MINIREVIEW

Nonenzymatic Glycation of Collagen in Aging and Diabetes (44264)

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Why should we be interested in nonenzymatic glycation of collagen? To investigators in other fields, it may seem quixotic to focus on a single post-translational modification of collagen, particularly one that occurs extracellularly and is not under gene control. However, this modification has far-reaching consequences, as it is implicated in the degenerative conditions associated with aging and diabetes. Unlike most post-translational modifications, nonenzymatic glycation of proteins serves no useful function in the organism and conforms to no principles of biological design. The process is driven by stochastic forces; hence, sugar adducts may undergo further reactions that are ultimately detrimental to the organism as long as they are energetically favorable. Over time, these reactions generate an array of complex colored and fluorescent compounds, called advanced glycation products.

While nonenzymatic glycation occurs on many proteins, glycation of collagen is of particular significance for several reasons. The long half-life of collagen, measured in years, allows time for the accumulation of enough advanced glycation products sufficient to alter many of the molecule's physical properties. Since collagen provides form and structure to almost every tissue and organ system, progressive changes in its properties are associated with progressive derangements in tissue and organ system functioning. The extracellular matrix also modulates many characteristics of resident cells, including migration, growth, proliferation, differentiation, and gene expression. Thus, physical changes in matrix components, such as nonenzymatic glycation of collagen, may affect any or all of these cell behaviors. The overall impact of matrix glycation is enormous, for wherever cells travel in the body they encounter collagen: in fibrillar arrays, in membrane meshworks, alone, and in complex conjunctions with other matrix components. In short, collagen is a uniquely important protein for it serves as the true common ground of an organism; by the same token, its very universality greatly amplifies the destructive effects of glyco-oxidative changes.

Since this topic was last reviewed, there have been shifts in the major areas of emphasis. Key themes now include the central role of oxidation, the diversity of early glycation pathways, and the role of specific cellular receptors for glycation products. Conceptual advances have been slower in other areas, such as structural characterization of advanced Maillard products, or the development of effective pharmacologic intervention. In the present review, recent work in all areas is discussed, with the aim of providing investigators in other fields with a useful construct for understanding the diverse and sometimes confusing literature. To this end, the review will begin with a look at the chemistry of nonenzymatic glycation; this section will include current concepts concerning initiation of nonenzymatic glycation as well as the biosynthesis and significance of structurally characterized glycation products. Regulation of nonenzymatic glycation has also been an area of intensive investigation; structural, enzymatic, and cellular mechanisms that may serve to modulate glycation reactions are discussed. The consequences of glycation are then reviewed, beginning with the effects on the molecule itself, and ending with a look at clinical consequences for the organism as a whole. Finally, progress is interventional strategies is reviewed. Two compounds are discussed in detail, illustrating possible approaches to intervention, as well as the inherent difficulties in this task.

The Chemistry of Nonenzymatic Glycation of Collagen

Initiation of Glycation. The first step in the nonenzymatic glycation of collagen has long been assumed to be

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the nonenzymatic condensation of a sugar aldehyde or ketone with the epsilon amino group of lysine or hydroxylysine, following which the resultant Schiff base may become stabilized *via* an Amadori rearrangement. Amadori products have been considered the initiators of nonenzymatic glycation because they are capable of undergoing degradation to form highly reactive sugar fragmentation products. These products, such as the deoxyglucosones, have been termed the propagators of the Maillard reaction (1). Recent studies have elucidated some of the specific pathways of Amadori product decomposition.

It is now apparent that nonenzymatic glycation of proteins, including collagen, may be initiated by various mechanisms, not solely or necessarily predominantly by the Amadori degradation pathway. Alternative pathways by which sugar initiates glycation of proteins includes glucose auto-oxidation, the polyol pathway, and the triose phosphate-methylglyoxal pathway. The interrelationship among these different pathways is shown schematically in Figure 1; it can be seen that the same product may arise from very different reactions. The predominant mechanism of glycation in a given instance most likely depends on many variables, including tissue, species, age of organism, and diet, as well as those variables that modulate oxidative stress. Recent data also now suggest that in some cases compounds other than sugars—specifically, ascorbate and polyunsatu-

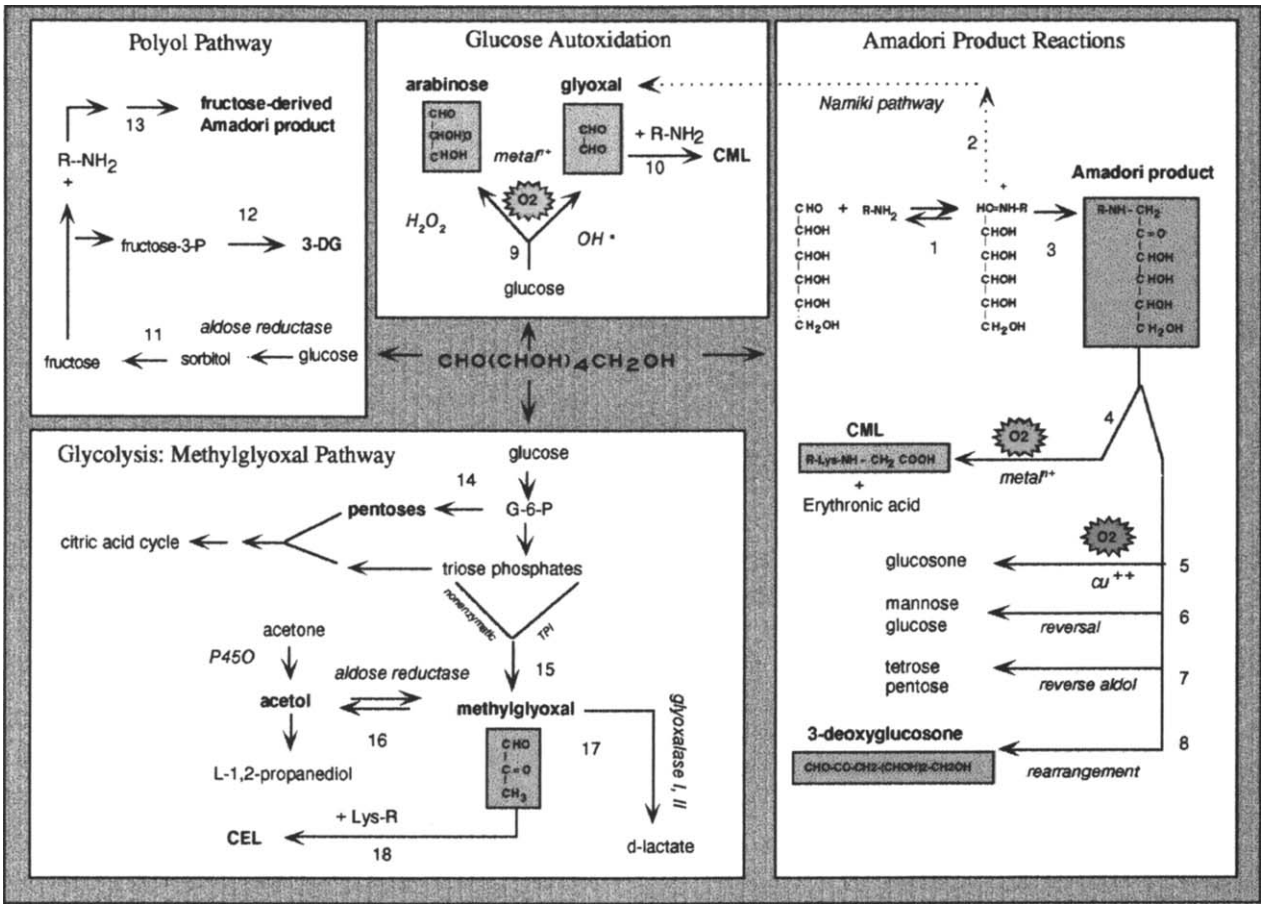

Figure 1. Initiation of glyco-oxidative cross-linking by glucose. Glucose is capable of initiating glyco-oxidative modification of collagen through diverse reaction pathways, shown here functionally categorized into four major groups:
I. Amadori product reactions: (Reaction 1) nonenzymatic condensation of glucose and a lysine or hydroxylysine residue; (Reaction 2) oxidative fragmentation of Schiff base go glycoxal; (Reaction 3) Amadori rearrangement; (Reaction 4) oxidative fragmentation of the Amadori product catalyzed by a transition metal, yielding carboxymethyllysine (CML) and erythronic acid; (Reaction 5) oxidative fragmentation of Amadori product in the presence of cupric ion, yielding glucosone; (Reaction 6) reversal of Amadori reaction, ± oxidation, yielding mannose and glucose; (Reaction 7) reverse aldol reaction, ± oxidation, yielding tetrose and pentose sugars; (Reaction 8) rearrangement, ± oxidation, yielding 3-deoxyglucosone. Further reactions of the Amadori decomposition products are not shown.
II. Glucose Autoxidation: In the presence of molecular oxygen and transition metal, glucose can undergo oxidation (Reaction 9) to form arabinose, a pentosidine precursor, and glyoxal, a CML precursor (Reaction 10). Reactive oxygen species, such as hydrogen peroxide and hydroxyl radical, are also generated during this process, causing oxidative damage to protein.
III. Polyol pathway: (Reaction 11) Glucose is a substrate for aldose reductase, ultimately yielding fructose; (Reaction 12) The metabolite fructose-3-phosphate is a precursor of 3-deoxyglucosone; (Reaction 13) Fructose can form fructose-derived Amadori products.
IV. Glycolysis: Methylglyoxal pathway: (Reaction 14) Alterations in flux through the pentose shunt can affect precursor availability for pentosidine and other glycoxidation products; (Reaction 15) The triose phosphates serve as precursors for methylglyoxal, which is produced primarily nonenzymatically; (Reaction 16) Methylglyoxal production *via* acetone and acetol, catalyzed by aldose reductase; (Reaction 17) Metabolism of methylglyoxal to lactate; (Reaction 18) Reaction of methylglyoxal with lysine residue to form advanced glycation product CEL.

rated fatty acids—may serve as the precursors of advanced glycation products.

Amadori product reaction pathways. The initial step in this group of reactions is the condensation of the carboxyl moiety on a reducing sugar with the epsilon amino group of a lysine or hydroxylysine residue (Fig. 1, Reaction 1), resulting in an unstable Schiff base–type compound. The Schiff base may undergo oxidative fragmentation before the Amadori rearrangement can occur, giving rise to glyoxal (Fig. 1, Reaction 2), the so-called Namiki pathway (2). This reaction occurs rapidly, whereas the Amadori product is formed much more slowly. Amadori products are relatively stable, and there appears to be a steady-state level of this compound. The metabolic fate of Amadori products is complex (Fig. 1, Reactions 4–8).

Three decomposition pathways may occur under both oxidative and anti-oxidative conditions (3): reversal (yielding glucose and mannose), rearrangement (yielding 3-deoxyglucosone), and reverse aldol reaction (yielding pentoses and tetroses). Under oxidative conditions and in the presence of a transition metal, the Amadori product yields carboxymethyllysine (CML)—as a protein adduct—and erythronic acid. However, if oxidative fragmentation occurs in the presence of reducible substrates, such as cupric ion, the Amadori product yields glucosone. Recent *in vitro* studies have revealed several factors capable of influencing Amadori product reactions, including phosphate concentration, pH, and oxidative stress (3). These and other factors may determine the metabolic fate of a given Amadori product. Most of the decomposition products have been detected *in vivo* in plasma and urine, thus providing indirect evidence that these reaction pathways occur *in vivo*. For example, Knecht *et al.* reported that 3-deoxyglucosone and its presumed metabolite 3-deoxyfructosone were detected in urine and plasma in amounts suggesting that several milligrams of 3-deoxyglucosone are formed in the body each day (4). Fujii *et al.* (5) reported detection of 2-keto-3-deoxygluconic acid, a highly reactive oxidation product of 3-deoxyglucosone in human erythrocytes and in plasma.

Glucose auto-oxidation. As initially reported by Wolff more than 10 years ago, glucose is capable of undergoing auto-oxidation, catalyzed by transition metals and molecular oxygen, resulting in a highly reactive dicarbonyl compound capable of reacting with protein amino groups (6, 1). It was speculated that this pathway might constitute a minor pathway for advanced glycation products formation (1). Recently, Baynes and coworkers have raised the provocative suggestion that Amadori products may in reality be "bystanders", while glucose auto-oxidation products comprise the major precursors of intermediate and advanced glycation products (7). In these *in vitro* studies, collagen was incubated with glucose for up to 5 weeks under oxidizing or antioxidizing conditions. The investigators found that while sugar adduct formation was reversible, reactions leading to glycoxidation products such as CML were not reversible. They also found that the rate of CML formation was not dependent on the rate of Amadori product formation and, furthermore, that formation of glyco-oxidation products could be uncoupled from formation of precursors. Under antioxidative conditions, glucose adduct formation (assessed as fructoselysine) was not affected, whereas the formation of pentosidine, CML, and fluorescence was inhibited. Antioxidative conditions also inhibited formation of high-molecular-weight compounds. If the collagen was subsequently washed to remove free glucose and then incubated under oxidative conditions, CML and pentosidine formed, but only about 10% as much as when collagen was incubated with glucose under oxidative conditions. From these data the investigators infer that auto-oxidation of glucose may be the primary route by which Maillard products are formed, at least under these *in vitro* conditions.

The extent to which sugar oxidation products generated prior to reaction with proteins serve as precursors of advanced glycation products *in vivo* remains unclear. Antioxidant defense systems and metal chelation may suppress auto-oxidation of glucose and Schiff base oxidative fragmentation, so that in most cases Amadori products may well constitute the major precursors of advanced glycation products.

It should also be noted that the source of the reactive species that drive protein cross-linking may be of less significance to the organism than the degree of collateral damage that is done to the protein backbone by the reactive oxygen species generated in the process. Very recently Baynes and colleagues have described the measurement of amino acid oxidation products (ortho-tyrosine and methionine sulfoxide) as a way of assessing oxidative damage to proteins independently of glyco-oxidative damage (8). Oxygen species may also play a specific role in cross-linking; a recent study by Monnier and colleagues (9) implicates hydrogen peroxide in this regard.

Polyol pathway. The polyol pathway may also serve as the initiating event in nonenzymatic glycation of proteins. Glucose can give rise to sorbitol and fructose; 3-phosphokinase then phosphorylates these compounds to form the inert compound sorb-3-phosphate and fructose-3-phosphate. The latter is a potent cross-linking agent and a source of 3-deoxyglucosone. Recently Lal *et al.* (10) have shown that polyol pathway metabolites are increased in lenses from aging rats, paralleling a large increase in nonenzymatic glycation of lenses (11). Hamada *et al.* (12) reported on increased levels of fructose-3-phosphate in diabetic erythrocytes; this level was decreased following treatment with an aldose reductase inhibitor.

Triose-phosphate-methylglyoxal pathway. Methylglyoxal is a toxic 2-oxo-aldehyde that is produced by most glucose-metabolizing cells. In microorganisms, synthesis is catalyzed by methylglyoxal synthase, serving as a bypass pathway for triose phosphate metabolism when inorganic phosphate levels are low. In animals there are several possible pathways for synthesis and degradation. Methylglyoxal can be produced by the oxidation of acetone, first to

acetol and then to methylglyoxal, catalyzed by a monooxygenase. Methylglyoxal can also be produced nonenzymatically from triose phosphates. Recently, Vander Jagt *et al.* (13, 14, 15) reported that methylglyoxal is a preferred substrate for aldose reductase. They have formulated a new model of the pathogenesis of diabetic complications that integrates the glucose-Amadori pathway and the glyoxal-acetol pathway. According to the integrative model, both pathways will be stimulated when diabetes is poorly controlled, but for different reasons. The triose phosphate pathway is stimulated by hyperglycemia, resulting in increased methylglyoxal production *via* the nonenzymatically mediated pathway. The enzymatically mediated acetone-acetol pathway is activated if ketoacidosis occurs, which is likely in poorly controlled diabetes. This pathway also culminates in increased methylglyoxal formation. Since methylglyoxal is a better substrate for aldose reductase than is acetol, it will be preferentially reduced to form more acetol, which will tend to accumulate. Since both methylglyoxal and acetol are much more reactive than glucose, the investigators postulate that it is these compounds that may be responsible for the covalent modifications of proteins seen in diabetes.

Baynes' group has in fact provided evidence that directly implicates methylglyoxal in cross-linking (Fig. 1, Reaction 18). Very recently they described a novel advanced glycation end product, N-epsilon-(carboxyethyl)lysine, that arises from the reaction of methylglyoxal with lysine residues (16). This compound, which they have given the acronym CEL, increases with age in human lens proteins in parallel with the increase in CML. Although glyoxal was found to be the most reactive precursor of CEL, other compounds could serve as precursors also, including pentoses, hexoses and ascorbate.

Ascorbate oxidation. Monnier and colleagues (17, 18) have reported that auto-oxidation of ascorbic acid generates compounds capable of glycating and cross-linking proteins *in vitro.* Oxidation of ascorbate leads to formation of the intermediates dehydroascorbic acid and diketogluconic acid and, ultimately, xylosone or l-threose. Threose was found to be the most active of the metabolites in its capacity to glycate and cross-link proteins. *In vitro* studies suggest that even very low levels of L-threose are capable of cross-linking proteins rapidly. Ortwerth *et al.* (19) have suggested that the rate-limiting step of protein glycation by threose is the rate of ascorbate oxidation. Recently they reported on the role of UV-generated oxygen species in these reactions (20).

Advanced Glycation Products. Nonenzymatic glycation reactions have, in the past, conventionally been divided into early, intermediate and late reactions. While the pathways generating reactive sugar fragmentation products might be considered early glycation reactions, the distinction between intermediate and late glycation reactions is no longer clearcut. Categorization of reaction pathways is further hindered by the fact that there are only a few structurally characterized advanced glycation products that are accepted by consensus as existing *in vivo.* These compounds, discussed in detail below, include carboxymethyllysine (CML) and related compounds, and pentosidine. Although there is convincing evidence that pyrraline exists *in vivo,* conflicting findings regarding this cross-link have been reported very recently. Periodic reports of new glycation products appear regularly in the literature, but their existence *in vivo* has yet to be confirmed by independent investigators. The apparently slow pace of progress in this area reflects the considerable difficulties inherent in investigating nonenzymatic glycation *in vivo.* Tools that are effectively used *in vitro,* such as maintenance of highly controlled reaction conditions, inactivation of metabolic intermediates with trapping reagents, and removal of reaction products at specific intervals, are not readily duplicated *in vivo.*

CML. CML is a colorless compound, first identified by Baynes and coworkers, resulting from oxidative fragmentation of Amadori products derived from glucose and lysine (21). A related product, carboxymethylhydroxylysine (CMhL) is generated from the reaction product of glucose and hydroxylysine. CML is a glycation end product, in that it does not undergo further reactions.

Given that CML is an oxidized adduct rather than a true cross-link and, furthermore, incapable of forming cross-links, why is it of interest? Concepts concerning the significance of CML have undergone considerable change over the past 10 years. When CML was first characterized (22), the reaction pathway was conceptualized as a kind of "safety valve" that prevented Amadori products from becoming advanced glycation products associated with tissue damage. This concept became less tenable as it could not be documented that the CML pathway actually diverted any Amadori products from advanced glycation product formation. Indeed, as it became apparent that CML accumulation inevitably paralleled the accumulation of advanced glycation products, and that both glycation and oxidation were required for its formation, CML came to be seen as a useful marker of glyco-oxidative damage rather than a protective mechanism. In very recent studies Baynes' group has reported that CML appears to be more than a fellow traveler of advanced glycation products; it is in fact a component of AGEs (23). Polyclonal antibodies to proteins containing advanced glycation products recognize CML, and, conversely, CML inhibits recognition of advanced glycation product sites. Perhaps even more surprising, CML appears to be a dominant component of advanced glycation products; on average, 30% of the original lysines present on a protein are incorporated into CML after glycation (23).

The reaction pathways that give rise to CML have been studied intensively recently. It is not apparent that precursor-product relationships for CML are far more complex than originally thought, as compounds other than Amadori products may serve as precursors of CML. Recent studies by Baynes' group and by Monnier's group illustrates how selective combinations of precursors, oxidation conditions,

and blocking agents may be used to tease out preferential reaction pathways (24, 23, 25). In one study by Monnier and coworkers (25), glucose/lysine mixtures, Amadori products, and glyoxal were used as starting materials, with addition of boric acid or aminoguanidine as blocking agents. CML formation was strongly dependent on oxidation when the glucose-lysine mixture or Amadori products served as precursors. Boric acid totally suppressed CML formation from Amadori product, but only partially suppressed it in the glucose/lysine system. In contrast, aminoguanidine had little effect on CML formation from Amadori product, but blocked 50% of CML production in the glucose/lysine system. These results suggest that glucose auto-oxidation must play a role. Since boric acid blocks Amadori products, it completely inhibits CML formation when the only possible precursor is Amadori product. However, when glucose is incubated with lysine, the Amadori product is not the only possible precursor, glucose can first auto-oxidize, and then react with lysine. Aminoguanidine, in contrast, does not block Amadori products; hence, it has little effect on CML Formation when Amadori product is the only possible precursor. However, in the lysine-glucose mixture, aminoguanidine will block the sugar fragmentation products that can serve as CML precursors. Based on these results, Monnier *et al.* have estimated that approximately 50% of the CML formed in a glucose/lysine mixture originates from oxidation of Amadori product, and 40%–50% originates from a pre-Amadori stage largely independent from glucose auto-oxidation. They suggest that this step may be related to the Namiki pathway of the Maillard reaction (the oxidative degradation of rapidly formed Schiff bases, described in the preceding section).

The role of glyoxal and glycolaldehyde in CML formation has also been the subject of recent studies. Monnier and coworkers reported that the initial rate of CML formation from glyoxal was not dependent on oxidation, and they suggested that this pathway might involve an intramolecular Cannizzaro reaction (25). However, Baynes and coworkers found that proteins need to be incubated with glyoxal under oxidative conditions if glyoxal is to serve as a precursor of CML. They showed that glyoxal was the only alpha-dicarbonyl sugar formed when glucose was incubated under oxidative conditions; arabinose was the only major product formed (26). Further reaction of glyoxal with protein yielded CML, whereas arabinose and protein resulted in formation of pentosidine. Metal chelators and dicarbonyl trapping reagents also inhibited formation of advanced glycation products. They also found that if lysine is added to a mixture of glucose and aminoguanidine, glyoxal-triazine formation increases significantly, thereby strongly suggesting that glucose auto-oxidation does not play a role in glyoxal-mediated CML formation (26).

Very recently, Baynes' group has reported that CML is formed during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein (27). They note that glyoxal can be formed during UV irradiation of polyunsaturated fatty acid, and during oxidation of linolenic acid in an iron ascorbate model system (28). These data raise several provocative issues, including the possibility that the majority of CML in tissue proteins could theoretically be derived from lipid peroxidation reactions. Baynes and coworkers suggest that the rapidity of extracellular protein cross-linking and the rate at which browning products form in experimental diabetes indicate that lipid peroxidation reactions might play a much bigger role than previously thought. Since intermediates of carbohydrate peroxidation reactions are similar to those of lipid peroxidation, the initiating events are difficult to determine *in vivo*. Determination of the relative contribution of carbohydrate peroxidation and lipid peroxidation to CML accumulation *in vivo* will require identification of compounds unique to each pathway.

Pentosidine. Pentosidine is a trifunctional fluorescent cross-link first identified and characterized by Monnier's group (29). Pentosidine has been found in collagen in almost all tissues analyzed, and is also present in erythrocytes. Even though it represents only a small fraction of total fluorescent advanced glycation products, pentosidine has proven to be useful as a marker of glyco-oxidative damage (30).

Pentosidine is derived from the reaction of lysine and arginine residues with a sugar, which was initially believed to be obligatorily a pentose. Subsequently, *in vitro* studies by Monnier and by Baynes have shown that many sugars can serve as precursors, including glucose, fructose, and sucrose (31, 32). Whatever sugar initiates the reaction, a key step appears to be the formation of a pentated protein (Fig. 2). Rates of formation of pentosidine are highly dependent on the reactivity of the precursor sugar, with pentoses being much faster than hexoses (31). Among monosaccharide hexoses, fructose reacts with protein much more quickly to form pentosidine than does glucose (32, 31). Other compounds can also serve as precursors of pentosidine, including ascorbate, Amadori compounds, and 3-deoxyglucosone. Pentosidine requires an oxidative environment; like CML, its formation can be inhibited or slowed by compounds that modulate levels of oxidative stress. Indeed, most of the experiments described in the preceding section included analysis of pentosidine formation along with CML formation. The kinetics of pentosidine formation were similar to those of CML; after a short lag phase, the rate of pentosidine formation proceeded independently of the amount of Amadori product present (7, 24).

Pyrraline. Pyrraline is the trivial name given to a glucose-derived pyrrole compound, 5-hydroxymethylcarbaldehyde norleucine. Pyrraline has been found in tissues and plasma, and has been shown to increase in aging and diabetes (33). Until very recently, pyrraline was detected *in vivo* exclusively through immunological assays. There has been controversy over the significance of these findings when one group was unable to replicate some of the results (34). They reported that they were unable to

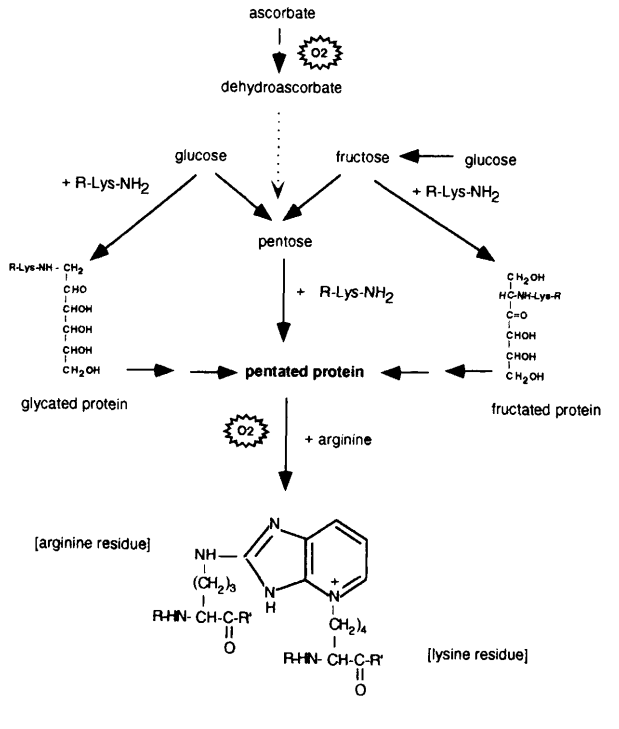

Figure 2. Pentosidine synthesis: Reaction pathways for pentosidine are shown. Although there is evidence that these reactions occur *in vivo*, the relative contributions of the different pathways is not known. A key step is the formation of a pentated protein; this compound reacts with arginine to form pentosidine. Pentated proteins may be generated in several ways. Amadori product derived from glucose or fructose may undergo subsequent reactions to form pentated proteins (see Fig. 1). Pentose may also be generated directly from glucose either through normal metabolic pathways (e.g., glycolysis, see Fig. 1) or it may be generated by auto-oxidation of glucose (which yields the pentose sugar arabinose). Ascorbate may also serve as a precursor; oxidation of ascorbate yields the highly reactive threose and xylose.

detect pyrraline in albumin from control and diabetic patients, using an ELISA and a Western blot. They also reported they were unable to detect pyrraline at any time point in *in vitro* studies in which bovine serum albumin was incubated with sugar *in vitro*.

To clarify this issue, Monnier's group developed a method for quantification of pyrraline by reverse-phase HPLC of alkaline hydrolysates (35). Time- and sugar concentration–dependent increases in pyrraline formation were observed in serum albumin incubated with either 100 m*M* glucose or 50 m*M* 3-deoxyglucosone. Formation of pyrraline from 3-deoxyglucosone was rapid at slightly acidic pH, confirming its synthetic pathway through this Maillard reaction intermediate. Low levels of pyrraline (<10 pmol/mg protein) were also detected in a pool of human skin collagen, although no age effect was apparent. Pyrraline-like material was detectable in human plasma proteins using a slight modification of the techniques used *in vitro*.

Monnier and coworkers have also investigated mechanisms of pyrraline cross-linking. They found that oxidation of pyrraline may result in the formation of ether bonds between pyrraline and cysteine residues on other proteins (36). It is possible that an absence of pyrraline immunoreactivity may be attributable to its oxidation and subsequent modifications.

AGEs. The term AGE, an acronym for advanced glycation end products, refers to a heterogeneous group of chromophores and fluorophores presumably arising from reactions between sugars and lysine residues on proteins. These compounds appear to increase with age in most tissues and species (1). There is no gold standard for defining these compounds or for quantifying them. The term AGE has sometimes been used to refer to those glycation products that react with antibodies prepared according to certain criteria (11); sometimes the term is used more loosely to refer to tissue fluorescence detected at defined excitation and emission wavelengths. For immunochemical assays, polyclonal and monoclonal antibodies are generally raised against a protein, usually BSA, that has been incubated with a sugar. These antibodies do not react with early glycation products and do not react with unmodified proteins (11). Evidence has been presented for the existence of a common AGE epitope; it has further been speculated that immunologically similar AGEs are formed when different proteins are incubated with sugars (37). The relationship between these findings and Baynes' report that CML is the dominant component of AGEs remains unclear.

Other glycation products. Periodically, new glycation products are characterized; some are proposed to be the major constituent of AGEs. Nagaraj and Monnier recently reported on a novel pyrrole resulting from the glycation of ascorbate degradation products. This compound, formyl threosyl pyrrole, is formed by the condensation of an e-amino of lysine with two molecules of threose, a highly reactive sugar resulting from the oxidative degradation of ascorbate (36).

Baynes and colleagues have reported on several new markers of glyco-oxidative damage (38). In addition to CEL, the analogue of CML arising from methylglyoxal and lysine described above, they describe the characterization of three additional compounds: (i) an imidazolone adduct formed by reaction of 3-DG with arginine residues; (ii) glyoxal-lysine dimer, and (iii) methylglyoxal-lysine dimer. These last two comprise imidazolium cross-links. Nakamura *et al.* have described two fluorophores, named crosslines, which they first identified *in vitro* in a model system consisting of glucose and lysine. They have detected crosslines immunochemically *in vivo*, and propose that they may play a role in diabetic complications (39).

Regulation of Nonenzymatic Glycation. Although the Maillard reaction is not enzymatically mediated, glycation of proteins appears to be regulated at several stages *in vivo*. Some of the mechanisms that modulate tissue levels of glycation products may have arisen in response to the effects of cumulative tissue damage from glycation; alternatively, regulation of glycation products may simply be

a serendipitous effect of a given mechanism, unrelated to its primary physiological purpose.

Structural regulation: Glycation limits and site specificity. Although glucose adduct formation increases when glucose concentration increases, there appears to be a glycation limit of 2–4 moles of sugar/protein molecule. Even very large molecules appear to be subject to these limits, despite the presence of many apparently available sites for glucose adduct formation. It is possible that this glycation limit is somehow related to site-specificity of glycation. Studies of several proteins subject to glycation have shown that adduct formation occurs only on specific residues, possibly those whose structural features help to catalyze the Amadori rearrangement (40–47). Glycation of collagen is also characterized by site specificity (48). Detailed comparisons of glycation of tropocollagen, alpha chains, and isolated cyanogen bromide peptides suggest that tertiary structure does not play a role in this phenomenon, and that specificity is conferred by primary structure considerations alone.

Enzymatic regulation: 2-oxoaldehyde metabolizing enzymes. It has been known for some time that endogenous enzyme systems capable of reducing 3-deoxyglucosone exist. Kato *et al.* described two NADPH-dependent reductases active against reactive oxoaldehydes: 2-oxoaldehyde reductase and aldose reductase (49, 50). Several investigators have reported that 2-oxoaldehyde reductase is identical to aldehyde reductase (51, 52). Although both 2-oxoaldehyde and aldose reductase are highly active against methyl glycoxal and phenylglyoxal, 2-oxoaldehyde differs from aldose reductase in its inactivity toward glucose and toward alcohols. Kato *et al.* have provided *in vitro* evidence that 2-oxoaldehyde prevents glucose-derived cross-linking *in vitro,* and suggest that 2-oxoaldehyde may serve as the predominant enzyme in the detoxification of 3-deoxyglucosone and glucose (50).

Vander Jagt *et al.* (13) have also examined the specificities of human aldose reductase and aldehyde reductase towards such substrates as trioses, triose phosphates, and related three-carbon aldehydes and ketones. Both enzymes are able to catalyze the NADPH-dependent reduction of all of the substrates. Aldose reductase shows more discrimination among substrates than does aldehyde reductase and is generally the more efficient catalyst. The best substrate for aldose reductase is methylglyoxal, as discussed in detail previously.

Cellular regulation: Receptors for glycation products. Defenses against glyco-oxidative damage also exist at very late stages of the Maillard reaction. A macrophage receptor capable of recognizing advanced glycation products was first described more than 10 years ago (53). Since then other types of cell-surface receptors capable of recognizing glycation products have been identified and, in some cases, structurally characterized (54). Cell-surface receptors are described later in more detail. Whereas it has been postulated that some of these receptors, particularly those associated with macrophages, constitute an important defense against glyco-oxidative damage, it is unclear to what extent tissue levels of glycation products are actually affected by them.

Very recently, Monnier and colleagues have described another line of defense against glycation, two isoenzymes capable of degrading glycated amines and amino acids (55, 56). These enzymes, named amadoriases, were isolated from an *Aspergillus* sp. strain selected on fructosyl adamantanamine as a sole carbon source).

Consequences of Collagen Glycation

Effects on Properties of Collagen. Nonenzymatic glycation affects the physical, chemical, and mechanical properties of collagen, as discussed previously (1). Over the past 6 years, there have been relatively few reports of novel findings in this area; most recent studies confirm earlier studies using new methodology, or report findings in a tissue or species not previously analyzed. For example, Oxlund and Andreassen reported that the increases in urea break time and tensile strength in diabetic tail tendon are attributable to higher levels of browning products, but not early glycation products (59). While these conclusions are not novel, having been reported in a previous study by the same investigators (58), the experimental design was different in that aminoguanidine was used to determine which types of glycation products modulated the mechanical properties of collagen.

The relationship between collagen glycation and solubility remains problematic, as studies are conflicting. Earlier studies provided convincing evidence that the striking increase in collagen insolubility seen in diabetes could not be attributed to either increased levels of early glycation products or to increased browning products. Recently, one investigator reported that solubility is correlated with pentosidine and fluorescence levels in lung collagen in aging rats (59). However, other investigators have found that the curve of age-associated changes in solubility is different from the curve describing increases in advanced glycation products and pentosidine. Pentosidine and collagen-associated fluorescence increase fairly slowly during much of the lifespan; during the last 10%–15% of the lifespan they increase very rapidly, whereas changes in solubility show a steadier increase with age (60). While it seems likely that no direct correlation exists between the accumulation of advanced glycation products on collagen and its solubility, the precise relationship remains unclear.

Nonenzymatic glycation also affects the morphology and ultrastructure of collagen. Charonis' group has reported on the effects of *in vitro* glycation on tubular basement membrane properties (61). Morphological analysis was performed using high-resolution, low-voltage scanning electron microscopy, which revealed several significant changes following glycation. These included a 58% increase in the amount of open areas in the basement membrane meshwork, almost exclusively due to increased numbers of large open-

ings (>12.5 nm). These data suggest that the increase in large pore area may play a role in the loss of barrier function in diabetic microangiopathy and nephropathy.

Consequences of Collagen Glycation: Cell-Matrix Interactions. *Effects of matrix glycation on cell behavior.* The accumulation of glycation products on collagen affects not only the properties of collagen, but also the behavior of resident cells. Cells grown on glycated matrix differ significantly from cells grown on control matrix with respect to growth, differentiation, motility, gene expression, and response to cytokines (1). Charonis' group, for example, has looked at the effects of basement membrane glycation, particularly the effects of glycated laminin and glycated type IV collagen, on endothelial cell behavior. They found that adhesion and spreading of endothelial cells were decreased when glycated collagen and laminin were used as substrates. They suggest that such glycation-induced changes may constitute a signal for other phenotypic modifications of cells in the microvasculature (62). Similar results were obtained with mesangial cells grown on isolated type IV collagen or on intact complexes of glomerular basement membrane and mesangial matrix, even after relatively modest glycation. Morphological differences between cells plated on glycated substrate and cells plated on control substrate could be seen within 5 min of plating (63). Matrix glycation is also capable of affecting cellular phenotype and intracellular signalling. Using Western blotting, Hasegawa *et al.* (65) found that tyrosine phosphorylation was decreased in a focal adhesion kinase that regulates phosphorylation of a focal adhesion protein (64). Actin filaments were also disorganized in cells grown on glycated matrix.

When cells are grown on a complex matrix, what happens if matrix components are glycated to different degrees? That is, does the glycation of some matrix components have more effect than glycation of others with respect to cell behavior? There are few systematic data on this point. However, some investigators have studied cells grown on complex matrices in which only a single component is glycated. For example, glycation of laminin alone appears to affect proliferation and spreading of endothelial cells (65).

Receptors for glycation products. How does matrix glycation affect cell behavior? Recent studies suggest that many of the effects are mediated by cell receptors, an area of intense investigation at present. Receptors capable of binding to glycation products have been identified on macrophages, monocytes, lymphocytes, endothelial cells, mesangial cells, and fibroblasts. Structural characteristics of several receptors for glycation products have been elucidated. At present there does not appear to be a consensus among investigators in the field regarding the relative distribution and importance of the different receptors identified thus far.

AGE receptor. Receptors for advanced glycation products were first identified on macrophages more than 10 years ago by Vlassara's group (53). They observed that the receptors recognize glycated proteins but not unmodified proteins or Amadori products, and that they stimulate the release of IL-1 and TNF-a. This group subsequently isolated two cell-surface proteins from a murine macrophage cell line: a 90-kD protein that recognized FFI (a synthetic compound structurally similar to AGEs) and a 60-kD protein that recognized albumin-AGE (54). The two cell-surface proteins appear to be closely associated; antibodies to either of them block binding of glycation products. Sequence data for the receptor proteins indicate that these cell-surface receptors are not the same as RAGE, the receptor described by Stern and coworkers (66). AGE-receptors have been detected on T lymphocytes as well as on monocytes and macrophages.

Scavenger receptor. Macrophages also have another type of receptor capable of binding to advanced glycation products, the so-called scavenger receptor, first reported by Goldstein (67). Initially this receptor was thought to be specific for binding to oxidized LDLs. Araki and colleagues have shown that, at least *in vitro,* the scavenger receptor also binds glycation products, and mediates its endocytic uptake and degradation (68). The extent to which this receptor contributes to endocytosis of AGE *in vivo* remains to be determined.

RAGE. Stern's group has reported finding three cell-surface proteins that recognize glycation products (69, 66). Based on sequence analysis, these receptors are distinct from the AGE receptor described by Vlassara *et al.* as well as from the scavenger receptor of Goldstein. The cell-surface proteins include a novel 35-kD polypeptide that is part of the immunoglobulin superfamily (based on 3 immunoglobulin-like regions), a 90-kDa polypeptide that is either closely related or identical to milk-derived lactoferrin (LF-1), and a 20-kDa polypeptide identical to bovine high mobility group 1 protein (70). Stern *et al.* have named the 35-kDa polypeptide RAGE (receptor for AGE), and report that it is part of a 404-kDa polypeptide containing a transmembrane spanning domain and a short, charged cytosolic tail (66). LF-1 forms a noncovalent, high-affinity bond to RAGE, which remains anchored to the cell surface. Antibodies to either LF-1 or to RAGE block binding of advanced glycation products. RAGE has been shown to be widely distributed among different tissues and different cell types (71).

Ligand binding by RAGE has many different effects, including induction of endothelial oxidant stress and increased vascular permeability (72). Both of these effects can be blocked by anti-RAGE IgG or by antioxidants. Activation of RAGE can also be blocked by infusing so-called soluble RAGE (sRAGE), the extracellular domain of the membrane-bound receptor (69). sRAGE blocks AGE ligands so that they cannot interact with cell-bound RAGE. Effects secondary to the increased oxidative stress, such as induction of the transcription factor NF-kB and increased expression of heme oxygenase 1, are also blocked. How does ligand binding produce these deleterious effects on

cells? Two mechanisms have been postulated for the induction of oxidative stress: physical proximity of the glycated protein, an oxidizing source, to the cell membrane, and stimulation of signal transduction pathways that generate intracellular oxidants. Increased vascular permeability may result in part from the increased expression of IL-6, tumor necrosis factor *a,* and other growth factors and cytokines that are induced by ligand binding.

Stern's group has also shown that RAGE in vascular endothelial cells is capable of binding circulating glycated proteins. Using radiolabeled probes, Stern's group showed that RAGE can bind glycated albumin, which is then taken up into intracellular vesicles and stored or transported to the abluminal cell surface (69).

Stern *et al.* have shown that cellular response to soluble AGEs differs markedly from response to immobilized AGE. Soluble ligand induces monocyte migration, probably in response to a concentration-gradient activation, while bound ligand slows monocyte migration. They speculate that matrix-bound glycation products could decrease wound healing response by slowing down migration of cells to the site of injury. Genetically diabetic mice, for example, have depressed wound healing in part because of decreased migration of monocytes, which could be due to increased glycation of matrix.

Clinical Correlations. *Collagen glycation and aging.* The relationship between aging and nonenzymatic glycation has been recognized and studied for more than 20 years. Most early studies, conducted before specific glycation products were identified, were largely descriptive (1). Descriptive studies continue to appear in the literature, particularly when new glycation products have been identified. Such studies may have value in confirming or expanding upon earlier reports in which nonenzymatic glycation was assayed by tissue fluorescence alone, and by elucidating the relationship between changes in fluorescence and changes in specific products. The fact that descriptive studies continue to be pertinent underscores the many difficulties inherent in studying nonenzymatic glycation in vivo.

Baynes and coworkers reported on age-associated changes in skin collagen glycation in subjects aged 20–85. They measured the specific glycation products CML, CMhL, and pentosidine as well as collagen-associated fluorescence (73); glucose adduct content was also measured. They observed a 5-fold increase in tissue content of all of the specific advanced glycation products as well as a 5-fold increase in fluorescence; in contrast, glucose adducts increased by only 33%. This study shows consistent effects of aging on the different markers of glyco-oxidative damage. The study also shows that glucose adduct concentration is not correlated with glyco-oxidative damage during aging. Previously there have been controversy regarding the effects of aging on glucose adduct content (1).

Marion and Carlson (74) have reported on the effects of aging on glucitolyllysine and pyrraline content of bovine lens capsule and Descemet's membrane. Using monoclonal antibodies, the investigators found that pyrraline increased approximately 3-fold in lens capsule, whereas glucitolyllysine did not. No significant changes were seen in Descemet's membrane. The investigators speculate that advanced products other than pyrraline may accumulate in Descemet's membrane, which could account for the significant increases in fluorescence and resistance to trypsin digestion.

In several recent studies on *in vivo* aging, glycation has been assessed solely by tissue fluorescence. Whereas such studies may be useful archivally, especially when the authors are attempting to do more than measure accumulation of glycation products, these studies are inherently limited by the nature of the assay itself, which does not identify or quantify a specific product. For example, Odetti *et al.* (75) studied the rate at which skin collagen fluorescence increased in subjects aged 42–78. They found that the increase was exponential; the rate was not correlated with sex, body weight, or vascular pathology. Odetti *et al.* (76) have also investigated the *in vivo* relationship between glycation and lipoperoxidation during aging. Activity in the reaction pathways was assessed by measuring fluorescence in skin at several different sets of wavelengths. Significant correlation coefficients were found between the age-adjusted fluorescence intensities, from which the authors conclude that a close relationship exists between glycation and oxidation. Although the conclusion is reasonable, it is unlikely that any detailed understanding of the complex relationship between glycation and peroxidation will be gained from inferential studies of fluorescence.

Collagen glycation and diabetes. As new assays for specific glycation products have been developed, they have allowed investigators to further explore the role of glycation products in long-term complications of diabetes. Recent studies in which specific glycation products are measured not only in tissue, but also in blood and urine, have contributed to our understanding of the complex etiology of complications.

Dyer *et al.* (77) reported on a study in which they measured fructoselysine (the initial glycation adduct), CML, pentosidine, and fluorescence in skin samples from type I diabetic subjects and age-matched control subjects. In diabetic subjects fructoselysine increased 3-fold, relative to age-matched controls, while CML, pentosidine, and fluorescence were increased 2-fold relative to controls. They also reported on the correlation between levels of glycation products and severity of diabetic complications in these patients (78). They found that fructoselysine, CML, pentosidine, and collagen-associated fluorescence levels were correlated with the severity of retinopathy, and were also elevated in early nephropathy. There was no association between levels of glycation products and the decrease in joint mobility. Fructoselysine and CML were found to be independently associated with retinopathy and nephropathy. The authors conclude that levels of glycation products in skin are correlated with functional abnormalities in other tissues.

Does tight glycemic control affect levels of glycation products, and are these changes correlated with clinical status? Lyons *et al.* (79) measured fructoselysine, pentosidine, collagen-associated fluorescence, CML, and CMhL in insoluble skin collagen before and after a 4-month period of intensive therapy to improve glycemic control. Glycated hemoglobin fell from 11.6% to 8.3%, accompanied by a significant decrease in fructoselysine. However, levels of pentosidine, CML, CMhL, and fluorescence were unchanged. From these data the authors infer that once cumulative damage to collagen has occurred, it cannot be reversed easily.

Beisswenger *et al.* (80) measured levels of advanced glycation products in skin samples from type I diabetic subjects, using an ELISA, to see if increased levels could be detected before the onset of microvascular complications. They found that levels of immunoreactive glycation products were increased in subjects during the premicroalbuminuric phase of nephropathy ($\leq$28 mg/24 hr), and continued to increase as subjects developed microalbuminuria and macroalbuminuria. Increased levels were also detectable during the earliest stages of retinopathy. In contrast, there were no detectable increases in pentosidine or tissue fluorescence during these earliest stages of microangiopathy.

The role of glycation products in the susceptibility of diabetic subjects to macrovascular disease has been investigated by Nakamura *et al.* (81). Using immunohistochemical techniques to detect advanced glycation products, they examined coronary arteries and atherosclerotic plaques in control and diabetic subjects. They found that immunoreactive glycation products (AGEs) were present in plaques from some diabetic subjects, but not in plaques from control subjects.

In addition to looking at tissue levels of glycation products, investigators have also reported on glycation products in blood and urine. Recently it has been reported that diabetic subjects have increased levels of circulating glycation products. Niwa *et al.* (82) found a marked elevation in serum levels of 3-deoxyglucosone, the highly reactive sugar fragmentation product, in uremic patients as compared with control subjects. Niwa *et al.* (83) have also reported that serum 3-deoxyglucosone was higher in diabetic patients with nephropathy as compared with diabetic subjects without nephropathy. Similar results have been found in experimental diabetes. Yamada *et al.* (84) reported that plasma-free 3-deoxyglucosone levels were significantly higher in STZ rats relative to controls. Advanced glycation products, detected immunochemically, have also been reported to be associated with hemoglobin; levels of such modified hemoglobin are increased in diabetic subjects (85). It has been suggested that Hb-AGE may be superior to glycohemoglobin as an index of long-term control, and hence, long-term tissue damage.

Peptides containing advanced glycation products, sometimes referred to as AGE-peptides, have been detected in the circulation, and are presumed to comprise the degradation products of glycated proteins. Recent studies have shown that levels may be increased up to 8-fold in diabetic subjects (54), a finding of particular pertinence, since these peptides are capable of inducing pathological changes in the vasculature, including increased permeability, monocyte extravasation, and blunting of response to vasodilatory agents (86). Cohen *et al.* showed that vascular damage in experimental diabetes could be prevented by infusing monoclonal antibodies capable of binding to circulating glycated protein (87). Mitsuhashi *et al.* (1997) described the use of lysozyme matrix to remove AGE-modified proteins from uremic sera; they suggest that depletion of toxic AGES from the circulation might attenuate associated complications (88).

Glycation products are also detectable in urine; recent studies have shown that urinary concentration of such products is increased in diabetic subjects. Baynes' group reported increased fructoselysine and CML in urine from diabetic subjects. The urinary concentration of fructoselysine correlated strongly with glycohemoglobin measurements (4). Monnier's group has reported that diabetic subjects excrete more than twice as much pentosidine per mole of creatinine as do diabetic subjects.

The effect of dialysis on circulating levels of glycation products as well as on tissue levels has recently been the subject of study. Makita *et al.* (89) observed that dialysis did not clear circulating AGE-peptides efficiently, whereas renal transplantation normalized serum AGE-peptide levels. Makita *et al.* also compared the efficacy of different dialysis treatments on circulating AGE-peptides. Diabetic subjects receiving so-called high flux hemodialysis had significantly lower levels of AGE-peptides than patients on conventional hemodialysis (89).

Friedlander *et al.* have raised questions concerning the effects of peritoneal dialysis fluid, which contains glucose, on tissue levels of glycation products (90). They found that peritoneal tissues in contact with fluid had increased levels of pentosidine; however, plasma levels of pentosidine were lower in patients on peritoneal dialysis as compared with patients on hemodialysis. They speculate that the peritoneal membrane may clear protein-associated pentosidine to some extent, thus accounting for lower steady-state levels.

Takahashi *et al.* have reported on another effect of glycation in diabetes that may contribute to the development of complications. They found that aldehyde reductase (52), the enzyme that degrades 3-deoxyglucosone, is glycated in diabetes, and that the glycated form is much less efficient than the nonglycated enzyme. This could result in increased levels of 3-deoxyglucosone, one of the propagators of the Maillard reaction.

Interventional Strategies

Glycation and oxidation damage many proteins, not just matrix macromolecules. Plasma proteins, red blood cells, lipids, DNA, intracellular transduction enzymes, and antioxidant enzymes all sustain glyco-oxidative damage over time. Damage catalyzes damage, as glycation and oxi-

dation generate reactive fragmentation products, free radicals, and charged molecules that amplify the original reactions. Thus, damage to one protein ultimately damages all proteins. As we learn more about glyco-oxidative damage, and how its web of reaction, interaction, and amplification extends to all levels of an organism's functioning, the need to find effective means of attenuating the damage has become increasingly important. There are several different strategies for doing this; to some extent they arise from different conceptual frameworks but are by no means mutually exclusive. The major types of strategies are discussed below. Two agents, aspirin and aminoguanidine, are then reviewed in more detail, as they illustrate some of the pitfalls inherent in this task.

Survey of Strategic Approaches. One of the earliest strategies for decreasing accumulation of glycation products consisted of blocking glucose adduct formation, on the assumption that Schiff base–Amadori products serve as precursors for most of the advanced glycation products. Many compounds have been shown to be effective at blocking glucose adduct formation, at least *in vitro,* including acetylsalicylic acid (91–96), acetic anhydride (91), ibuprofen (97), glutathione (93, 97), diclofenac (98), and the dibasic amino acids lysine and arginine (91, 99, 100). One of the most extensively studied adduct blockers is aspirin, discussed in detail below. Recent studies have suggested that blockade at this early stage may not be the most effective approach for blocking formation of advanced glycation products. There is at present a considerable body of evidence suggesting that formation of advanced glycation products is not rate dependent on glucose adduct formation; it is also clear that alternative pathways for advanced glycation product formation exist that bypass Amadori products entirely. Thus, it may be more effective to block the sugar fragmentation products, which may represent—if not the final common pathway—at least a common pathway. One such agent is aminoguanidine, discussed in detail below. The polyol pathway has been another interventional target, exemplified by inhibitors of aldose reductase. However, despite extensive studies of this class of agents, the approach does not appear to have lived up to its initial promise. An alternative approach to intervention consists of blocking a recurrent type of reaction rather than blocking a specific reaction pathway. To this end, agents with antioxidant properties have received attention recently.

Very recent studies by Brownlee's group have shown that Vitamin E, for example, prevents the formation of intracellular advanced glycation products in the arterial wall of STZ diabetic rats (101). The natural antioxidant lipoic acid has also received attention as a potentially promising therapeutic agent (102). Dietary restriction represents yet another strategy for attenuating glyco-oxidative damage, and, based on animal studies, the most replicable and effective. Recent studies of lifetime calorie restriction in rats, mice, and monkeys showed that calorie restriction was associated with a significant decrease in collagen-associated fluorescence, pentosidine, and CML in several tissues (60, 103). Effects varied between tissues and between species. It is of particular interest that two studies have shown that even lifetime caloric restriction does not manifest its effects on glyco-oxidative damage until late in the lifespan. The mechanisms underlying the remarkable effects of calorie restriction are still unknown. Despite vast literature on the subject (104–106), there is no consensus as to the mechanisms by which dietary manipulation affects lifespan or even how it affects individual physiological systems.

Two Specific Agents. *Aspirin.* Aspirin has been extensively studied as an agent capable of blocking protein glycation by acetylating lysine residues. Among the substrates investigated are type-I collagen (92), basement membrane collagen (95), albumin (91), lens crystallins (94), and hemoglobin (91). Studies have shown that pretreatment with aspirin can prevent some of the changes in physical properties of collagen associated with increased glycation; for example, Yue *et al.* showed that tail tendon treated with aspirin and then incubated with glucose did not manifest the increase in thermal rupture time associated with increased glycation. However, subsequent studies by Yue *et al.* (92) raised questions concerning the validity of extrapolating from *in vitro* results to *in vivo* results. They found that both aspirin and sodium salicylate were effective in blocking glycation of tail tendon collagen *in vitro,* and in preventing the increase in thermal rupture time associated with increased glycation. However, administration of aspirin and sodium salicylate to diabetic rats *in vivo* prevented the increase in thermal rupture time in the diabetic animals *without* blocking the increase in nonenzymatic glycation of collagen. Yue *et al.* speculated that aspirin and salicylate might be modulating the effects of hyperglycemia on collagen by other mechanisms; they raised the possibility that other known actions of aspirin, such as modulation of prostaglandin metabolism, might play a role, or that aspirin might block a later stage of the Maillard reaction *in vivo* by an as yet undetermined mechanism. Rendell *et al.* (91) also reported on aspirin administration. They questioned Yue's interpretation, suggesting that since collagen is not exposed to circulating aspiring, the drug may need to be administered for a longer period than was done by Yue *et al.* for its effect on glycation to be appreciated.

Recently, Baynes and coworkers attempted to resolve some of these conflicting data (107). Through a series of carefully controlled *in vitro* studies, they found that while aspirin did not affect formation of sugar adducts, it did block later stages of the Maillard reaction—it inhibited formation of cross-linking, fluorescence, and the glyco-oxidation products CML and pentosidine. The investigators conclude that it is aspirin's antioxidant activity that is responsible for its efficacy. They further speculate that the results reported earlier in the literature concerning the ability of aspirin to acetylate lysine may be attributable to any number of factors, particularly maintenance of pH, as the experiments were technically demanding.

Baynes' *in vitro* findings are consistent with a very recent clinical study of aspirin. Contreras *et al.* reported on a one-year study of aspirin administration (200 mg/day) to type-II diabetic subjects. Skin biopsies were obtained at the start and end of the study and analyzed for glycation products. Subjects who received aspirin showed no change in glucitolyllysine levels of skin collagen whereas pentosidine content was significantly decreased (108).

Aminoguanidine. Aminoguanidine is a small hydrazine compound, first reported more than 10 years ago (53) to block formation of advanced glycation products. Although some studies since then have reported that aminoguanidine inhibits the accumulation of collagen-associated fluorophores in skin, lens proteins, kidney, tail tendon, bladder, and aorta in experimental diabetes, other studies have reported conflicting results (109–114). The lack of consensus over efficacy of aminoguanidine stems, in part, from the lack of consensus over how to assess its efficacy, or indeed the efficacy of any strategy for attenuating glyco-oxidative damage. This is hardly surprising in a field where investigators cannot even agree on the structure and characterization of the glycation products they wish to affect. In the case of aminoguanidine, studies differ with respect to virtually every conceivable parameter, including aminoguanidine administration (dose, route of administration, frequency of administration, and duration), experimental model (species, genetic versus induced diabetes, dose and frequency of administration of STZ), timing of the initiation of aminoguanidine administration relative to induction of diabetes, range of hyperglycemia values in the experimental model acceptable for the study, methodology used for assessing severity of diabetes and/or complications such as nephropathy, and—most striking—specific methods used for measuring advanced glycation products. For example, differences in the technique used to solubilize tissue for measuring fluorescence could alone account for some of the discrepancies. Solubilizing agents ranged from highly purified collagenase, specific for collagen, to a mixture of pepsin and proteinase K, a technique capable of solubilizing other matrix proteins including elastin, which also accumulates fluorophores. Given that these differences in a single step of one assay can have profound effects on the results, the cumulative effect of differences between studies at virtually every stage of the experimental design can hardly be overemphasized.

The mechanism of action of aminoguanidine has also been the subject of controversy. Although it was initially proposed that aminoguanidine inhibited the formation of advanced glycation products by binding to Amadori products (115), more recent studies have suggested that it binds to glucose fragmentation products (116, 117). Aminoguanidine exerts other pharmacologic effects, such as inhibition of nitric oxide synthase, that may also affect glyco-oxidation; the relative importance of these effects is the subject of controversy.

Summary and Conclusions

How can we summarize the progress that has been made in the field since this topic was previously reviewed? Should we feel optimistic that the pieces of the puzzle are falling into place, as suggested recently (118), or do we have a disquieting sense that as we work out the details in one area—all the reaction pathways, products, side reactions—the perspective shifts and the puzzle of glycation, like a fractal, regenerates complexity? Perhaps both are appropriate. Glyco-oxidative damage represents the interface between the orderly workings of genomic intelligence and the disorganizing force of stochastic events. This interface, the cusp of our mortality, is the Faustian bargain of evolution. The passage from the unconscious immortality of unicellular existence to the complexities of sentient life required the development of an oxidative metabolism, the slow fire that consumes us. Until now we have only been able to watch it and study it; whether we can change it remains to be seen.

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