MINIREVIEW

Nonenzymatic Glycation of Collagen in Aging and Diabetes (43158C)

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Collagen biosynthesis is a complex process characterized by extensive posttranslational modifications. Intracellular events include the hydroxylation of certain lysine and proline residues and enzymatically mediated glycation of hydroxylysine; after the collagen is secreted from the cell, C-terminal and N-terminal extension peptides are cleaved and tropocollagen molecules become stabilized by covalent cross-linking into fibrillar arrays or networks. Nonenzymatic glycation of certain lysine and hydroxylysine residues also occurs in the extracellular matrix. This modification appears to have direct effects on collagen structure and function as well as indirect effects that arise as a result of further reactions of the glycated residues.

The first step in glycation of collagen involves the condensation of a sugar aldehyde or ketone with the ϵ-amino group of lysine or hydroxylysine; the resultant aldimine may undergo an Amadori rearrangement to form the more stable Amadori product. These two compounds, the Schiff-base and the Amadori rearrangement product, comprise the so-called "early glycation products" (Fig. 1). There is considerable evidence suggesting that Amadori products undergo further reactions *in vivo*, ultimately generating an array of fluorophores and chromophores known collectively as advanced Maillard products or browning products.[1]

[1] There is little consensus as yet concerning terminology in this field. The nonenzymatic addition of sugars to protein amino groups has been referred to as nonenzymatic "glycosylation," "glucosylation," and "glycation." In this review, the term glycation will be used, as recommended by Roth (124). "Early glycation products" will refer to the Schiff base and its Amadori rearrangement product. The fluorophores and chromophores resulting from reactions of these initial products have been referred to as "browning products," "advanced Maillard products," and "advanced glycosylation end products" or AGEs. As there is controversy (discussed in the review) concerning the origin of the fluorophores and chromophores that have been observed to accumulate in connective tissue *in vivo*, the more inclusive term browning products will be used to describe these compounds. The term Maillard product will be used only when discussing the products of specific Maillard reactions.

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Although Maillard reactions have been of considerable interest to food chemists since the turn of the century, it has only been relatively recently that attention has focused on nonenzymatic glycation of proteins *in vivo*. The effects of glycation on long-lived macromolecules, such as collagen, have been of particular interest, especially in studies of connective tissue changes in aging and diabetes. The present review begins with a brief history of the field, which includes a survey of the effects of aging and diabetes on glycation of collagen. It next focuses on the *relationship* between collagen glycation and (i) specific physicochemical changes in collagen observed in both aging and diabetes mellitus and (ii) clinical features of diabetic subjects. The next portion reviews evidence for *regulatory mechanisms* governing glycation of collagen *in vivo* that might be amplified or, alternatively, modified pharmacologically in order to attenuate the pathophysiologic changes of connective tissue associated with aging and diabetes.

Historical Perspective

The possible role of nonenzymatic glycation in collagen cross-linking was first suggested by Bensusan (1). With considerable presience, he hypothesized that hexoses might form Schiff base-type adducts with the ϵ-amino group of lysine residues and subsequently become stabilized by undergoing an Amadori rearrangement. He also postulated several pathways by which these compounds might form advanced Maillard products. In 1968 Bookchin and Gallop (2) reported that the variant hemoglobin that was increased in diabetic subjects contained a hexose linked to N-terminal valine. This finding stimulated further studies suggesting that the nonenzymatic addition of sugar occurred in many proteins *in vivo*, and that nonenzymatic glycation of proteins increased under hyperglycemic conditions, a topic comprehensively reviewed by Bernstein (3). Glycation of collagen was reported to be increased

about 2- to 4-fold in both diabetes mellitus and experimental diabetes in every tissue analyzed, including basement membrane from both kidney and lens capsule (4–8), aorta (9), tail tendon (10), and skin (11, 12).

An association between increased glycation of collagen and aging was reported at almost the same time when Bailey *et al.* (13) observed that cow skin hydrolysates contained unknown reducible compounds that increased with age. Shortly thereafter, these compounds were identified as lysine-carbohydrate condensation products containing a Schiff base-type bond (14, 15). Other investigators soon reported that content of early glycation products increased throughout the life span in collagen from human bone, tendon, cartilage, skin, and glomerular basement membrane (16–19). However, more recent studies have not confirmed the earlier reports. Several investigators have found no evidence for age-related increases in early glycation products in human skin or basement membrane (11, 20); others have found only slight increases in glycation of human tendon and aorta that were strongly correlated with age-associated increases of glycemia (21). In our own studies, we have found no significant age-associated increase in early glycation products of skin collagen from either monkeys or rats over their life spans.

These conflicting results are in some cases most likely due to differences in the specificity of the analytical techniques used. For example, in many of the earlier studies, the investigators used the thiobarbituric acid assay, which is subject to interference by a number of compounds, including free glucose and glycosidically bound carbohydrates (22–24). In later studies, which suggest that early glycation products do not increase with age, more specific techniques have been used for quantification, including the furosine assay (23), or affinity chromatography followed by high-performance liquid chromatography to isolate the glycated residues (12, 25, 26).

As interest developed in the biologic consequences of nonenzymatic glycation of proteins, attention was also focused on maturational products of glycated residues. Although the accumulation of yellow-brown chromophores and fluorophores ("browning products") in aging collagen was first reported more than 25 years

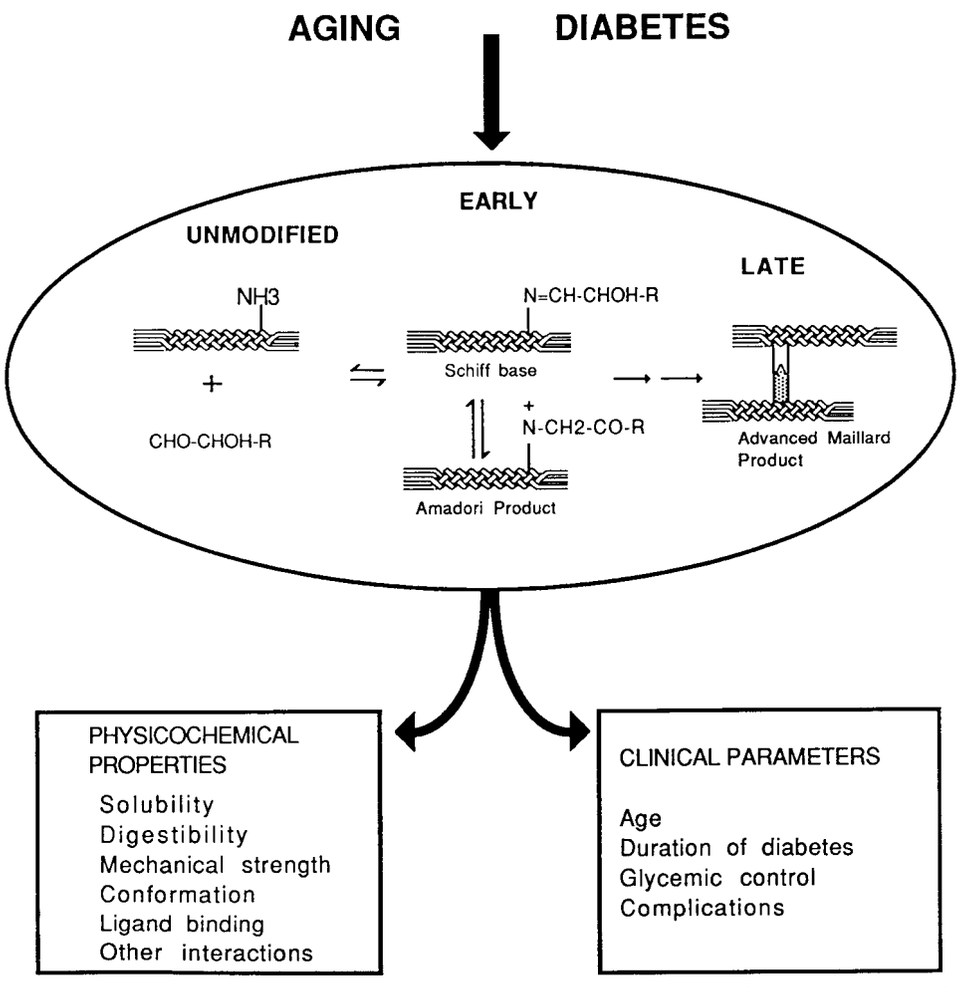

Figure 1. Effects of aging and diabetes on nonenzymatic glycation of collagen and its relationship to physicochemical changes and clinical parameters. Unmodified collagen is schematically shown reacting with glucose to form early and late glycation products.

ago, these compounds were initially thought to arise from oxidative reactions of tyrosine residues, since tyrosine content of tissues appeared to decrease in parallel with the increase in fluorescence (27). The association between browning products and nonenzymatic glycation of collagen has only been investigated relatively recently, stimulated by reports that incubation of lens crystallins with glucose *in vitro* led to accumulation of pigmented and fluorescent cross-links with spectral properties similar to those of Maillard products (28, 29). These observations prompted investigations of other long-lived proteins, particularly collagen. Monnier *et al.* (30) studied the accumulation of browning products in collagen from dura mater in normal and diabetic subjects ranging in age from 19 to 89 years. In control subjects they found a linear correlation between age and accumulation of fluorescent compounds. Insulin-dependent diabetic subjects had a significantly higher content of browning products in the dura mater than did age-matched controls. Browning products have also been found to be increased with age in collagen from tendon, skin, and cartilage (31, 32).

In these studies, the fluorophores and chromophores were inferred to be Maillard products from indirect evidence, such as spectral similarities to known Maillard products (30) or chromatographic behavior (32). Some investigators have questioned whether these browning products are derived exclusively, or even predominantly, from nonenzymatically glycated collagen via Maillard reactions (33). Although attempts have been made to characterize specific browning products, isolation and analysis of such compounds as they exist *in vivo* has proven very difficult, owing to low yields, difficulties in isolating the desired compounds, and the ease with which artifacts may be generated, particularly if hydrolysis is involved. For example, a fluorophore [2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole] was isolated and characterized that was postulated to be a late-stage intermediate of an advanced Maillard product (34). Subsequent studies strongly suggest that this particular compound is an artifact of hydrolysis (35, 36). Only one advanced Maillard product in collagen *in vivo* has been isolated and fully characterized, a trifunctional fluorophore consisting of an imidazo-pyridinium ring containing lysine and arginine side chains (37). The authors suggest that the two amino acids are cross-linked through a Maillard reaction with pentose; the compound has hence been named pentosidine. Pentosidine content of human dura mater increased linearly throughout the life span. Skin also showed an age-related increase in pentosidine, although absolute values were not as high as in dura mater. In diabetic patients, skin content of pentosidine was highest (38) in those with renal failure; nondiabetics with renal failure also had elevated levels (38).

Several partially characterized compounds believed to represent late glycation products present on collagen *in vivo* have also been described: these include "L1," which increases in rat aorta with aging (39) and "Compound M," which is present in insoluble human dura mater (40). "Compound P" (40) has subsequently been fully characterized as pentosidine (37).

Collagen Glycation and Connective Tissue Changes in Aging and Diabetes

In both aging and diabetes, there are widespread changes in connective tissue in virtually every organ system: collagen becomes more insoluble and more resistant to digestion, and such properties as thermal rupture time and mechanical strength are also altered. These changes in the physicochemical properties of collagen are believed to contribute, in part, to the development of long-term complications seen in diabetic subjects, including cardiovascular disease, retinopathy, nephropathy, thickened and indurated skin, and joint stiffness (41, 42). Can such changes be accounted for by increased collagen content of glycation products? This question has been addressed directly, by analyzing the relationship between collagen glycation content and specific properties, and indirectly, through examining correlations between collagen glycation content and clinical features of diabetic subjects.

Correlations between Collagen Glycation and Alterations in Specific Properties. *Mechanical properties.* Mechanical stiffness of collagen has been observed to be increased in diabetes (43) and is postulated to result from increased cross-linking. Andreassen *et al.* (44) examined the effects of glycation on several mechanical properties of rat tail tendon collagen. Mechanical stability was not affected by increased content of early glycation products, and increased only when browning products were formed. Kent *et al.* (45) found that mechanical strength increased and solubility decreased in rat tail tendon collagen incubated in glucose, observations that they attributed to formation of glucose-derived cross-links (presumably late Maillard reaction products). The presence of such cross-links was inferred from CNBr peptide maps showing accumulation of high molecular weight material in the glycated collagen.

Yue *et al.* (46) reported that thermal rupture time of rat tail tendon collagen showed a strong correlation with content of early glycation products *in vitro* and *in vivo*. Yue *et al.* (10) subsequently performed a more detailed investigation of this relationship. They found that when normal tail tendon collagen was incubated with glucose *in vitro*, both aspirin, which theoretically should block the lysine residues via acetylation (47), and salicylate (not an acetylator) were effective in preventing increased glycation and increased thermal rupture time. However, aspirin or salicylate administered *in vivo* to diabetic rats prevented increased rupture time

without preventing increased collagen content of early glycation products. The *in vivo* experiments suggest that the effects on thermal rupture time are not mediated through alterations in glycation content. Yue *et al.* (10) suggest that, alternatively, aspirin and salicylate could have selectively blocked glycation of a key subset of lysine residues involved in alterations in rupture time; this small decrease in glycation might not be detectable when glycation of the whole molecule is measured.

Kohn *et al.* (48) also examined the effects of *in vitro* glycation on thermal rupture time of collagen. Rat tail tendon was incubated with glucose, ribose, or glucose-6-phosphate; both early glycation products and accumulation of browning products were measured. Increase in break time was correlated with the increase in browning products, which accumulated most rapidly and in the greatest amounts with ribose, but not with early glycation products.

Solubility. The relationship between acid solubility and collagen glycation has been investigated in detail by Brennan (49). She provided evidence that the striking increase in insolubility observed in tail tendon collagen in diabetic rats cannot be attributed to either early glycation products or to browning products. Brennan (44) has suggested that the changes in solubility may reflect qualitative alterations in lysyl oxidase-mediated cross-linking, perhaps through maturation of difunctional cross-links into as yet uncharacterized mature forms, rather than an increase in total cross-links (50). Williamson *et al.* (51, 52) have also found that collagen solubility is not apparently correlated with nonenzymatic glycation. For example, they found that in diabetic rats islet cell transplantation or castration prevented diabetes-induced decreases in solubility in newly synthesized granulation tissue collagen without preventing increased nonenzymatic glycation. Particularly intriguing were the results of a study of experimental galactosemia in which they found that solubility of granulation tissue collagen was increased in galactosemic rats, as was nonenzymatic glycation (53).

Susceptibility to digestion with collagenase does not appear to be correlated with collagen content of early glycation products. Perejda *et al.* (54) found no difference in digestion of unmodified Type I collagen prepared from chick embryo tendon as compared with collagen that was glycated *in vitro*; similar results were reported by Kennedy and Lyons for human skin collagen (55). Incubations were not carried out long enough in either study to examine the relationship between browning products and collagenase digestion.

Ligand binding. Several investigators have examined the relationship between collagen glycation and ligand binding. Tarsio *et al.* (56–58) have examined the effects of glycation on collagen-enhanced binding of heparin to either laminin or fibronectin, an *in vitro* model of potentially important interactions that occur in the basement membrane. Heparin binding was significantly decreased when Type IV collagen or laminin were glycated *in vitro*; when both laminin and collagen were glycated, heparin binding was only 6% of maximal binding (58). Similar results were found for fibronectin (56, 57). These effects on ligand binding appear to be directly correlated with content of early glycation products; the experiment was not designed to examine the effects of browning products. The authors speculate that such glycation-induced alterations in the association of basement membrane components might lead to the structural and functional abnormalities seen in diabetic nephropathy (56).

Brownlee *et al.* (59) found that significantly greater amounts of L-lysine, serum albumin, and IgG bound to Type I collagen (prepared from soluble calf skin) that had been glycated *in vitro* as compared with unmodified collagen. In this study, the authors assume that the increase in ligand binding to glycated collagen results from the presence of browning products containing reactive carbonyl groups. The relative roles of early glycation products and browning products in altering ligand binding were not specifically investigated. The collagen-bound ligands were still able to form immune complexes, consistent with *in vivo* observations that glomerular basement membrane (GBM) from diabetic rats has almost five times the amount of IgG bound to it as control GBM (60). Brownlee *et al.* (61) have also observed that cross-linking of low density lipoprotein to collagen that has been glycated *in vitro* increases linearly with the amount of browning products present on the collagen. These data are consistent with *in vivo* observations that 2.5 times the amount of lipoprotein is cross-linked to aorta collagen in diabetic animals as compared with controls (42).

The interaction between collagen in blood vessels and circulatory components such as platelets may be altered in diabetes. LePape *et al.* (62) have shown that rat tail tendon collagen glycated *in vitro* or obtained from diabetic rats was more effective in aggregating platelets than control collagen. They suggest that this abnormal interaction with platelets may contribute to the risk of thrombotic disease in diabetic subjects. This effect appears to be due to early glycation products rather than to the accumulation of browning products.

Conformational changes. The relationship between glycation of collagen and conformational changes has been a subject of considerable interest. Tanaka *et al.* (63) used x-ray diffraction to study changes in tail tendon collagen induced by *in vitro* glycation with ribose. Expansion occurred perpendicular to the axes, suggesting that intermolecular spacing of the molecules was increased. Although molecular expansion could be attributed to the direct effects of the sugar moieties on electrostatic changes, steric volume, or hydration, the

authors speculate that the most likely mechanism is the formation of specific cross-links (late glycation products) between residues at one or two key sites on the molecule. In a subsequent study, the authors described the partial purification of fluorescent dimers that may represent α chains cross-linked by such sugar-derived cross-links (64).

In contrast, conformation of Type IV collagen may be directly affected by early glycation products (65). Tsilibary *et al.* (65) found that the free dimeric C-terminal globular domain of Type IV collagen (NC1) loses its ability to inhibit lateral aggregation of helical Type IV collagen after glycation *in vitro*. The resultant structural abnormalities have been observed by rotary shadowing electron microscopy (66). Using gel electrophoresis, the investigators examined the glycated protein for evidence of glucose-derived cross-linking. The small amount of high molecular weight material present was too small to be the sole cause of the impaired binding between NC1 and Type IV collagen. Although such cross-link adducts may contribute to the observed structural abnormalities, the investigators conclude that the glycation of certain key lysine residues is sufficient to alter binding properties.

Enzymatically mediated cross-links. The structure and stability of collagen within the extracellular matrix is largely conferred by enzymatically mediated cross-links (for recent comprehensive reviews see refs. 67 and 68). Since glycation and lysyl oxidase-mediated cross-linking both involve lysine and hydroxylysine residues, it is reasonable to speculate that some interaction exists between these two very different cross-linking pathways. Although reducible cross-links have been reported to be decreased or unchanged in diabetes (50, 69), Williamson and coworkers (70, 71) have provided indirect evidence that total cross-link content may be increased. In a recent study of skin collagen from young diabetic subjects, evidence was presented suggesting a strong correlation between increased content of a specific lysyl oxidase-mediated cross-link and increased content of early glycation products (12). There are a number of possible mechanisms by which glycation might affect lysyl oxidase-mediated cross-linking. For example, changes in steric hindrance due to addition of sugar moieties might play a role in altering the nature of lysyl oxidase-mediated cross-linking; enzymatic glycation has been shown to exert such an effect in bone collagen (72). Changes could also be induced by perturbations of fibrillogenesis, a prerequisite for formation of lysyl oxidase-derived cross-links. Guitton *et al.* (73) have suggested that early glycation products may interfere with fibrillogenesis by perturbing ionic interactions. Hyperglycemia itself may also affect fibrillogenesis by inhibiting linear assembly of fibrils (74).

Correlations between Collagen Glycation and Clinical Parameters in Diabetes Mellitus. In general, there appear to be few correlations between collagen content of early glycation products and most clinical parameters in diabetes mellitus. Uitto *et al.* (75) found no correlation between GBM glycation and severity of nephropathy; Lyons and Kennedy (76) found no correlation between glycation of skin and joint mobility. In a more comprehensive study, Vishwanath *et al.* (11) investigated correlations between early glycation products in skin collagen and age, duration of diabetes, retinopathy, nephropathy, arterial stiffness, and joint stiffness in Type I diabetic subjects ranging from 29 to 52 years of age. Glycation was significantly increased in the skin from the diabetic subjects, but showed no correlation with any of the parameters except glycohemoglobin measured at the time of biopsy. Similar findings were reported in a recent study of young Type I diabetic subjects, ranging in age from 6 to 20 years of age (12). Skin content of early glycation products was correlated with glycohemoglobin measured up to 12 months previously, but was not correlated with age, joint stiffness, or retinopathy. There was, however, a weak correlation with duration of diabetes, microalbuminuria, and with clinical assessments of skin thickening and tightening. Of interest also was the observation that glycation was correlated with abnormalities in lysyl oxidase-mediated cross-linking.

In the first comprehensive, cross-sectional study of the relationship between collagen content of browning products and severity of diabetic complications, Monnier *et al.* (31) measured fluorescence in the collagenase-soluble fraction of skin from insulin-dependent diabetic subjects. All patients had fluorescence levels greater than age-matched controls and there was a linear correlation between these values and duration of diabetes. There was also a significant correlation between age-adjusted fluorescence and degree of retinopathy, arterial and joint stiffness, and blood pressure. The most significant variables were age and the presence of retinopathy. In a subsequent report, Monnier *et al.* (77) investigated the relationship between overall and individual browning rates (i.e., accumulation of fluorophores in skin collagen) and the severity of retinopathy. Overall browning rate of subjects without retinopathy was not significantly different from that of controls, whereas those with retinopathy had rates that were significantly higher. Furthermore, when browning rates were plotted as a function of age, they decreased with age in subjects without retinopathy but increased in subjects with retinopathy. There were no significant differences between glycohemoglobin levels and glycation of skin collagen among the various categories. These data suggest that, within a given category of diabetic subjects, the browning process occurs at a constant rate; furthermore, it appears that some diabetics either are more resistant to the browning process or have a greater capacity to remove collagen modified in this way. Collagen-linked

fluorescence in skin has also been studied in a group of younger diabetic subjects, ranging from 17 to 30 years of age. Fluorescence levels were correlated with age, duration of diabetes, and retinopathy but not with glycohemoglobin levels (78); these results are thus consistent with the study of older diabetic subjects.

Current Concepts of Biosynthesis and Regulation of Nonenzymatic Glycation of Collagen *In Vivo*

The effect of increased glycation on the structural and functional properties of collagen have stimulated interest in investigating *in vivo* biosynthesis pathways, including intrinsic regulatory mechanisms. Conventionally, glycation reactions are divided into three groups (79), shown schematically in Figure 2. "Early Maillard reactions" (Fig. 2A) include formation of the Schiff base and its rearrangement into the more stable Amadori product. "Intermediate reactions" (Fig. 2B) include the degradation and dehydration of Amadori products to form highly reactive compounds that serve as "propagators" of nonenzymatic glycation. "Advanced reactions" (Fig. 2C) involve the reaction of these intermediate products with amino groups to form fluorescent, pigmented cross-links. Advanced Maillard products may undergo extensive polymerization reactions *in vitro*, yielding deeply colored compounds referred to as melanoidins (see Fig. 2C). These reactions are unlikely to occur *in vivo*, however (32).

Early Maillard Reactions. The first step in the Maillard reaction in collagen involves the nonenzymatic condensation of a sugar aldehyde or ketone with the ϵ-amine group of either lysine or hydroxylysine. Although most studies have focused on reactions of glucose with proteins, there is evidence that other sugars may participate in nonenzymatic glycation *in vivo*. Both fructose and pentose react with collagen *in vitro* to form Maillard products far more rapidly than does glucose (48, 80–82). Recently, Suarez (83) has presented data suggesting that fructation of collagen occurs *in vivo*. Eaton (84) has also suggested that fructose, which is biosynthesized via the polyol pathway and hence is increased in diabetes, is present in the extracellular matrix and available to react with collagen. Evidence for the participation of pentose in Maillard product formation in collagen *in vivo* has been provided by Sell and Monnier (37). These authors speculate that the most likely precursor of pentose is ribose or one of its metabolites, such as might arise from ADP-ribosylation reactions.

For most proteins, including collagen, there ap-

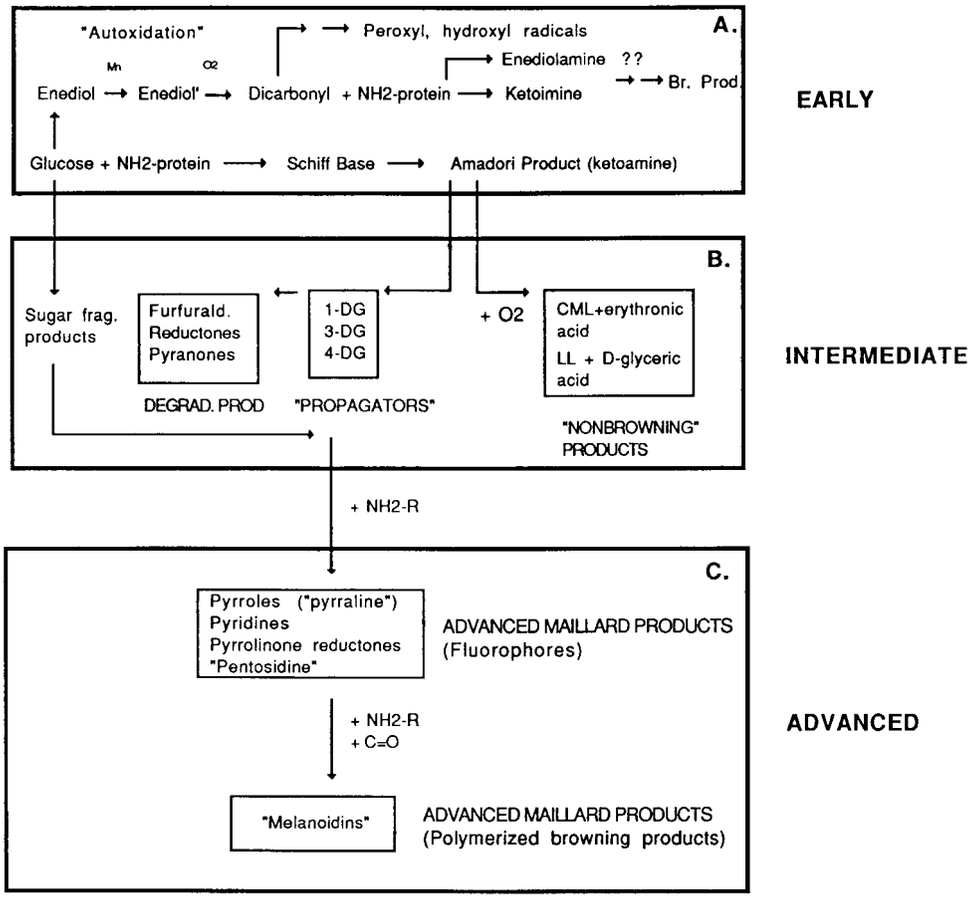

Figure 2. Scheme of the Maillard reaction.

pears to be a glycation limit of 2–4 mol of glucose/mol of protein (85) even when the proteins are incubated with sugar *in vitro* for extended periods of time. The rate of glycation drops to less than 2% of the initial rate following addition of the first few glucose molecules. Bitensky *et al.* (85) have speculated that the reduction in rate may be secondary to the increase in negative charge associated with glycation.

Within this glycation limit, the extent of glycation appears to be dependent on several variables, including pH, temperature, protein and glucose concentrations, and duration of exposure to glucose (41). Since pH and temperature are closely regulated *in vivo*, it has been suggested that degree and duration of hyperglycemia are the most important variables governing glycation levels. However, other factors may also play a role in regulating the addition of sugars to the collagen chain. For example, Wolff and co-workers (86, 87) have suggested that glycation content may also be modulated by glucose oxidation (Fig. 2A). They have provided evidence that through sequential reduction of a transition metal and molecular oxygen glucose auto-oxidizes to a highly reactive dicarbonyl compound that can bind to proteins. *In vitro* experiments show that addition of the metal-chelating agent diethylenetriaminepenta acetic acid to the incubation mixture will reduce binding of glucose to bovine serum albumin by about 45%. These data suggest that there are two pathways by which proteins bind to glucose: metal catalyzed (involving generation of reactive ketoaldehydes through auto-oxidation) and metal independent (formation of Schiff-base adducts followed by enolization to the Amadori product.) Diethylenetriaminepenta acetic acid also decreases the production of chromophores and fluorophores in parallel with its inhibition of glycation, suggesting that browning products may form from the ketoimine; whether or not such reaction pathways are similar to those followed by the Amadori products remains to be elucidated. In addition to its contribution to formation of glucose-protein adducts, glucose auto-oxidation also generates peroxides and free radicals that may in themselves alter protein conformation, thus contributing to the change in properties associated with nonenzymatic glycation. Wolff suggests that antioxidant agents may be useful in ameliorating the consequences of auto-oxidation seen in diabetes and aging.

It has been suggested that rates of glycation of proteins may also be affected by location of glycation sites (48). Although site specificity of glycation has been well documented in many proteins, such as albumin, hemoglobin, low density lipoprotein, fibrin, and RNase (3, 88–93), collagen glycation sites have not been studied in detail. We have recently reported on evidence for preferential glycation sites on a cyanogen bromide peptide (CB3) from Type I collagen, both *in vivo* and *in vitro* (94). We observed that the higher the glycation content, the greater the proportion of glycated residues located at the favored site. Studies by LePape *et al.* (95) and Tsilibary *et al.* (65) have provided indirect evidence that glycation does not occur randomly on Type I collagen chains. Possible mechanisms responsible for site specificity of glycation have been reviewed recently by Baynes *et al.* (96). In general, increased lysine reactivity with glucose is associated with relatively decreased pK values for the ϵ-amino group of the lysine side chain (3). Proximity to carboxylic acid residues or high affinity binding sites for phosphate ion or organic phosphates also may favor glycation (90, 93).

Once the sugar molecule has reacted with the amino group to form a Schiff base, it may be stabilized by undergoing an Amadori rearrangement. This reaction may be catalyzed by neighboring acidic or basic residues, such as carboxylic acid residues (88, 90) and histidine or arginine residues (93). The kinetics of the equilibrium between aldimines (Schiff base) and ketoimines (Amadori rearrangement) have been extensively studied in hemoglobin by Huisman *et al.* (97) and by Higgins and Bunn (98). In hemoglobin, the Schiff-base adduct is a highly unstable compound whose rate of dissociation is 60 times greater than the rate of formation of the Amadori rearrangement product. At any given concentration of glucose, there will be a rapidly equilibrating level of protein in the aldimine form; indeed, in diabetics the percentage of glycohemoglobin in the aldimine form relative to the ketoimine form is significantly higher than that of controls (97). Although such kinetic data are not available for nonenzymatically glycated collagen, it seems likely that a similar increase in the relative proportion of the aldimine form may also occur under hyperglycemic conditions.

Hyperglycemia leads to a fairly rapid increase in collagen glycation; is this change reversible following restoration of euglycemia? McLennan *et al.* (99) found that 8 weeks of optimal insulin therapy initiated 4 weeks after diabetes induction could not reverse content of early glycation products or increased rupture time in tail tendon collagen, even though insulin treatment initiated at the onset of diabetes induction prevented the abnormalities from developing. Furthermore, *in vitro* work suggests that once a protein has been glycated, it will undergo cross-linking reactions for 2 to 3 weeks even after the sugar has been removed from the incubation medium (100). These studies clearly suggest that short-term elevations in blood sugar that lead to increases in early glycation products may have long-term physiologic consequences that are not necessarily reversible.

Intermediate Maillard Reactions. Since there is evidence from kinetic studies that formation of advanced Maillard products is primarily dependent on Amadori product concentration, potential regulatory mechanisms controlling concentration of this com-

pound are of considerable interest (32). One such mechanism may involve a degradation pathway that converts Amadori products to inert "nonbrowning products" (Fig. 2B). Ahmed *et al.* (101) reported the presence, *in vivo* and *in vitro*, of a degradation pathway in which oxidative cleavage between C-2 and C-3 of the carbohydrate chain of the Amadori product results in formation of carboxymethyllysine, derived from the protein after fragmentation of the sugar moiety, and erythronic acid. The reaction is pH dependent and sensitive to phosphate, chelators, and radical scavengers. The investigators have detected carboxymethyllysine *in vivo* in lens protein and in tendon collagen. Carboxymethyllysine has long been known to be present in urine; the authors speculate that it may be derived from the oxidation of Amadori adducts to lysine residues in protein. In a subsequent report, Ahmed *et al.* (102) identified a second set of oxidative cleavage products, resulting from cleavage between C-3 and C-4: 3-(*N*-lysine)lactic acid and D-glyceric acid. These compounds have been detected in urine and human lens protein. Since all of these oxidative products are colorless, the authors refer to these oxidative pathways as nonbrowning pathways of the Maillard reaction. The authors further suggest that oxidative cleavage of Amadori adducts may serve to limit potential damage resulting from accumulation of advanced Maillard products, since the oxidation products are chemically inert and do not contribute to further cross-linking.

The sequence of reactions that Amadori products undergo to form Maillard products is complex and not fully understood *in vivo*, especially as it pertains to collagen. For a review of Maillard chemistry, the reader is referred to a recent comprehensive symposium on this subject (103). A key step appears to involve the degradation of the initial Amadori product into one of several α-dicarbonyl groups, such as the deoxyglucosones, that are more reactive than their precursors and thus serve as propagators of the Maillard reaction. Deoxyglucosones have been extensively studied *in vitro*; detailed reviews of recent work in this area are available (39, 79, 104). These compounds may react with free amino groups to form pyrrole-based pigmented and fluorescent adducts and cross-links (32). A recent report suggesting that liver enzymes may metabolize 3-deoxyglucosone suggests that a potential control mechanism may exist at this stage of the reaction (105).

Advanced Maillard Reactions. Although advanced Maillard reactions have been extensively studied in model reaction systems *in vitro*, elucidation of biosynthesis pathways and characterization of specific cross-linking compounds present *in vivo* has proven remarkably difficult. Pentosidine is the only fully characterized Maillard product known to be present on collagen (37); however, its biosynthetic pathway *in vivo* has not yet been elucidated. A pyrrole compound derived from 3-deoxyglucosone has been identified in human albumin using an immunoassay (106, 107); this compound, given the trivial name "pyrraline," is increased in albumin from diabetic subjects (Fig. 2C). Indirect evidence suggests that pyrraline may also be present in collagen: Scott *et al.* (108, 109) have shown that an Ehrlich's chromogen, which they infer to be a pyrrolic compound, is present in a three-chain cross-linked peptide derived from Type III and Type IV collagen. The chromogen is also present in preparations of bone, tendon, skin, cartilage, and periosteum (108).

Even though advanced Maillard reactions appear to be chemically irreversible, there is some evidence that tissue content of advanced Maillard products may be regulated *in vivo*. Recent studies by Vlassara *et al.* (110) have reported on the characterization of a high affinity macrophage receptor that mediates the uptake and degradation of proteins that have been glycated *in vitro* or isolated from diabetic rats. This receptor appears to be distinct from previously described scavenger receptors (111). Although the receptor does not appear to recognize Amadori products, it has a high affinity for proteins glycated *in vitro*. Additional studies have suggested that the receptor recognizes a specific type of structure that is homologous to 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (112). There is evidence that the macrophage system may be involved in remodeling of aging tissue, since two monokines, tumor necrosis factor and interleukin 1, are released by macrophages when they bind proteins containing Maillard-type adducts. As more is learned about regulation of this receptor in aging and diabetes, it may be possible to develop strategies for modulating its effectiveness.

Biosynthesis of Browning Products via Non-Maillard Reactions. There is evidence that early glycation products might participate in cross-linking of collagen through pathways other than the Maillard reaction. Hicks *et al.* (113) measured thermal rupture time of control and glycated rat tail tendon collagen with and without the presence of decomposing lipid hydroperoxides. They found that the glycated collagen had a significant increase in rupture time in the presence of the lipid peroxide products that could not be accounted for only by reactions involving malondialdehyde. Since serum from diabetic patients has been shown to have increased levels of lipid peroxidation products (114), the authors suggest that cross-links arising through interactions between glycated collagen and oxidizing lipids may contribute to some of the alterations in connective tissues observed in diabetes, particularly in lipid-rich structures such as blood vessels.

Other studies have also shown that oxidizing lipids are capable of generating cross-links in collagen and other proteins with fluorescence spectra similar to those of Maillard products (115). These reactions may not involve glycated residues. For example, Lunec *et al.*

(116) suggest that free radical-induced denaturation of proteins oxidizes constituent aromatic amino residues to form highly fluorescent hydroxylated derivatives and kynurenines (116). Sundholm *et al.* (117) also showed that incubation of gelatin with oxidizing lipids led to increased cross-linking.

Pharmacologic Intervention. Despite the presence of regulatory mechanisms at various stages of Maillard product formation, accumulation of browning products does occur even in normal aging. In diabetic subjects collagen content of both early and late glycation products may be increased. Given the possible effects both early and late glycation products may have on collagen structure and function (discussed earlier in the text), there has been considerable interest in pharmcologic intervention at various stages in the glycation process.

Sensi *et al.* (118) reported that D-lysine reduced collagen content of early glycation products *in vitro*. They suggest that, *in vivo*, D-lysine could theoretically compete with circulating and structural proteins for sugar residues. Eble *et al.* (100) showed that free lysine could inhibit polymerization of glycated proteins, presumably competing with unmodified proteins for the active cross-linking sites on glycated proteins.

Data suggesting that fructation of collagen may contribute to accumulation of browning products has led to an interest in the use of aldose reductase inhibitors to inhibit biosynthesis of fructose. Odetti *et al.* (119) and Suarez *et al.* (120) found that browning products were decreased in diabetic rat collagen following administration of an aldose reductase inhibitor, whereas Cohen and Klepser (121) found that an aldose reductase inhibitor had no effect on fluorescence in diabetic GBM. Differences in experimental design may be responsible for these conflicting results.

Recently, Cerami and co-workers (122) have reported that a hydrazine compound, aminoguanidine, can bind to the reactive aldehyde on Amadori products, thus theoretically preventing their progression into more advanced Maillard products. Cerami *et al.* (123) showed that thickening of the arterial wall and accumulation of collagen-linked fluorescence could be prevented in diabetic rats by aminoguanidine administration. Aminoguanidine also reportedly prevents IgG trapping by basement membranes (123). Odetti *et al.* (119) recently reported that aminoguanidine treatment decreased skin collagen fluorescence in diabetic rats. Phase II clinical trials of aminoguanidine are now in progress; within the next few years clinical data should be available.

Summary and Conclusions

Considerable progress has been made in our understanding of nonenzymatic glycation of collagen, and the relationship between glycation of collagen and changes in connective tissue associated with aging and diabetes. Recent studies surveyed in this review suggest the following conclusions:

1. Collagen content of early glycation products does not appear to increase throughout the life span in normal human subjects, although small increases may occur that are linked to glycemic changes. These products are increased, relative to age-matched controls, in experimental diabetes and in diabetes mellitus in collagen from virtually all tissues analyzed.

2. Collagen content of browning products increases with aging and appears to be higher in diabetic subjects than in age-matched controls. Rates of accumulation may be accelerated in subpopulations of diabetic subjects at high risk for developing complications.

3. Increases in early glycation products do not appear to be associated with alterations in collagen solubility, thermal rupture time, or mechanical strength, nor is there an association with most diabetic complications. Alterations in these products may, however, affect conformation, ligand binding, lysyl oxidase-mediated cross-linking, and interactions between collagen and other macromolecules in the extracellular matrix.

4. Increased content of browning products is associated with many physicochemical changes in collagen as well as with long-term complications in diabetes mellitus.

5. Regulatory mechanisms have been identified *in vivo* that may serve to control or limit the formation of glycation products.

7. Pharmacologic agents have been identified that may be able to reduce collagen content of late glycation products.

Despite the progress that has been made in this field, many areas of uncertainty and controversy exist. For example, there is not yet a consensus that the browning products associated with collagen exclusively comprise advanced Maillard products derived from nonenzymatically glycated residues. There is evidence that oxidative reactions involving lipids also play a role in generating fluorophores and chromophores that may alter properties of collagen. Thus, in the extracellular matrix collagen may be continuously modified by at least three very different processes: Maillard reactions, interactions with oxidizing lipids, and enzymatically mediated cross-linking. The interrelationships between these and possibly other posttranslational modifications remain a poorly understood area of great complexity.

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