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The Maillard reaction in vivo

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Summary: The Maillard or browning reaction between reducing sugars and protein contributes to the chemical deterioration and loss of nutritional value of proteins during food processing and storage. This article presents and discusses evidence that the Maillard reaction is also involved in the chemical aging of long-lived proteins in human tissues. While the concentration of the Amadori adduct of glucose to lens protein and skin collagen is relatively constant with age, products of sequential glycation and oxidation of protein, termed glycoxidation products, accumulate in these long-lived proteins with advancing age and at an accelerated rate in diabetes. Among these products are the chemically modified amino acids, N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxymethyl)hydroxylysine (CMhL), and the fluorescent crosslink, pentosidine. While these glycoxidation products are present at only trace levels in tissue proteins, there is strong evidence for the presence of other browning products which remain to be characterized. Mechanisms for detoxifying reactive intermediates in the Maillard reaction and catabolism of extensively browned proteins are also discussed, along with recent approaches for therapeutic modulation of advanced stages of the Maillard reaction.

Zusammenfassung: Die Maillard- oder Bräunungsreaktion genannten Umsetzungen zwischen reduzierenden Zuckern und Eiweiß führen zur chemischen Zerstörung der Aminosäuren und zum Verlust der Proteinqualität während der Lebensmittelbearbeitung und -lagerung. Der vorliegende Beitrag zeigt Befunde auf, daß die Maillardreaktion auch im Gewebe des Menschen bei der Alterung von Proteinen mit langer biologischer Halbwertszeit auftritt. Die Konzentrationen an den sogenannten Amadori-Produkten, die im Initialstadium der Maillardreaktion aus Glucose und den Proteinen der Augenlinse oder dem Kollagen der Haut entstehen, erwiesen sich als relativ konstant, auch mit zunehmendem Alter. Die Produkte der Glycosylierung und nachfolgenden Oxidation der Proteine, auch Glycoxidationsprodukte genannt, häufen sich dagegen im Alter an, und zwar bei Diabetikern in vermehrtem Maße. Zu diesen Produkten gehören die Aminosäurenderivate N-(carboxymethyl)-lysin (CML), N-(carboxymethyl)-hydroxylysin (CMhL) sowie das fluoreszierende Quervernetzungsprodukt Pentosidin. Während diese Glycoxidationsprodukte in den Körpergeweben nur in Spuren vorkommen, gibt es deutliche Hinweise auf die Anwesenheit weiterer Bräunungsprodukte, deren Charakterisierung jedoch noch aussteht. Es werden Möglichkeiten zur „Entgiftung" der reakti-

ven Zwischenprodukte aus der Maillardreaktion sowie zum Abbau extrem gebräunter Proteine diskutiert sowie neuere Möglichkeiten zur therapeutischen Modulierung fortgeschrittener Stadien der Maillardreaktion aufgezeigt.

Key words: advanced glycosylation endproducts (AGE), ag(e)ing, aminoguanidine, ascorbate, autoxidation, biomarker, browning reaction, chemical modification of proteins, diabetes, glycation, glycoxidation, nonenzymatic glycosylation, oxidation, Maillard reaction

Schlüsselwörter: Aminoguanidin, Ascorbat, Autooxidation, Biomarker, Bräunungsreaktion, chemische Veränderung von Proteinen, Diabetes, Glycosylierung, Glycoxidation, nichtenzymatische Glycosylierung, Oxidation, Maillardreaktion

Introduction

The human body may be viewed at one level as a low temperature oven with a relatively long, approximately 75 year cooking cycle. While metabolism is under stringent enzymatic control during this time, the body's proteins, nucleic acids, lipids and carbohydrates are constantly subject to random endogenous and environmental stresses. These stresses include non-enzymatic reactions such as spontaneous hydrolysis, oxidation reactions initiated by reactive species such as hydrogen peroxide, hypochlorous acid and superoxide or hydroxyl radicals, and other chemical modifications caused by adventitious interactions between biomolecules. Among the latter reactions are the carbonyl-amine reactions (1) characteristic of the Maillard reaction in food science. There are also inhibitory mechanisms for limiting the damage from these reactions in biological systems, and efficient mechanisms for either repairing damaged molecules or degrading and recycling their useful components. However, while most biological molecules may be repaired or replaced, there are some proteins in the body which have unusually long lifespans. These include the crystallins in the lens of the eye, myelin proteins of the nervous system, and collagens and elastin in the extracellular matrix of connective tissues throughout the body. These long-lived proteins are exposed to damaging chemical insults throughout their life, and undergo gradual physical and chemical changes with age. Thus, qualitative and quantitative analysis for modified amino acids in long-lived proteins can yield a unique insight into the rate, extent and types of damage to which proteins and other biomolecules are exposed in living systems, including information on the pathways and progress of the Maillard reaction *in vivo*.

This article will discuss the evidence for cumulative chemical modification and browning of human tissue proteins by glucose and the role of these chemical modifications in the development of pathophysiology in normal aging and the accelerated development of age-like complications in diabetes. The focus will be on the nature of permanent, glucose-induced chemical modifications of protein, with emphasis on the description of a group of compounds recently termed "glycoxidation" products (2), which are chemical modifications and crosslinks formed in proteins as a result of sequential glycative and oxidative modifications. These compounds (Figure 1) accumulate with age in tissue proteins and at an accelerated rate during hyperglycemia in diabetes.

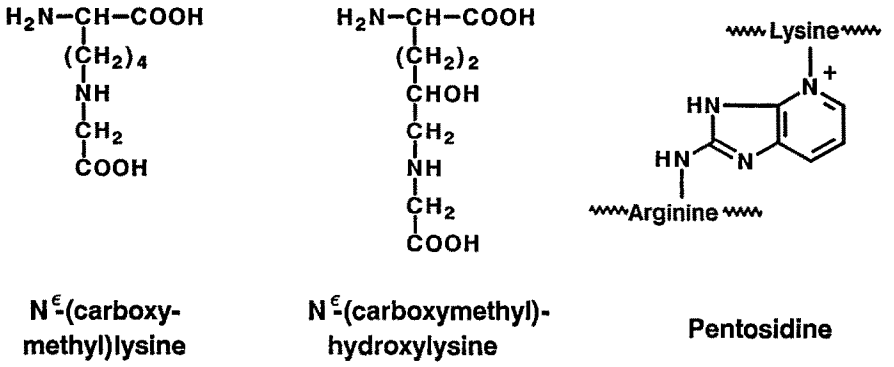

Fig. 1. Products of the Maillard reaction which have been shown to accumulate in tissue proteins. N^{ε}-(carboxymethyl)lysine is formed by oxidative cleavage of the Amadori compound, fructoselysine (FL), between C-2 and C-3 of the glucose chain. N^{ε}-(carboxymethyl)hydroxylysine is formed similarly by oxidation of glycated hydroxylysine in collagen. Oxygen is also involved in the formation of the lysine and arginine containing ribose- or glucose-derived crosslink, pentosidine, also known previously as Maillard Fluorescent Product #1 (MFP-1). The fluorescent compound, L_1, (not shown) is derived from reaction of protein with 3-deoxyglucosone and its relationship to pentosidine is unknown. 3-(N^{ε}-lysino)-lactic acid (not shown) is formed on oxidative cleavage of FL between C-3 and C-4 of the glucose chain. While it is formed in concert with CML, its accumulation in tissue proteins has not been confirmed.

Relationships between age, diabetes and glycation of protein

The first step in the Maillard reaction *in vivo* is termed non-enzymatic glycosylation or glycation of protein in order to distinguish it from enzymatic glycosylation which leads to the formation of glycosidic bonds and complex oligosaccharides. The Amadori adduct to amino groups in protein is the first stable product of glycation, the major product being the adduct of glucose to lysine residues, known as fructoselysine (FL). While at one time it was thought that this Amadori compound accumulated in proteins with age, recent work has shown that the extent of glycation of proteins is fairly constant with age in man (3, 4, 5). This is illustrated in Figure 2 which shows that the glycation of lens proteins is constant with age in the adult population, and that there is only a modest change in glycation of skin collagen with age. The lower extent of glycation of crystallins, compared to collagen, is consistent with the lower glucose concentration in the lens, compared to blood and extravascular fluids (6). These results indicate that there is a life-long, steady state relationship between the ambient glucose concentration and the extent of glycation of protein in the body.

Lens crystallins and skin collagen are long-lived, metabolically inert proteins, so that the kinetics of turnover of the proteins are not a significant factor in determining their extents of glycation. Thus, the constant glycation of these proteins with age must result either from a reversible equilibrium between rates of formation and dissociation of the Amadori adduct or from a forward reaction in which the Amadori adduct

rearranges, decomposes or reacts to regenerate lysine and other products. These pathways can be distinguished by the fact that the reverse reaction should lead to epimerization of glucose at C-2 and formation of both mannose and glucose. Studies with the model Amadori compound, N^{α}-formyl-N^{ε}-fructoselysine, have shown in fact that this compound decomposes in physiological buffers to form mannose, glucose and lysine in amounts consistent with reversal of the Amadori rearrangement (7). Several laboratories have also shown that the amount of Amadori adduct to a protein declines with time of incubation in glucose-free medium under physiological conditions *in vitro,* usually with a half-life of several days to more than a month, depending on the protein (reviewed in 8). However, the formation of mannose in these reactions has not yet been confirmed, so that reversibility of glycation of proteins either *in vitro* or *in vivo* has not been rigorously established.

Free lysine could also be produced from the Amadori compound through forward reactions, such as those leading to release of deoxyglucosones (DG) (9). While DGs are well-known intermediates in Maillard reactions *in vitro,* they have not been detected in biological systems. This is probably the result of their high reactivity as well as the presence of enzymatic pathways for their inactivation, e.g., oxidation to deoxygluconic acids or reduction to deoxy sugars. There is indirect evidence for formation of 3-deoxyglucosone (3-DG) *in vivo* since the fluorescent compound, L_1, which is a product of reaction of 3-DG with protein, has been identified in human tissue proteins (10); its concentration also increases with age and in diabetes in various tissues (10, 11). Kato and colleagues

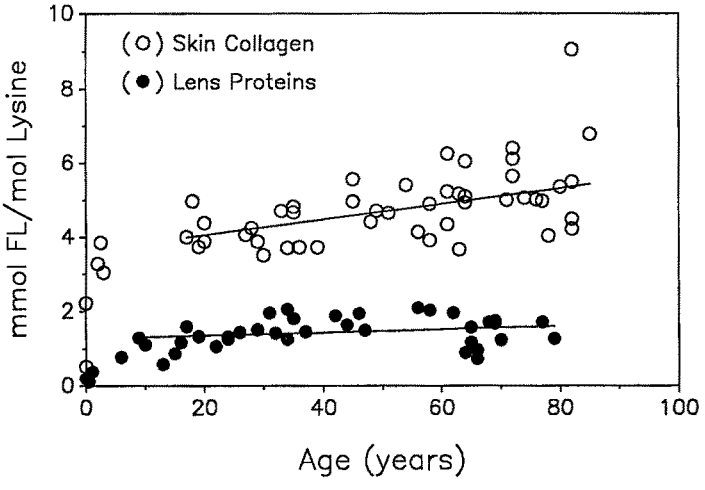

Fig. 2. Comparative extents of glycation of human lens proteins and skin collagen. Glycation of dialyzed human lens proteins and insoluble skin collagen was determined by measurement of furosine released on acid hydrolysis of the protein, using selected ion monitoring gas chromatography – mass spectrometry (3, 5). The regression lines are fitted to samples from donors of age ≥10. The correlation between glycation and age is insignificant for the lens samples ($p > 0.1$). A 33 % increase in glycation of skin collagen is observed between ages 20 and 80 ($p < 0.01$).

have also identified 3-deoxyfructose (3-DF) as a product of reduction of 3-DG by hepatic enzymes (12), and Knecht and Feather (13) have recently identified 3-DF in human urine. Thus, based on the identification of a reaction product and metabolite (L_1 and 3-DF, respectively), the formation of 3-DG is likely to occur in biological systems in much the same way that it is formed in food systems. There is still no evidence for the formation of 1- or 4-DG (9) *in vivo,* but it is likely that, as with 3-DG, there will be unique reaction products, as well as protective enzymatic pathways for limiting damage from reactions of these intermediates. In summary, the steady state glycation of proteins *in vivo* is probably a balance between reverse and forward reactions from the Amadori product, although neither of these reaction pathways have been adequately studied in biological systems.

The extent of glycation of collagen in skin and other tissues, such as aorta, kidney and dura matter, normally amounts to significantly less than 1 % of the lysine residues in the protein (Figure 2) (5, 8). During long-term hyperglycemia in diabetes, glycation may increase by 4-fold or more, to 2–3 % of the lysine residues in the protein. As a point of reference, this is comparable to the extent of modification of casein by lactose in heat-treated milk (14). Since there are ~100 moles of lysine (including hydroxylysine) per mole of triple stranded collagen ($M_r = 300$ kD) in skin, and lysine and hydroxylysine are glycated to similar extents (Dyer DG and Baynes JW, unpublished), there is a range of 0.5–2.5 moles of Amadori adduct per mole of collagen in non-diabetic and diabetic skin, respectively. In diabetic skin collagen this amounts to ~1 mole of Amadori adduct per collagen strand. The known reactivity of these glucose adducts in Maillard reactions *in vitro* provides a reasonable foundation for the hypothesis that the Maillard reaction is a source of the age-dependent increase in browning, fluorescence and crosslinking of collagen (15).

Comparative browning of protein by glucose *in vitro* and *in vivo*

One characteristic common to all long-lived proteins in the body is that they become less soluble, less elastic, less digestible by enzymes, and more crosslinked, brown and fluorescent with age (15). Figure 3 illustrates visually the gradual, age-dependent browning of human costal cartilage; similar age-related changes are observed in purified collagens extracted from other tissues of the body (15). The rate of browning of collagen is accelerated in diabetes (15) and the role of the Maillard reaction in this process is supported by the 3-D fluorescence spectra shown in Figure 4. Thus, the spectrum of insoluble skin collagen (from a 50-year-old diabetic donor) is remarkably similar to that of a protein, such as albumin, browned by incubation with glucose under physiological conditions of pH and temperature. When corrected for endogenous protein fluorescence (primarily tryptophan), similar spectra are obtained on incubation of a number of other proteins with glucose *in vitro.* Some differences should be observed between the spectra of browned proteins and collagen because of the presence of enzymatically-derived fluorescent crosslinks in collagen, but the similarities between the spectra in Figure 4 are impres-

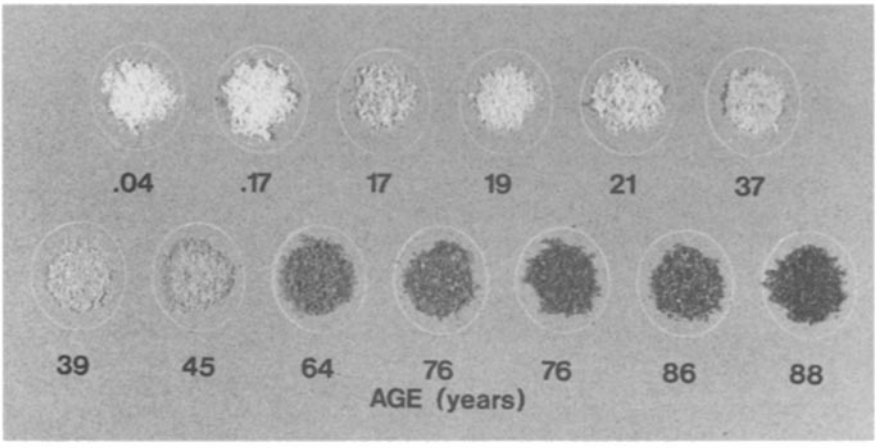

Fig. 3. Appearance of costal cartilage (~50 % collagen) isolated at autopsy from donors of various ages. The cartilage was isolated by dissection from the rib cage, cleaned of adventitious tissue, diced into ~1 mm cubes, washed with phosphate buffered saline, extracted by homogenization in chloroform:methanol (2:1), then dried under air.

sive and argue strongly that the Maillard reaction is relevant to the aging of tissue proteins *in vivo.*

Chemical evidence for advanced stages of the Maillard reaction *in vivo*

Several laboratories are studying the nature of the chemical modifications, fluorescent compounds and crosslinks formed in proteins incubated with glucose *in vitro* in order to develop assays for detecting and quantifying these compounds in proteins naturally aged *in vivo.* Our laboratory has described two products of oxidative cleavage of the Amadori adduct to lysine residues in protein, N^{ε}-(carboxymethyl)lysine (CML) and 3-(N^{ε}-lysino)lactic acid (LL) (Figure 1). These compounds were originally detected in proteins incubated with glucose under aerobic conditions *in vitro,* and were subsequently detected in human lens proteins, skin collagen and urine (7, 16). The related compound, N^{ε}-(carboxymethyl)hydroxylysine (CMhL), formed on oxidation of glycated hydroxylysine residues, has also been detected in skin collagen (5). CML, LL and CMhL are formed in metal catalyzed oxidation reactions (also termed "autoxidation" reactions because of the role of molecular oxygen as the oxidizing agent), and their formation from glycated proteins *in vitro* can be inhibited by strong iron or copper chelators such as diethylenetriaminepentaacetic acid and desferrioxamine (7, 16). Otherwise, CML accumulates in proteins during glycation under aerobic conditions *in vitro,* and as shown in Figure 5, CML also accumulates with age in human lens crystallins and skin collagen (3, 5). As in food science (14, 24) the concentration of CML in tissue proteins may be useful as an index of cumulative damage via the Maillard reaction. Thus, the higher concentration of CML in lens protein

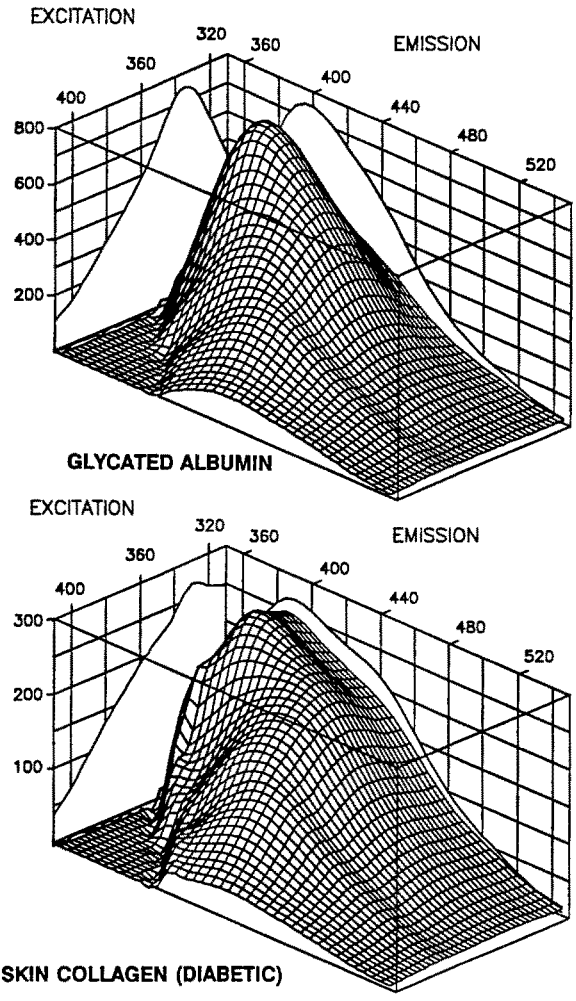

Fig. 4. Comparative three-dimensional fluorescence spectra of browned bovine serum albumin and human skin collagen. Fluorescence spectra were recorded at 10 nm excitation wavelength increments between 310 and 410 nm. Emission spectra were recorded between 350 and 550 nm, using a Shimadzu RF5000 Spectrofluorimeter interfaced to a laboratory computer. Data were collected using the program, Lotus Measure, and the three dimensional plots produced using Plot-It, Version 1.5 (Scientific Programming Enterprises, Haslett, Michigan). A. Bovine serum albumin (20 mg/ml) was incubated under air in 0.2 M phosphate buffer, pH 7.4, containing 250 mM glucose for 28 days at 37 °C. The incubation mixture was dialyzed against 0.5 M acetic acid adjusted to pH 2 with 6 M HCl, then diluted to 1 mg protein/ml solution in the same buffer. B. Human buttock skin collagen was obtained by surgery, cleaned of extraneous tissue, extracted sequentially with 1 M NaCl, chloroform:methanol (2:1) and 0.5 M acetic acid, then lyophilized (5). A sample (1 mg/ml) was solubilized by digestion with pepsin (4 %) in 0.5 M acetic acid. A small amount of insoluble debris (<2 % of the hydroxyproline content of the collagen) was removed by centrifugation and the sample adjust to pH 2 for recording the fluorescence spectra.

compared to skin collagen (Figure 5), despite the lower concentration of FL in the lens (Figure 2), suggests a higher level of oxidative stress in the lens, possibly the result of exposure to ultraviolet radiation. The concentration of CMhL in skin collagen is also strongly correlated with age and with the CML content of the collagen (5). Both CML and CMhL are increased in skin collagens from diabetic, compared to non-diabetic patients (Dunn JA and Baynes JW, unpublished), providing evidence for increased glycative and oxidative (glycoxidative) modification of protein in diabetes.

We have also observed recently that in addition to their formation by autoxidation of glycated proteins, both CML and LL are also formed on "autoxidative glycosylation" of protein by ascorbate (17). Autoxidative glycosylation is a term introduced by Wolff (18, 19) to describe the glycation of a protein by products of autoxidation of sugars. As originally described, the term referred to the spontaneous oxidation of glucose to a 1,2-dicarbonyl sugar (glucosulose), followed by its reaction with protein to form a ketimine adduct. Subsequent reactions, including further autoxidation of the ketimine and glycation of protein by sugar fragmentation products, were proposed as a route for the accelerated browning of proteins by glucose in diabetes (20). The biological relevance of this pathway of autoxidative glycosylation is uncertain (21, 22) because of the lack of unambiguous evidence for formation of the glucosulose and ketimine intermediates *in vivo*. The pathway for autoxidative glycosylation of protein by ascorbate has been worked out in more detail (Figure 6) (17), and involves the oxidation of ascorbate to dehydroascorbate, fragmentation of dehydroascorbate to form threose, glycation of protein by threose, and

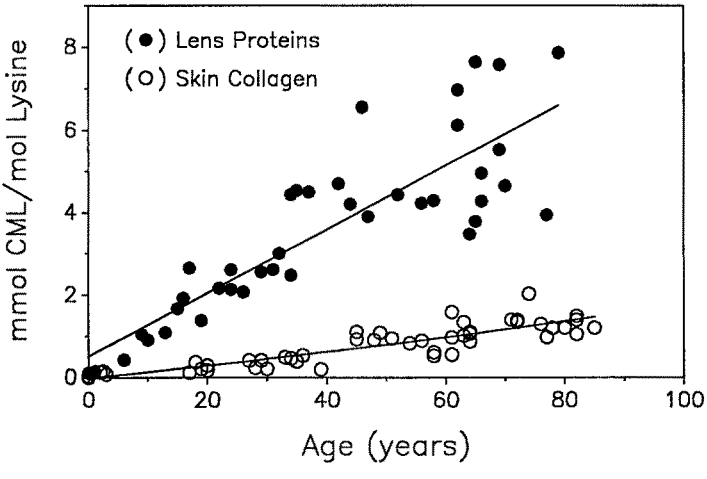

Fig. 5. Comparative levels of CML in human lens proteins and skin collagen. CML was measured, following acid hydrolysis of the protein, as the trifluoroacetyl methyl ester using selected ion monitoring gas chromatography – mass spectrometry (3, 5). For both lens protein and skin collagen there is about a 6-fold increase in CML concentration between ages 10 and 80.

then a second oxidative reaction in which threuloselysine is cleaved to yield CML or LL. It is now uncertain whether the CML in tissue proteins and urine is derived uniquely from oxidation of glucose- or also from ascorbate-derived adducts to proteins and possibly from other sources. Studies on human urine have shown that there is a significant correlation between the concentrations of FL and CML in diabetic urine (23), but that the absolute concentration of CML is only slightly increased in diabetic, compared to non-diabetic urine. These results are difficult to interpret with respect to the source of urinary CML since changes in blood glucose concentration in diabetes are also accompanied by changes in the kinetics of protein turnover, ascorbate and dehydroascorbate concentration, and the level of oxidative stress (17, reviewed in 2). The role of ascorbate as a source of CML *in vivo* is being tested separately by evaluating the effects of dietary ascorbate deficiency and supplementation on the level of CML in tissue proteins and urine. Importantly, although the source of CML, LL (and CMhL) in living systems remains uncertain, all of these compounds are products of glycoxidative damage to proteins via the Maillard reaction

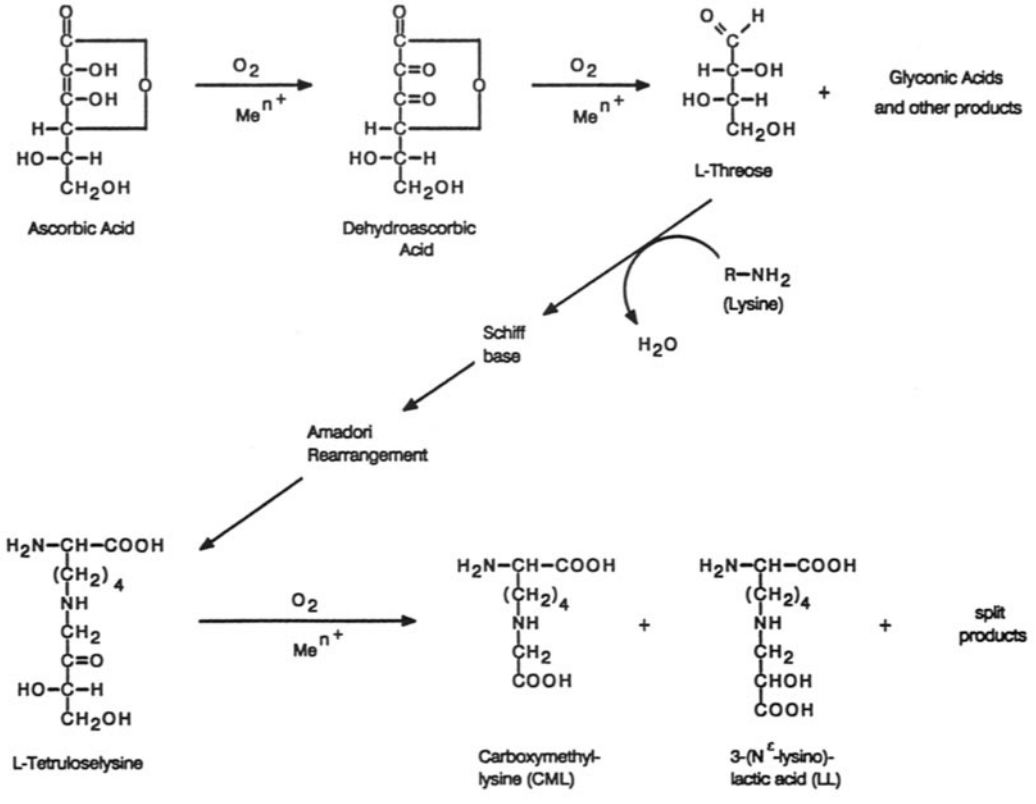

Fig. 6. Autoxidative glycosylation of protein by ascorbate, leading to the formation of CML in protein (17). The reaction pathway illustrates the glycosylation of protein by threose, a product of autoxidative degradation of ascorbate. A second, metal-catalyzed oxidation of threuloselysine leads to formation of CML.

and accumulate with age in tissue proteins and at an accelerated rate in diabetes. Both reducing sugars (glucose, lactose) and ascorbate may also serve as a source of CML in food products. Dr. Helmut Erbersdobler (at the Christian-Albrechts-Universität zu Kiel) has suggested (personal communication) that ascorbate may be the primary source of CML in foods fortified with ascorbate as an antioxidant. Thus, in some foods the concentration of CML may exceed that of Amadori adducts (24).

Maillard reaction crosslinks in tissue proteins

Sell and Monnier (25, 26) identified the fluorescent Maillard product, pentosidine (Figure 1), in human tissue proteins. Pentosidine is crosslink formed between lysine and arginine residues in protein, and is also a glycoxidation product, based on its structure and synthetic studies. Synthetic pentosidine was prepared initially by reaction of ribose with lysine and arginine (25), suggesting a role for ribose in the chemical modification of proteins, particularly in diabetes with complicating renal disease (26). Baynes and colleagues (27, 28) independently identified Maillard Fluorescent Product #1 (MFP-1) which was eventually shown to have spectrometric, fluorimetric and chromatographic properties similar to those of pentosidine, but was prepared synthetically by reaction of glucose, rather than ribose, with lysine and arginine or with intact proteins. While MFP was originally thought to be a glucose-derived crosslink related to pentosidine, recent analyses by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and liquid chromatography – mass spectrometry (Dyer DG and Baynes JW, in preparation) indicate that pentosidine and MFP-1 are identical compounds. Thus, since pentosidine contains only 5 carbons from the sugar component (Figure 1), it formation from glucose must involve the loss of a carbon atom either from glucose itself or from a later intermediate in the reaction. Further reference to MFP-1 will be dropped in deference to the name, pentosidine, introduced by Sell and Monnier (25).

At this point, while its origin from ribose vs. glucose and other sugars may be uncertain, there is agreement that pentosidine is a prominent carbohydrate-derived fluorescent crosslink in proteins and that it increases in lens protein and collagens with age and at an accelerated rate in diabetes. The only other fluorescent compound known to increase in tissue proteins with age is the fluorescent compound, L_1 (10, 11), formed by reaction of 3-DG with protein, but its structure is unknown and the requirement for oxygen (or oxidation) in its formation has not been reported. While pentosidine and L_1 are prominent age- and diabetes-related fluorescent products in proteins, they are probably present in proteins at only trace levels. Thus, pentosidine is present in collagen at ≤ 1 mole per 100 moles of triple-stranded protein. Its concentration in lens protein is even lower, closer to 1 mole per 1000 moles of lens crystallin. It is unknown whether pentosidine is present in food proteins, although it is formed spontaneously in aerobic incubations of protein with ribose or glucose *in vitro*.

Because of its trace concentration in proteins, it is difficult to argue convincingly that pentosidine crosslinks are significant in the develop-

ment of pathology in either aging or diabetes. However, studies on the development of fluorescence and crosslinking of proteins *in vitro* provide an interesting insight into what may be occurring *in vivo*. Thus, protein dimer formed during prolonged incubation of lysozyme with glucose (Figure 7A) must contain at least one mole of covalent crosslink per mole

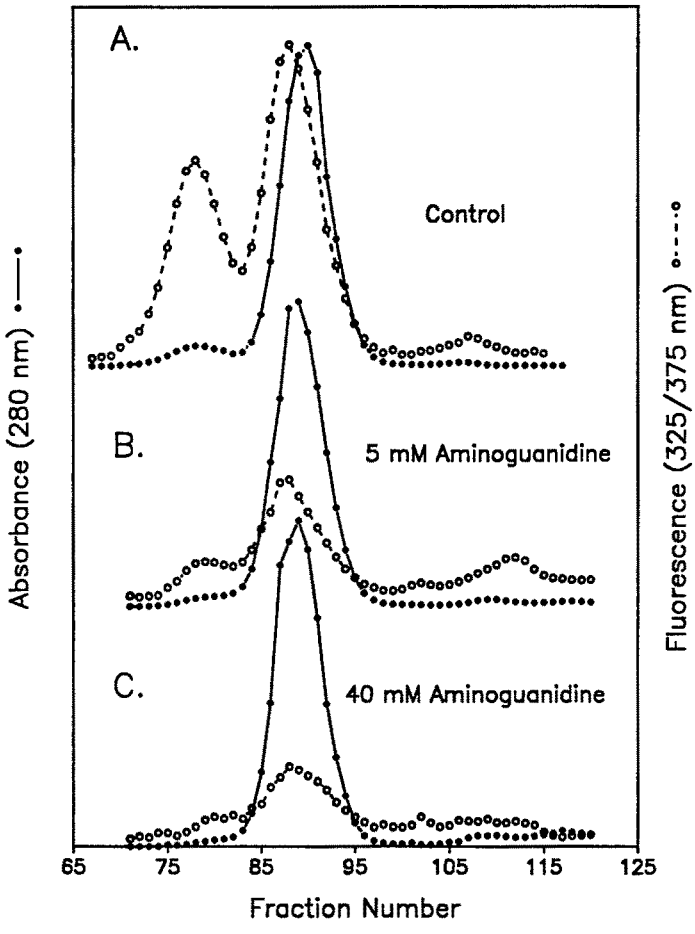

Fig. 7. Formation of fluorescent products and crosslinks in protein by glucose and inhibition by aminoguanidine.
(A) Hen egg white lysozyme (20 mg/ml) was incubated under air in 0.2 M phosphate buffer, pH 7.4, containing 250 mM glucose for 28 days at 37 °C. Following dialysis, an aliquot of the incubation mixture was chromatographed on a 1 × 150 cm column of Sephadex G-75 superfine; individual fractions were collected for measurement of absorbance and fluorescence. There was negligible fluorescence or dimerization of lysozyme in an incubation without glucose (data not shown). The lysozyme dimer peak accounted for ~7 % of the total protein in the control incubation in the presence of glucose.
(B, and C) Chromatography of an incubation mixture identical to that in A, except for the presence of 5 or 40 mM aminoguanidine, respectively.

of dimerized protein. Compared to monomer, the dimer is typically 5-10-fold enriched in fluorescence at the excitation and emission maxima of pentosidine (Ex: ~325 nm; Em: ~375 nm), suggesting that pentosidine is involved in the intermolecular crosslinking of the protein. The lower level of fluorescence in the mononer fraction probably results from limited intra-molecular crosslinking of the protein. Since pentosidine has been synthesized from radioactive sugars and amino acids of known specific radioactivity, a conversion factor has been obtained for relating fluorescence to mass, so that the concentration of pentosidine in protein can be measured accurately. Surprisingly, HPLC analyses reveal that there is only about 0.001 mole of pentosidine per mole lysozyme dimer. However, since there must, by definition, be at least one mole of crosslink per mole of dimer, there must be an additional 1000 currently unidentified crosslinks in the dimerized protein for each mole of pentosidine which can be detected in the dimer fraction. This is a potentially significant observation since, if it also applies to the measured concentration of pentosidine in collagen or lens proteins, then there may be a significant level of unknown, colorless and non-fluorescent crosslinks in browned proteins *in vivo,* as *in vitro.* Under these circumstances the age- and diabetes-dependent increase in pentosidine and total fluorescence in collagen should be taken as a *biomarker,* i.e., the tip of an iceberg, indicative of more extensive, underlying damage to the protein via the Maillard reaction. By analogy, in the food sciences, trace levels of volatile, undesirable flavor components may be used as indicators of extensive heat damage to food products. From this perspective glycoxidation products may be equally useful as reporter groups for assessing the extent of chemical damage to proteins via the Maillard reaction *in vivo.*

Repair of damage from the Maillard reaction *in vivo*

Vlassara and colleagues (29, 30) have described a high-affinity receptor on tissue macrophages and blood monocytes which recognizes extensively browned proteins. This receptor is termed the AGE receptor, the acronym/pun, AGE, standing for **A**dvanced **G**lycosylation **E**ndproduct or late-stage Maillard reaction products in protein. The AGE receptor is proposed to have a role in the turnover of browned proteins, even those with long biological lifespans. Thus, while proteins such as collagen in the extracellular matrix and myelin basic protein in tissues of the nervous system are normally considered to be long-lived proteins, their catabolism may be accelerated by wounding. By analogy, the extensive browning, or "molecular wounding" of proteins via the Maillard reaction may induce a localized inflammatory response involving recognition of the browned protein via the AGE receptor, followed by endocytosis and degradation of the protein. The ingestion of AGEd proteins by macrophages is also accompanied by the secretion of growth-promoting cytokines which induce the synthesis of collagens and the remodeling of tissue. Thus, the metabolic removal of browned protein is coupled to the restructuring of the tissue. A similar receptor has also been described on endothelial cells (31). While the AGE receptor on macrophages may have a beneficial function in the remodeling of AGEd tissues, the binding of AGEd proteins

to the endothelial receptor causes a potentially dangerous increases in the permeability and pro-coagulant activity of the vascular wall. Thus, increased interactions of AGEd proteins with endothelial cells may play a role in the development of vascular pathology in diabetes. Thus far, the characterization of the AGE receptor has relied largely on the use of proteins extensively browned by prolonged incubation with high concentrations of glucose *in vitro*. Because of the heterogeneity in chemical modification of these proteins, the identity of the specific ligands recognized by the AGE receptor is unknown. The quantitative role of the AGE receptor in limiting damage from the Maillard reaction *in vivo* and accelerating vascular changes in diabetes is also unknown.

Therapeutic inhibition of the Maillard reaction *in vivo*

In the food sciences there are numerous approaches to limiting browning reactions. These include control of pH, moisture content, temperature and oxygen tension, chelation of redox metal ions, and the use of additives such as bisulfite (32, 33). The fact that all of these approaches would have toxic or lethal consequences *in vivo* has necessitated the development of alternative approaches for controlling the Maillard reaction in biological systems. In effect, this has meant limiting the progress of secondary and tertiary stages of the reaction, i.e., those occurring after formation of the Amadori compound. Thus, Brownlee et al. (34) have introduced the use of the compound, aminoguanidine (AG), as an inhibitor of browning stages of the Maillard reaction *in vivo*. Although clinical trials are under way, the published information on biological applications of AG is limited to experiments in animal models of diabetes, primarily rodents. These studies show that, while AG had no effect on the increase in glycation of aortic collagen in diabetic rats, it does inhibit the accelerated development of fluorescence and crosslinking of the collagen (34). Similar results have been obtained in studies on development of fluorescence in tail collagen of diabetic rats (Baynes JW, unpublished). Thus far, experiments with AG in animal models of diabetes have been limited to less than six-months' duration, so that long-term toxicity has not been excluded.

Studies with model proteins have revealed that AG also inhibits the development of fluorescence and intermolecular crosslinking of lysozyme during incubation with glucose *in vitro* (Figure 7B and C), concomitant with inhibition of formation of pentosidine (data not shown). While its mechanism of action is still unknown, AG does not appear to have significant activity as an antioxidant or chelator since it does not inhibit the metal catalyzed oxidative degradation of FL to CML or the production of superoxide on autoxidation of FL (Richardson JM and Baynes JW, unpublished). It is likely that AG acts by trapping dicarbonyl intermediates, such as glucosones, deoxyglucosones or sugar fragmentation products, which are the probable precursors of fluorescent and crosslinking compounds, particularly those involving arginine residues. There is indeed a long history of use of dicarbonyl compounds, such as phenylglyoxal and butanedione, to titrate or chemically modify the guanidino group of arginine residues in proteins (35). Preliminary studies in our laboratory have shown that semicarbazide and *ortho*- aromatic diamines are also

effective inhibitors of crosslinking of proteins by glucose (Katz BM and Baynes JW, unpublished). The development of new drugs and therapeutic approaches for limiting the browning stages of the Maillard reaction *in vivo* is certain to be a major direction for future research in aging and diabetes.

Role of oxygen as fixative of chemical damage in the Maillard reaction

With the possible exception of L_1, all of the Maillard products which have been shown to accumulate in protein thus far, including CML (3, 5), CMhL (5) and pentosidine (25–28), are products of oxidation reactions. We have termed these compounds "glycoxidation products" (2) because they are formed by sequential glycative and oxidative modification of proteins, and suggest that oxygen may be considered as a "fixative" of chemical damage to protein via the Maillard reaction *in vivo*. The chemical basis for this argument derives from the strong oxidation potential of molecular oxygen and reactive species such as the hydroxyl radical. Thus, like the rusting of iron, the oxidation of Maillard products is an essentially irreversible process. This is especially true when the oxidation reaction causes chemical cleavage of a carbon chain, for example, in the formation of CML, LL or CMhL.

In food chemistry most of the well characterized products of the Maillard reaction are volatile or easily volatilized compounds, and their characterization has been facilitated by the relative ease with which they can be analyzed by high resolution gas chromatographic and mass spectrometric techniques. While the nonvolatile browning products, known as pre-melanoidins and melanoidins are clearly visible and account for a greater fraction of the total mass of the browned food, these compounds are poorly characterized because of the difficulty in their isolation and analysis. Similarly, it may be fortuitous that the first Maillard products known to accumulate in tissue proteins are primarily, if not exclusively, glycoxidation products (Figure 1). Perhaps the ready detection of glycoxidation products may also result from their low molecular weight, stability to conditions of acid hydrolysis used for analysis of proteins, and the ease with which they may be detected and characterized by gas or liquid chromatography and mass spectrometry. Pentosidine is also readily detected because of its intense fluorescence. Although it may account for only a small fraction of the total crosslinks in proteins, its presence implies the existence of other compounds which are precursors to the crosslink. Indeed, continued incubation of glycated proteins in the absence of glucose leads to a further increase in both fluorescence and crosslinking of the proteins (36, 37). Browning and crosslinking of protein by reducing sugars also occurs in the absence of oxygen, and short-chain fragments, such as glyceraldehyde and glycolaldehyde, formed on cleavage of sugars by reverse aldol reactions, are potent mediators of browning and crosslinking reactions under anaerobic conditions. Thus, in addition to biomarkers such as glycoxidation products, there are undoubtedly a range of other crosslinks and chemical modifications of protein resulting from the Maillard reaction which remain to be detected and quantified in tissue proteins. Among these may be conjugated Schiff bases, iminopropenes

(38) and heterocyclic derivatives which would be unstable to acid hydrolysis.

Summary and conclusion

There is increasing evidence for accumulation of a variety of Maillard reaction products in long-lived tissue proteins with age, and thus that the Maillard reaction is one aspect of the non-enzymatic, chemical aging of proteins in the body. The accelerated accumulation of Maillard products in diabetes is also consistent with the development of age-like complications in this disease. Most, if not all, of the Maillard compounds presently known to accumulate in proteins with age belong to a class of molecules termed glycoxidation products, i.e., products of sequential glycation and oxidation reactions. These compounds account for only trace modification of proteins in the body, but there is indirect evidence for more extensive modification and crosslinking of protein by other, as yet unidentified compounds. Research on the Maillard reaction in biological systems will continue to focus on the identification of new reaction products in tissue proteins, development of assays for their quantitative analysis, evaluation of their quantitative role in structural and functional alterations of tissue proteins in aging and diabetes, and design and application of pharmaceutical agents for limiting the natural course of the reaction. Unique aspects of the research in biological systems will include the characterization of metabolic pathways for detoxifying reactive intermediates in the Maillard reaction and for removal of end-products, once they are formed. In addition, studies on the interplay between glycation and oxidation of proteins may clarify the importance of these chemical reactions in the aging of tissue proteins. The broad base of information available on the Maillard reaction in food science and technology provides a strong foundation for the work to be done in biological systems, as indeed recent progress on the Maillard reaction in biological systems has stimulated new approaches to the chemical analysis and nutritional evaluation of food products.

Abbreviations used

AG, aminoguanidine; AGE, Advanced Glycosylation End-product; CMhL, N^{ε}-(carboxymethyl)-hydroxylysine; CML, N^{ε}-(carboxymethyl)-lysine; 3-DG, 3-deoxyglucosone; 3-DF, 3-deoxyfructose; FL, N^{ε}-fructoselysine; L_1, fluorescent Maillard product identified by Kato and colleagues (10); LL, 3-(N^{ε}-lysino)-lactic acid; MFP-1; Maillard fluorescent product #1 (12, 28).

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