemia plays a role in the pathogenesis of these complications, its mechanism of action might be only indirectly or partly related to nonenzymatic glycosylation and browning. The status of the research does not allow us to attribute unequivocally the changes observed above to the Maillard reaction per se or to the activation of the polyol pathway or any other mechanism.

Because of the slow progress in understanding the chemistry of the advanced Maillard reaction under physiologic conditions, its occurrence in vivo is based primarily on circumstantial evidence. However, regardless of whether this reaction plays a role in the initiation of diabetic and age-related complications, it is reasonable to postulate that damage mediated by reducing sugars to long-lived molecules will increase the susceptibility to developing complications and decrease the chances of reversibility through extensive cross-linking.

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