

# Protein Glycation, Diabetes, and Aging

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## ABSTRACT

Biological amines react with reducing sugars to form a complex family of rearranged and dehydrated covalent adducts that are often yellow-brown and/or fluorescent and include many cross-linked structures. Food chemists have long studied this process as a source of flavor, color, and texture changes in cooked, processed, and stored foods. During the 1970s and 1980s, it was realized that this process, called the Maillard reaction or advanced glycation, also occurs slowly *in vivo*. Advanced glycation endproducts (AGEs) that form are implicated, causing the complications of diabetes and aging, primarily via adventitious and crosslinking of proteins. Long-lived proteins such as structural collagen and lens crystallins particularly are implicated as pathogenic targets of AGE processes. AGE formation in vascular wall collagen appears to be an especially deleterious event, causing crosslinking of collagen molecules to each other and to circulating proteins. This leads to plaque formation, basement membrane thickening, and loss of vascular elasticity. The chemistry of these later-stage, glycation-derived crosslinks is still incompletely understood but, based on the hypothesis that AGE formation involves reactive carbonyl groups, the authors introduced the carbonyl reagent aminoguanidine hydrochloride as an inhibitor of AGE formation *in vivo* in the mid 1980s. Subsequent studies by many researchers have shown the effectiveness of aminoguanidine in slowing or preventing a wide range of complications of diabetes and aging in animals and, recently, in humans. Since, the authors have developed a new class of agents, exemplified by 4,5-dimethyl-3-phenacylthiazolium chloride (DPTC), which can chemically break already-formed AGE protein-protein crosslinks. These agents are based on a new theory of AGE crosslinking that postulates that  $\alpha$ -dicarbonyl structures are present in AGE protein-protein crosslinks. In studies in aged animals, DPTC has been shown to be capable of reverting indices of vascular compliance to levels seen in younger animals. Human clinical trials are underway.

## I. Introduction

The nonenzymatic reaction of the amino groups of amino acids, peptides, and proteins with reducing sugars, ultimately resulting in the formation of complex brown pigments and protein-protein crosslinks, was first studied under defined conditions by L.C. Maillard in the early 1900s (Maillard and Gautier, 1912). Thus, it came to be known as the Maillard reaction. Ironically, Maillard's speculation that this process might be important in diabetes was an insight too far ahead of the technology capable of demonstrating it. Food chemists soon recognized the

practical importance of the Maillard reaction in explaining brown color formation and loss of protein quality in cooked and stored foods. However, realization of the importance of Maillard-like reactions *in vivo* began in the mid-1970s. This started with our studies of hemoglobin A1c (HbA1c), a naturally occurring minor human hemoglobin that is elevated in diabetics. HbA1c was known to be a post-translational adduct of glucose with the N-terminal valine amino group of the  $\beta$  chain of hemoglobin, in which the glucose was thought to be attached via a nonenzymatically formed Schiff base structure. We found that measurement of the elevation of HbA1c in diabetics allowed assessment of the degree of glucose control integrated over several weeks (Koenig *et al.*, 1976). However, the idea that HbA1c was a Schiff base was troubling, since Schiff bases are generally hydrolytically unstable. Subsequent work (Koenig *et al.*, 1977) revealed that the carbohydrate in HbA1c was attached as a 1-deoxy-1-fructosyl residue to the N-terminal valine nitrogen, derived from an initially formed Schiff base via a chemical transformation known as the Amadori rearrangement (Figure 1) (Hodge, 1955).

It was not until around 1980 that researchers began recognizing the significance of the complex, late-stage Maillard processes as mediators of the complications of diabetes (Bunn *et al.*, 1978) and aging (Monnier and Cerami, 1981). Subsequently, proteins bearing Amadori product have come to be referred to as glycated proteins (distinguishing them from enzymatically glycosylated proteins), while the process of Amadori product formation is termed glycation. The later-stage, complex pigments and crosslinks formed from glycated protein during the *in vivo* Maillard reaction have come to be known as advanced glycation end-products, or AGEs (Bucala *et al.*, 1992). Throughout the 1980s and 1990s, a large body of evidence has accumulated implicating AGEs as mediators of various complications of diabetes and aging. Pharmacological agents have been developed and placed in clinical trials to combat AGE processes in man. This chapter will summarize developments in this field and consider its future direction.

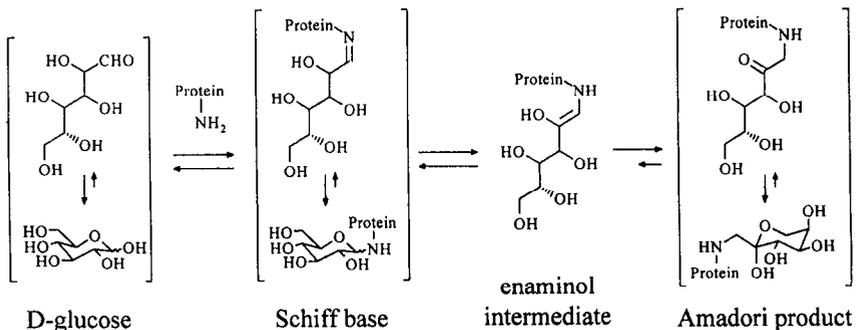


FIG. 1. Formation of the glucose-protein Schiff base and the Amadori rearrangement.

## II. Chemical Background of Glycation and AGE Formation Under Physiological Conditions

### A. THE MAILLARD REACTION

Reducing sugars (e.g., glucose, fructose, galactose, mannose, ribose) and certain other carbohydrate relatives (e.g., ascorbic acid) are inherently reactive toward nucleophiles. Their reactivity toward nucleophilic nitrogen bases in protein underlies the Maillard reaction. Glucose is, in fact, the least reactive of the common sugars, perhaps leading to its evolutionary selection as the principal free sugar *in vivo* (Bunn *et al.*, 1978). Nevertheless, glucose can react with a free amino group, such as the  $\epsilon$ -amino group of a protein lysine residue, to form an adduct commonly referred to as a Schiff base. In a Schiff base, the aldehydic carbon-oxygen double bond of the sugar is converted to a carbon-nitrogen double bond with the amine (Figure 1). As is the case with free sugars, the open-chain, double-bonded form of the Schiff base adducts of hexoses or pentoses are thermodynamically disfavored with respect to the equilibrium cyclized pyranose or furanose forms, or glycosylamines (Paulsen and Pflughaupt, 1980) (Figure 1). To simplify mechanistic discussions of subsequent reactions, the adducts are frequently depicted as the more-reactive, open-chain forms. This convention will generally be followed here.

In the 1920s, Amadori showed that glucose-aniline Schiff bases could be converted to isomeric products that were not glycosylamine anomers (Amadori, 1925) but were subsequently found to be 1-anilino-1-deoxyfructose derivatives. It was not until the 1950s that it was realized that Amadori products can form from aliphatic amines such as amino acids, not just from aromatic amines, and that the Amadori rearrangement was a key early step in the Maillard reaction (Hodge, 1955). The Amadori rearrangement of the Schiff base to the Amadori product is believed to occur via an intermediate, open-chain enol form (Figure 1).

Formation of the Schiff base from sugar and amine is relatively fast and highly reversible. Formation of Amadori product from the Schiff base is slower but much faster than the reverse reaction, so that the Amadori glycation product tends to accumulate on proteins. The Amadori rearrangement of a lysine-glucose Schiff base is thought to be facilitated if there is a histidine side-chain or another lysine amino group about 5 Å from the amino group on which the Schiff base has formed, due to localized acid-base catalysis (Acosta *et al.*, 2000). It is subsequent to the Amadori rearrangement that the less-well-understood, late-stage, advanced glycation processes begin. Despite considerable research by many laboratories, these slow, *in vivo*, AGE-forming processes are incompletely understood. It is known that the products include protein-protein crosslinking structures as well as various noncrosslinked structures. Many, but not all, of these products incorporate ultraviolet-visible and/or fluorescent chromophores.

In recent years, it has become increasingly clear that  $\alpha$ -dicarbonyl compounds are key intermediates in the formation of AGEs. The Amadori product can break down via its enol form to reactive, free  $\alpha$ -dicarbonyl glyoxal compounds such as 3-deoxyglucosone, methylglyoxal, and glyoxal (Figure 2) (Thornalley *et al.*, 1999, and references therein). These compounds are known to crosslink proteins and have been detected *in vivo*. The Amadori product also can dehydrate at the 4-position to yield a 1-amino-4-deoxy-2,3-dione (Amadori dione) (Figure 3) (Huber and Ledl, 1990; Chen and Cerami, 1993). This substance can further dehydrate at the 5-position to yield an unsaturated dione, the Amadori ene-dione (Figure 3) (Estendorfer *et al.*, 1990). Evidence for crosslinking of proteins by the Amadori ene-dione, as depicted in Figure 3, will be discussed.

## B. FLUORESCENT AGE CROSSLINKS

Reactions of  $\alpha$ -dicarbonyl compounds with basic amino acid residues or with Amadori intermediates are thought to be involved in formation of many of the known or suspected AGE crosslinking structures shown in Figure 4. Protein-protein crosslinks by such structures *in vivo* are thought to be responsible for a major share of the deleterious effects of AGEs in diabetes and aging. Along with brown color, fluorescence is one of the qualitative properties classically used to estimate

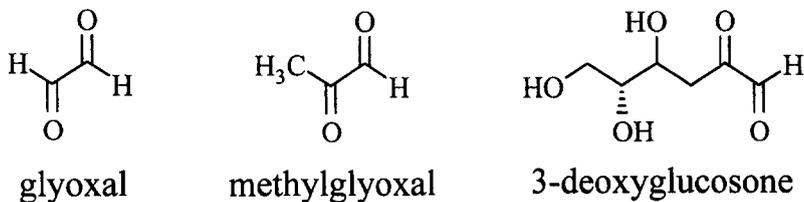


FIG. 2. Some  $\alpha$ -dicarbonyl glyoxal derivatives formed during glycation.

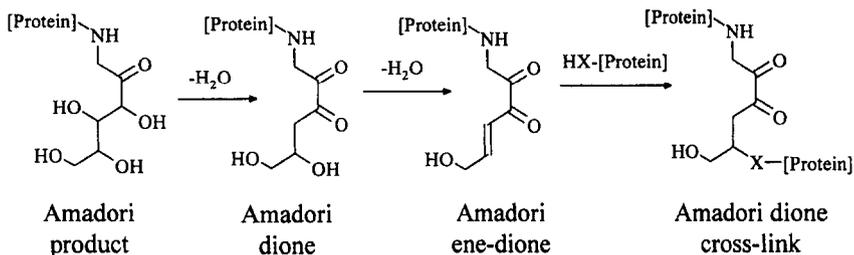


FIG. 3. Dehydration of the Amadori product to form Amadori dione and Amadori ene-dione and conjugate addition of a protein nucleophile to form a protein-protein crosslink.

AGE formation. Many of the AGEs in Figure 4 were detected and isolated based on their fluorescent properties. The fluorescent AGE crosslink pentosidine was first isolated and identified from dura mater collagen and has since been identified in many tissues (Sell *et al.*, 1991). Pentosidine is unusual in that it can be formed by the reaction of lysine and arginine with any of several carbohydrate precursors, including glucose, ribose, ascorbic acid, and 3-deoxyglucosone. Workers in a number of laboratories have isolated a variety of other fluorescent crosslinks in model reactions under physiological conditions: crosslines A and B, vesperlysines A, B, and C; AGE-X1; pentodilysine; and pyrropropidine or FPPC. Of these, the crosslines (Obayashi *et al.*, 1996) and vesperlysines (Tessier *et al.*, 1999) have been detected *in vivo*.

### C. NONFLUORESCENT AGE CROSSLINKS

Although their ease of detection makes them useful markers of AGE formation, the fluorescent AGE crosslinks are thought to account for only one percent or less of the total crosslinking structures formed under physiological conditions (Dyer *et al.*, 1991). Thus, the major AGE structures responsible for protein-protein crosslinking *in vivo* are nonfluorescent structures that have not yet been conclusively identified. Recently, criteria other than fluorescence have been utilized to find new, nonfluorescent crosslinking AGE candidates that might represent the major *in vivo* AGE crosslink(s). These are depicted in Figure 5 and will be discussed individually in the following paragraphs.

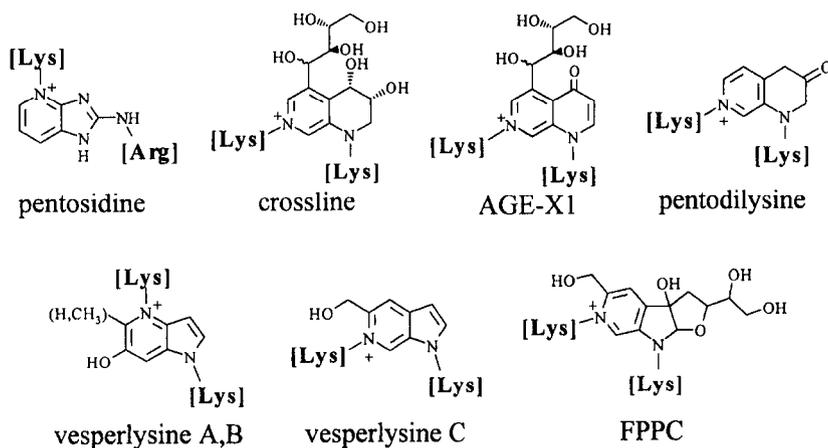


FIG. 4. Fluorescent AGE crosslinks formed under physiological conditions. [Lys] represents an  $\epsilon$ -norleucyl group (a desamino-lysine residue); [Arg] represents a  $\delta$ -norvalyl group (a desguanidino-arginine residue).

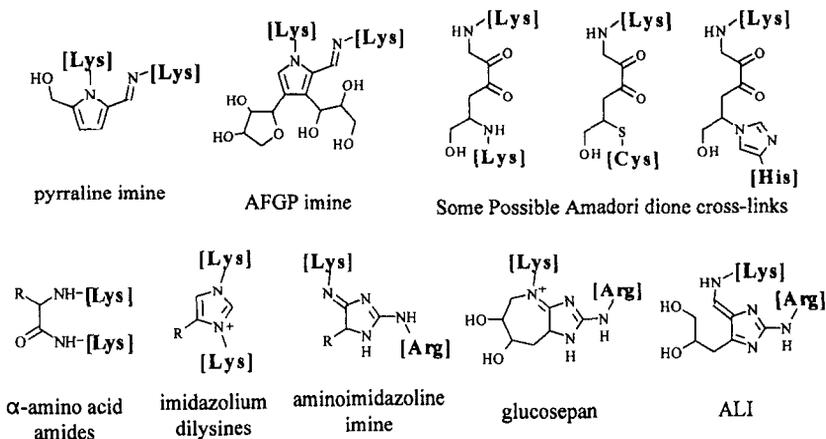


FIG. 5. Known and suspected nonfluorescent AGE crosslinks of physiological relevance. [Lys] represents an  $\epsilon$ -norleucyl group (a desamino-lysine residue); [Arg] represents a  $\delta$ -norvalyl group (a desguanidino-arginine residue). [Cys] and [His] represent  $\beta$ -substituted alanine portions of cysteine and histidine residues, respectively.

### 1. Imidazolium Dilyssine Crosslinks

Nonfluorescent imidazolium dilyssine crosslinks, also known as GOLD/MOLD crosslinks, have been isolated from reaction of two molecules of glyoxal derivative with two lysine residues *in vitro* and also have been detected *in vivo* (Brinkmann Frye *et al.*, 1998, and references therein). A related 2-acylimidazolium dilyssine crosslink was proposed some years ago by our group, based on the isolation of presumed breakdown product 4-furanyl-2-furoylimidazole (FFI) from acid hydrolysis of AGE-modified bovine serum albumin (Pongor *et al.*, 1984). Formation of FFI under these conditions is now thought to occur during isolation (Njoroge *et al.*, 1988). However, AGEs of unknown structure crossreactive with FFI are detectable in AGE proteins *in vitro* and *in vivo* (Chang *et al.*, 1985; Nakayama *et al.*, 1988; Palinski *et al.*, 1995). Such 2-acylimidazolium compounds are now known to be unstable toward hydrolytic loss of the acyl group, yielding 2-unsubstituted imidazolium derivatives, and thus are probable precursors of the imidazolium dilyssine crosslinks (Al-Abed *et al.*, 1996). The requirement of two moles of a glyoxal derivative for formation of a single imidazolium crosslink represents a mechanistic limitation on the role of imidazolium dilyssine crosslinks *in vivo*. Moreover, formation of an imidazolium dilyssine crosslink requires intermediacy of a double Schiff base of a glyoxal derivative with two lysine residues, which may alternatively rearrange to an  $\alpha$ -amino amide crosslink in a competing reaction (see below). Nevertheless, the imidazole dilyss-

ines are reported to be present at levels 10- to 50-fold higher than the fluorescent crosslink pentosidine in tissue proteins (Degenhardt *et al.*, 1998).

## 2. Alkyl Formyl Glycosyl Pyrrole (AFGP) Crosslinks

With James Farmar, we isolated AFGPs (alkyl formyl glycosyl pyrroles) as noncrosslinking AGEs that form in the reaction of two sugar molecules with one alkylamine molecule representing a lysine residue (Farmar *et al.*, 1988). The  $\alpha$  positions of the side-chains attached to pyrrole ring carbons in AFGPs are susceptible to nucleophilic attack by thiols (Klein *et al.*, 1992) and by lysine amino groups (J. Farmar and A. Cerami, unpublished data) (Figure 5), indicating that AFGPs are likely to be able to crosslink proteins. The requirement of two moles of sugar to form the AFGP structure and the fact that AFGPs are minor products in the absence of sulfite as a trapping agent (Farmar *et al.*, 1988) suggest that AFGP crosslinks may be of limited importance *in vivo*.

## 3. Pyrraline Crosslinks

*N*-Alkyl-5-hydroxymethyl-2-pyrrolaldehyde, or pyrraline, is a monomeric AGE that may form on lysine residues *in vivo* (see below). The aldehyde of pyrraline can form a Schiff base with another amino group (Nissl *et al.*, 1995) (Figure 5), which might lead to lysine-lysine crosslinking *in vivo*. However, this has not been demonstrated. Such crosslinks might be of limited stability with respect to hydrolytic reversion to their precursors.

## 4. $\alpha$ -Amino Acid Amide Crosslinks

Sugars and Amadori compounds have been shown to react with primary amines to form *N,N'*-dialkyl-alanine or -glycine amides (Figure 5). These represent a mode of lysine-lysine crosslinking that may occur *in vivo*; glyoxal derivatives from Amadori product breakdown are probable intermediates in their formation (Büttner *et al.*, 1997). Such  $\alpha$ -amino amides may be difficult to isolate from proteins because they lack distinctive chromophores and are likely to be cleaved under protein hydrolysis conditions to give products (carboxymethyllysine and carboxyethyllysine) that are already known to form via other AGE-related processes *in vivo* (see below). Nevertheless,  $\alpha$ -amino acid amides are interesting contenders for a significant role in protein crosslinking *in vivo*.

## 5. Aminoimidazoline Imine Crosslinks

A new class of aminoimidazoline imine AGE protein crosslinks (Figure 5) has recently been reported. These are thought to be derived from reaction of arginine and an  $\alpha$ -oxoaldimine Schiff base of a lysine residue and glyoxal or methylglyoxal or 3-deoxyglucosone (Lederer and Klaiiber, 1999, and references

therein). The latter product can cyclize to a bicyclic crosslink the authors named glucosepan (Figure 5). These nonfluorescent crosslinks are potentially significant players in AGE crosslinking *in vivo*, as they require only one mole of carbohydrate-derived dicarbonyl component for their formation and they form relatively efficiently in model systems.

#### 6. Arginine-Lysine-Imidazole (ALI) Crosslink

This recently described, nonfluorescent crosslink (Al-Abed and Bucala, 2000) (Figure 5) presumably derives from reaction of the Amadori dione (Figure 3) with an arginine residue. The ALI structure was isolated in a model system employing a model dipeptide (Arg-Lys), so that formation of the crosslink results in closure of a macrocyclic ring. ALI has not been reported as an intermolecular crosslink. It contains an enamine-like attachment that might be hydrolyzable. On the other hand, the authors showed that ALI has a potentially close immunochemical relationship with AGE structures on glucose-modified bovine serum albumin using an anti-AGE antibody that has been well characterized in prior studies in our laboratory (Makita *et al.*, 1992). Thus, it is possible that ALI may be more important as an entropically stabilized, *intramolecular* protein crosslink than as an intermolecular crosslink; this question awaits further research.

#### 7. Amadori Dione Crosslink

Conjugate addition of a nucleophilic protein side-chain to a protein-bound Amadori ene-dione would result in a protein-protein crosslink containing an  $\alpha$ -diketone structure in the linker (Figure 3). Figure 5 shows possible adducts for lysine, cysteine, and histidine. Precedent for conjugate addition of these amino acid residues to such an activated double bond is seen in the adduction of lysine, cysteine, and histidine to a structurally analogous site in the lipid peroxidation product 4-hydroxynonenal (Nadkarni and Sayre, 1995). We have postulated the existence of such an  $\alpha$ -dione-containing AGE crosslinking structure as shown in Figure 3. The current evidence for the existence of such crosslinks is that certain compounds we designed to destroy  $\alpha$ -dicarbonyl AGE intermediates were found to break a substantial proportion of the AGE crosslinks themselves, returning proteins experimentally crosslinked with AGEs to a noncrosslinked state (Vasan *et al.*, 1996). The agents that do this are thiazolium salts, whose mechanism of action is based on the catalytic chemistry seen for the co-enzyme vitamin, thiamin, which has a thiazolium structure. These agents and their therapeutic effects will be discussed in detail later. One such agent, 4,5-dimethyl-3-phenacylthiazolium chloride, or DPTC, has been found to reverse certain sequelae of diabetes and aging *in vivo* (Wolffenbuttel *et al.*, 1998; Asif *et al.*, 2000). Other than the activity of agents like DPTC, there is as yet no direct chemical demonstration of the existence of the Amadori dione crosslink. It is also possible that agents such as

DPTC might break other types of AGE crosslinks by as-yet-unknown mechanisms.

D. NONCROSSLINKING, PROTEIN-BOUND AGE STRUCTURES OF IMPORTANCE *IN VIVO*

Besides the crosslinking AGEs, which have profound effects on protein structure and function, a number of noncrosslinking AGE structures are found under physiological conditions. These may have deleterious effects as precursors of crosslinks or as biological receptor ligands inducing a variety of adverse cellular and tissue changes. [Such activities will be discussed in Section II.] Some of the more-important, noncrosslinking AGEs, shown in Figure 6, are described in the following paragraphs:

1. *Pyrraline*

Pyrraline is a pyrrole aldehyde AGE reportedly found *in vivo* (Hayase *et al.*, 1989; Niwa, 1999). Pyrraline is derived from reaction of 3-deoxyglucosone with lysine residues. Pyrraline may form crosslinks between proteins, as discussed earlier.

2. *1-Carboxyalkyllysines*

AGEs in which a 1-carboxyalkyl group is attached to a free amino group of an amino acid residue, such as *N*<sup>ε</sup>-(carboxymethyl)lysine (CML) and *N*<sup>ε</sup>-(1-carboxyethyl)lysine (CEL), are found in proteins and in free form *in vivo* (Liardon *et al.*, 1987; Krook *et al.*, 1993; Ahmed *et al.*, 1997). They may arise either from reaction of lysine residues with glyoxal derivatives (Glomb and Monnier, 1995) or from autoxidation of early-stage AGEs such as the Amadori product (Brinkmann Frye *et al.*, 1998). Carboxymethyl derivatives of α-amino groups of N-terminal amino acids are also possible, such as *N*-carboxymethylvaline in hemoglobin (Cai and Hurst, 1999). CML formation seems to be a major pathway *in vivo*,

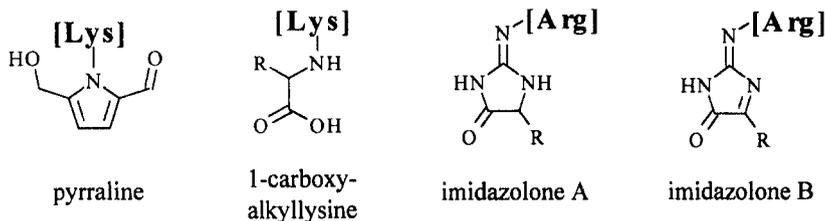


FIG. 6. Some noncrosslinking AGEs of physiological relevance. **[Lys]** represents an ε-norleucyl group (a desamino-lysine residue); **[Arg]** represents a δ-norvalyl group (a desguanidino-arginine residue).

as urinary levels of CML in humans average 30 to 40 percent of levels of the lysine Amadori product (Knecht *et al.*, 1991).

### 3. Imidazolones

The reaction of glyoxal or methylglyoxal (Paul *et al.*, 1998, and references therein) or 3-deoxyglucosone (Niwa *et al.*, 1997, and references therein) with the guanidino group of arginine can lead to formation of AGEs called imidazolones (Figure 6). These are structurally related to, but are probably not precursors of, aminoimidazoline imine crosslinks. Imidazolones of type A (Figure 6) undergo slow oxidation to imidazolones of type B. 3-Deoxyglucosone-derived imidazolone A and traces of B are found *in vivo*.

## E. FORMATION OF AGEs ON DNA

Like protein, DNA contains amino groups. The 2-amino group of guanosine is the most reactive. Reaction of glucose with guanosine yields *N*<sup>2</sup>-glucopyranosylguanosine (Knerr *et al.*, 1994) and a 1-carboxyalkyl derivative of N-2 from glucose-derived 3-deoxyglucosone (Knerr and Severin, 1993). A 1-carboxyethyl N-2 adduct of guanosine results from reaction with methylglyoxal (Papoulis *et al.*, 1995) or glucose. Glyoxal and its derivatives also can form five-membered ring cyclic derivatives bridging N-1 and the C-2 amino of guanosine crosslink (Shapiro *et al.*, 1969). None of these have been identified in *in vivo* sources. However, lysine and glucose-6-phosphate have been noted to form a reactive intermediate, not yet identified, that binds covalently to DNA (Lee and Cerami, 1987). Labeling studies show that the bound species contains the lysine component. The presence of AGEs on DNA causes unusual transpositional rearrangements (Lee and Cerami, 1990; Bucala *et al.*, 1993a) [see Section II].

## F. ROLE OF OXIDATION IN FORMATION OF AGEs

Both the Amadori product and later-stage AGEs undergo autoxidation and have pro-oxidant effects on other molecules (Baynes and Thorpe, 1999). Enol structures such as the enaminal precursor of the Amadori product (Figure 1) or the 2,3-enol of the Amadori product may undergo one-electron oxidation to a radical species. Further oxidation of the radical can lead to fragmentation products, such as carboxymethyllysine (Brinkmann Frye *et al.*, 1998). Alternatively, the AGE radical may abstract a hydrogen atom from a nearby biomolecule, converting it to a radical, leading to its autoxidation. The latter process is well demonstrated by the effect of glycation on lipoprotein complexes such as LDL (Bucala *et al.*, 1994,1995). These effects will be discussed in more detail in Section II.

## II. Biological Aspects of AGE Formation

AGE precursors were first discovered *in vivo* on a hemoglobin. The glycation of hemoglobin increases its oxygen affinity and makes it more susceptible to oxidation (Bucala *et al.*, 1992). However, effects of this type on soluble proteins are generally small and limited to the percentage of protein molecules actually glycated, which, in turn, depends on the half-life of the protein. Thus, most of the primary effects of AGEs leading to the complications of diabetes and aging are due to AGE formation on long-lived proteins. Collagen has a very low turnover rate and is a prime target. Especially damaging are the effects of AGEs on vascular collagen, which may contribute to atherosclerosis and coronary disease, kidney damage, retinal pathology, poor peripheral circulation, and other lesions. Lens crystallins are also a long-lived target, leading to cataracts. AGE damage to DNA may cause birth defects.

### A. EFFECTS OF AGE FORMATION ON CONNECTIVE TISSUE

During formation of normal collagen fibrils, some collagen lysine residues and hydroxylysine residues are oxidized by lysyl oxidase, which converts their  $\epsilon$ -amino groups to aldehydes. These aldehydes then undergo crosslinking reactions with lysine or hydroxylysine residues in adjacent collagen molecules in the fibrils. This process is self-limiting in that lysines in closely crosslinked collagen molecules are no longer sterically accessible to lysyl oxidase. Evolution has tailored this process to produce collagen fibrils with an optimal balance of strength and flexibility. Unfortunately, AGE crosslinking, being mediated by small molecules, is not subject to such limitations and can proceed even in mature collagen molecules. AGE-mediated overcrosslinking of connective tissue molecules (e.g., collagen molecules) to each other causes loss of bulk elasticity and flexibility and increased brittleness. Another example of damage to connective tissue by AGEs is the crosslinking of exogenous molecules to collagen, which can lead to pathological surface changes and overactivation of tissue repair mechanisms.

#### 1. Effects of AGE Formation on Vascular Connective Tissue

Collagen has been shown to react with other collagen molecules or with other plasma proteins (e.g., albumin, immunoglobulin, low-density lipoprotein (LDL)), presumably through AGE crosslinking reactions (Brownlee *et al.*, 1983). AGE crosslink formation in collagen contributes to diabetic circulatory complications such as vascular stiffening (Sims *et al.*, 1996) and myocardial dysfunction (Aven-dano *et al.*, 1999). The accumulation of the plasma proteins on structural proteins is believed to be a factor leading to the thickening of basement membranes and the development of atherosclerotic lesions (Bucala and Cerami, 1995). The possible involvement of AGE-modified LDL in this process is discussed below.

## 2. *Effects of AGE Formation on Nonvascular Connective Tissue*

In both animals and humans, there is a significant correlation between the stiffening of structural collagen (e.g., tendon collagen and dura collagen) with age, the development of yellow-brown discoloration and fluorescence, and the levels of AGEs present. This can be mimicked by incubation of collagen sample with sugars *in vitro* (Yue *et al.*, 1983; Kohn *et al.*, 1984). These changes are accelerated in diabetes.

### B. EFFECTS OF AGE FORMATION ON SOLUBLE PROTEINS

#### 1. *Effects of AGE Formation in Lens Proteins*

Human lens crystallin proteins are very long lived, possibly lasting an entire lifetime. Although crystallins have evolved to retain optical clarity for an extended period, yellow-brown pigments with the spectral and fluorescent properties of AGEs do form in the lens as a function of age (Monnier and Cerami, 1983). These pigments can become so dark as to form cataracts, leading to blindness. In the normal lens, individual lens proteins associate via hydrophobic interactions to form aggregates of several million daltons. Substances such as glucose or cyanate can modify amino groups in crystallins, causing conformational changes. These changes expose previously buried sulfhydryl groups, which can then autoxidize to form intermolecular disulfide bonds (Monnier *et al.*, 1979). The resulting disulfide-linked superaggregates of the aggregated lens crystallins can be of sufficient size to scatter light and produce a cataract. The importance of disulfide bonds in human cataract formation is indicated by studies showing that light scattering by cataracts in lens from older individuals can be significantly decreased by addition of reducing agents. However, a residual amount of protein crosslinking is present that is not disulfide related (Harding, 1973). AGEs may be directly implicated in this crosslinking.

#### 2. *Effects of AGE Formation on Lipoproteins*

AGE modification of LDL can occur both on amino groups of the apoprotein component (Bucala *et al.*, 1995) and on aminolipid components such as phosphatidylethanolamine (Bucala *et al.*, 1993b). AGE formation on the apoprotein component can lead to crosslinking of LDL to the collagen layer of the blood vessel wall (Brownlee *et al.*, 1983) and can increase the half-life of LDL in serum by blocking the recognition site for its receptor-mediated uptake (Bucala *et al.*, 1995). This increases the probability of autoxidation of the lipid component (Bucala *et al.*, 1993b). Like AGE formation, such oxidation of LDL can lead to loss of recognition by cellular LDL receptors and can induce its preferential uptake by macrophage scavenger receptors (Witztum and Steinberg, 1991). Macrophages

attracted to AGEs on vessel wall collagen may thus accumulate modified LDL, leading to their conversion to foam cells that are thought to be central to the atherosclerotic process. The ability of AGEs present on LDL to initiate free-radical reactions (Bucala *et al.*, 1993b) is of considerable importance because it provides an alternative to the hypothesis that metals are responsible for LDL autoxidation. Previous work on the initiation of LDL oxidation generally has implicated metal ions (e.g., iron, copper) as oxidation initiators. Unactivated oxygen does not normally react with most organic compounds. Through one-electron redox chemistry, iron or copper ions can induce the radical addition of oxygen to the olefinic bonds of unsaturated fatty acids to form hydroperoxides. Further reactions of these hydroperoxides with metals can form hydroxyl- and peroxy-radicals that eventually cleave the fatty acids to form aldehydes. However, free (unchelated) metals usually are not present in significant amounts *in vivo*. The source of oxidation initiation for substrates like LDL has therefore been puzzling. The ability of AGEs to initiate such oxidative reactions in the absence of metals provides a new explanation for lipoprotein lipid peroxidation *in vivo* (Bucala *et al.*, 1994).

### 3. AGE Formation in $\beta$ -Amyloid Plaques

There is evidence that AGEs play a role in abnormal amyloid aggregation in Alzheimer's disease (AD). Amyloid plaque material like that from AD patients is also found in low quantity in age-matched human controls. Analysis of plaque material from both sources revealed that the plaque from AD patients had three times the AGE content per milligram amyloid as the amyloid from controls (Vitek *et al.*, 1994). Since we know that AGE levels increase over time in normoglycemic individuals, one interpretation is that, in AD patients, a mechanism for removal of amyloid plaque is defective. The persisting plaque undergoes AGE crosslinking, gaining further resistance to proteolytic degradation and removal processes. *In vitro* studies have found that formation of insoluble, fibrillar aggregates from AGE-modified  $\beta$ -amyloid peptide was much faster than for control  $\beta$ -amyloid peptide.

## C. AGE-INDUCED OXIDANT DAMAGE

The role of oxidative processes in the formation of carboxymethyllysine from the Amadori product, and the pro-oxidant effect of AGE formation on LDL, have been discussed in previous sections. These are part of a larger area of what have been termed "glycooxidation" reactions (Baynes and Thorpe, 1999) in which AGE formation processes and oxidative processes synergistically interact to produce deleterious tissue modifications, with the possible involvement of redox-active metals. These processes have some overlap with lipid peroxidation processes. The case of LDL oxidation already has been discussed, as has the chemical similarity

of the Amadori ene-dione to the lipid oxidation product, 4-hydroxynonenal, both of which may crosslink proteins. Furthermore, glycoxidation and lipid peroxidation both can form glyoxal and other similar, small-molecule crosslink precursors.

#### D. AGE-INDUCED DNA REARRANGEMENT

As noted earlier, AGEs can form on DNA, particularly on guanine residues. The most-dramatic effect of AGE formation on DNA reported to date is the induction of unusual DNA rearrangements (Lee and Cerami, 1990,1991; Bucala *et al.*, 1993a). Strains of *E. coli* that accumulate high levels of glucose-6-phosphate, which is particularly active in forming AGEs, demonstrate increased levels of the gamma delta transposition, which is the relocation of the transposable element gamma delta (Lee and Cerami, 1991). In mammalian cells, evidence has been found that AGE formation on DNA may be responsible for insertions containing repetitive sequences of the Alu family that have been found to disrupt human genes (Bucala *et al.*, 1993a). The possibility that AGEs may induce genetic rearrangements *in vivo* has important implications; for example, as a possible cause of congenital malformations in infants of poorly controlled, insulin-dependent, diabetic mothers (Lee *et al.*, 1995).

#### E. EFFECTS OF AGEs IN FOOD

Early studies showed that intravenous administration of exogenous, AGE-modified rat serum albumin (AGE-RSA) to rats caused complex vascular dysfunction, compared to rats receiving control RSA. These effects included significant increase in vascular permeability, enhanced mononuclear cell migration into subendothelial and periarteriolar spaces, and defective vasodilatory responses (Vlassara *et al.*, 1992). Although ingestion of AGEs formed during the cooking or processing of food could be a potential source of exogenous AGEs, the possible importance of this was discounted due to the presumed poor absorption of food AGEs. However, it has since been found that food AGEs can be absorbed after eating. In diabetics with impaired renal function, food-derived AGEs with crosslinking activity can accumulate in serum to a significant degree (Koschinsky *et al.*, 1997), implying that normal kidney function is important to protect from dietary AGEs.

#### F. RECEPTORS FOR AGEs AND THEIR POSSIBLE ROLE IN NORMAL REMOVAL OF AGEs

Scavenger receptors with an affinity for AGEs have been found on many cells such as macrophages, lymphocytes, and barrier cells (e.g., endothelial cells, mesangial cells) (Esposito *et al.*, 1989; Skolnik *et al.*, 1991). Phagocytic cells expressing these receptors can internalize and digest senescent, AGE-modified pro-

teins, releasing AGE-modified peptides similar to those absorbable from food and normally excreted by the kidneys. The uptake of AGE proteins can signal the synthesis and release of certain cytokines and growth factors that can stimulate re-synthesis of proteins that have been phagocytically removed (Vlassara *et al.*, 1988). If this process is not well regulated, serious problems can develop (e.g., tissue overgrowth). High levels of AGEs on matrix proteins in the diabetic kidney can induce mesangial cells to produce excessive amounts of matrix proteins, resulting in a thickened basement membrane incapable of sustaining normal kidney filtration processes (Makita *et al.*, 1991).

### III. Pharmacological Intervention Against AGE Formation *in Vivo*

The development of agents against AGE-related complications has followed two main approaches (Figure 7). First, inhibition of AGE formation processes by carbonyl-blocking agents such as aminoguanidine, and second, more recently, the cleavage of already formed AGE protein-protein crosslinks by dicarbonyl-breaking compounds like DPTC.

#### A. PHARMACOLOGICAL INHIBITION OF AGE CROSSLINK FORMATION

In the early 1980s, when it became clear that advanced glycation reactions were a potential cause of complications of diabetes and aging, we began to think

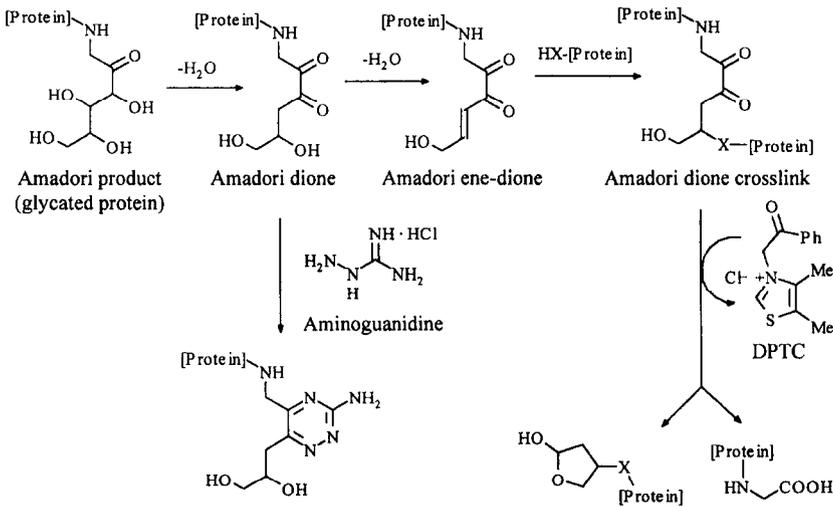


FIG. 7. Possible sites of post-Amadori crosslink prevention by aminoguanidine and crosslink cleavage by DPTC (ALT-711).

about ways to interfere with this process pharmacologically. Unfortunately, little was known at that time about the pathways of AGE formation in the Maillard reaction *in vivo*. We focussed our attention on the structure of the Amadori product. We reasoned that the ketone group of the 1-amino-1-deoxyfructose residue in the Amadori product was the key to subsequent AGE-forming reactions and that chemical deactivation of this ketone group would prevent AGE formation. We therefore sought to identify an agent that would react rapidly with ketones, rendering them chemically inert, while being relatively nontoxic. A number of factors focussed our attention on aminoguanidine. It is a low-molecular-weight, highly nucleophilic compound that very readily reacts with ketones and aldehydes. There are some published reports of its experimental use in humans for unrelated purposes, without significant side-effects. We therefore selected aminoguanidine for preclinical study (Brownlee *et al.*, 1986). In the intervening years, research in our laboratory and in others around the world has shown the effectiveness of aminoguanidine in inhibiting AGE formation *in vivo* in a wide variety of systems and tissues (Table I). As discussed earlier,  $\alpha$ -dicarbonyl intermediates, and not the initial Amadori product, are now believed to be the targets of aminoguanidine inhibition.

TABLE I

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*Effect of Aminoguanidine on Diabetes-related Complications or AGE Formation in Vivo*

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*Nephropathy*

- Inhibition of basement membrane thickening (Ellis and Good, 1991)
- Prevention of albuminuria (Soulis-Liparota *et al.*, 1991)
- Stabilization of IGF system (Bach *et al.*, 2000)

*Retinopathy*

- Normalization of endothelial cell proliferation (Hammes *et al.*, 1991)
- Restoration of retinal nNOS neurons (Roufail *et al.*, 1998)

*Vasculopathy*

- Reduction of vasodilatory impairment (Vlassara *et al.*, 1992)
- Preservation of vascular elasticity (Huijberts *et al.*, 1993)
- Improvement of erythrocyte deformability (Brown *et al.*, 1993)
- Anti-atherogenic effect (Panagiotopoulos *et al.*, 1998)

*Neuropathy*

- Retention of nerve conduction velocity (Kihara *et al.*, 1991; Miyauchi *et al.*, 1996)
-

A phase III clinical study of aminoguanidine (also known by the generic name, pimagedine) in diabetic patients was completed recently. Patients in the study showed significantly decreased LDL levels, decreased urinary albumin levels (an important indicator of diabetic nephropathy), decreased progression of retinal degeneration, and a trend toward prevention of loss of kidney function. Further trials will be necessary before aminoguanidine can be approved for clinical use.

## B. PHARMACOLOGICAL CLEAVAGE OF AGE CROSSLINKS

Like the outcome of the Diabetes Control & Complications Trial, the activity of aminoguanidine against diabetic complications is another important piece of evidence supporting the AGE theory of diabetic complications in humans. However, there is a major limitation to the utility of AGE formation inhibitors like aminoguanidine: they cannot affect already-formed AGE damage, particularly crosslinking. A drug that could break already-formed crosslinks is a more desirable goal, as it would allow direct restoration of AGE-damaged tissue to its original functionality, without relying on the body's much-slower, more-problematical removal and repair mechanisms. In the absence of a clear understanding of the specific chemical mechanisms of AGE crosslink formation, developing such a drug seemed an unattainable goal. However, in the course of designing a new class of AGE inhibitors, we devised a new theory of AGE crosslinking that suggested the possible existence of crosslinking structures that could be chemically broken.

Alpha-dicarbonyl compounds such as  $\alpha$ -oxoaldehydes (glyoxal derivatives) (Figure 2) and their Schiff bases are thought by many researchers to be key reactive intermediates in AGE formation *in vivo*. Somewhat less studied have been  $\alpha$ -dicarbonyl (diketone) intermediates of the Amadori dione pathway, resulting from dehydration of the Amadori product with the amino acid still attached (Figure 3). We noticed that the Amadori ene-dione contained a structural motif that would allow it to form a protein-protein crosslink via conjugate addition of another nucleophilic side-chain to the carbon-carbon double bond in a Michael reaction (Figure 3). Such a conjugate addition by lysine, histidine, and cysteine residues has been reported for a chemically related structural motif in the lipid peroxidation product, 4-hydroxynonenal (Nadkarni and Sayre, 1995). The resulting Amadori dione AGE crosslink structure (Figures 3 and 5) would contain an  $\alpha$ -diketone.

We designed a new class of anti-AGE agents that contained a thiazolium structure, which could chemically break  $\alpha$ -dicarbonyl compounds (e.g., glyoxals,  $\alpha$ -diketones) by cleaving the carbon-carbon bond between the carbonyls. We thought that these agents would inhibit AGE processes by destroying glyoxal derivatives and the Amadori diones. However, it was difficult to assess the

prospects for a major physiological role for  $\alpha$ -dicarbonyl-containing crosslink structures that would be cleavable by these agents. Thus, it was a pleasant surprise when we found that the thiazolium agent 3-phenacylthiazolium bromide (PTB) was able to cleave a major proportion of AGE crosslinks formed under physiological conditions (Vasan *et al.*, 1996). Subsequently, we have developed a more-stable and more-active derivative, 4,5-dimethyl-3-phenacylthiazolium chloride (DPTC), which has shown a variety of promising effects in preclinical studies. For example, at a dose of 1 mg/kg/day for 1-3 weeks in streptozocin-diabetic rats, DPTC reversed the large artery stiffness resulting from diabetes, as assessed by the criteria of systemic arterial compliance, aortic impedance, and carotid artery compliance. DPTC also reduced the diabetes-related increased crosslinking of tail tendon collagen, as measured by susceptibility to proteolytic degradation (Wolfenbittel *et al.*, 1998). In a more-recent study in aged dogs, invasive monitoring was used to measure end diastolic pressure and volume pre- and post-treatment. Treatment with DPTC at 1 mg/kg/day for 4 weeks led to a 40 percent lowering of left ventricular stiffness, essentially restoring the elasticity of the heart in aged animals to levels seen in much-younger ones (Asif *et al.*, 2000).

The finding that already-formed AGE crosslinks can be pharmacologically severed and attendant pathology thereby reversed has broad implications for the therapeutics of the complications of diabetes and diseases of aging. The prospect of reversing the effects of AGEs was previously only contemplated in the context of stimulating the body's own removal and repair mechanisms. Because of the unique mechanistic aspects of agents like DPTC, further chemical, preclinical, and clinical studies with these AGE-crosslink-breaking agents has the potential to establish new paradigms in the management of diabetes and aging diseases. Such research also will increase the understanding of how AGEs cause pathology and the extent of AGE involvement in the various complications of diabetes and aging, compared to other mechanisms proposed for these processes.

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