Nonenzymatic Glycosylation and the Pathogenesis of Diabetic Complications

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Glucose chemically attaches to proteins and nucleic acids without the aid of enzymes. Initially, chemically reversible Schiff base and Amadori product adducts form in proportion to glucose concentration. Equilibrium is reached after several weeks, however, and further accumulation of these early nonenzymatic glycosylation products does not continue beyond that time. Subsequent reactions of the Amadori product slowly give rise to non-equilibrium advanced glycosylation end-products which continue to accumulate indefinitely on longer-lived molecules. Excessive formation of both types of nonenzymatic glycosylation product appears to be the common biochemical link between chronic hyperglycemia and a number of pathophysiologic processes potentially involved in the development of long-term diabetic complications. The major biological effects of excessive nonenzymatic glycosylation include: inactivation of enzymes; inhibition of regulatory molecule binding; crosslinking of glycosylated proteins and trapping of soluble proteins by glycosylated extracellular matrix (both may progress in the absence of glucose); decreased susceptibility to proteolysis; abnormalities of nucleic acid function; altered macromolecular recognition and endocytosis; and increased immunogenicity.

OVER THE LAST 15 years, data from many clinical, morphologic, and biochemical investigations have established that hyperglycemia is the single metabolic consequence of insufficient insulin action most responsible for the development of chronic diabetic complications (1-3). Clinicians have responded to this emerging consensus by advocating intensive diabetic treatment regimens as a preventive therapeutic measure (4). Unfortunately, recent clinical trials suggest that when complications are already present, improvement of glycemic control alone may not be sufficient to prevent the continued progression of these pathologic processes (5, 6). For this common clinical problem, optimal future therapy may require pharmacologic agents that directly interfere with the self-perpetuating component of hyperglycemia-initiated tissue damage. Before such new drugs can be developed, the biochemical basis of progressive diabetic complications must be better understood.

There appear to be two general pathophysiologic mechanisms by which hyperglycemia leads to irreversible tissue damage. Intracellular hyperglycemia can result, by increased flux through different metabolic pathways, in altered steady-state levels of metabolites and synthetic products that may ultimately affect function adversely. This mechanism gives rise to quantitative and qualitative changes in glomerular basement membrane glycoprotein and proteoglycan components, biochemical alterations in peripheral nerve myelin composition, disturbances in platelet prostanoid production, and abnormalities in somatomedin and growth hormone secretion. A particularly well-studied example of this mechanism is the polyol pathway. In lens and nerve, two tissues that do not require insulin for glucose transport, increased activity of this glucose-consuming pathway has been implicated in the pathogenesis of acute diabetic cataracts and early peripheral neuropathy (7, 8). Increased polyol pathway activity results in several metabolic changes, including decreased levels of NADPH, glutathione, and myoinositol. Each of these components may have a role in the development of some diabetic complications. These processes and others that may contribute to the development of diabetic complications are reviewed elsewhere (3).

The other major consequence of hyperglycemia is excessive nonenzymatic glycosylation of proteins. In this process glucose chemically attaches to proteins without involvement of enzymes. The stable products thus formed accumulate inside insulin-independent cells, and outside on cell membrane proteins, circulating proteins, and structural proteins. Formation of glycosylated hemoglobin inside erythrocytes is the best known example of nonenzymatic protein glycosylation in vivo (9). Elucidation of the biochemical and physiologic factors involved in glycosylated hemoglobin formation led to the rapid development of clinical assays that provide previously unobtainable information about mean blood glucose levels in diabetic patients. Glycosylated adducts (the Amadori product) similar to that formed on hemoglobin have been subsequently reported in a large number of mammalian proteins (10).

Recent chemical studies of nonenzymatic glycosylation have focused on important new glycosylation adducts, advanced glycosylation end-products, which form very slowly from the Amadori product through a series of further reactions and rearrangements. In contrast to the Amadori product, these adducts, once formed, are irreversible, and continue to accumulate indefinitely on longer-lived proteins. New biological investigations over the past few years have explored major functional consequences of excessive nonenzymatic glycosylation. In this review, similarities and differences between the familiar Amadori products and the less well-known advanced glycosylation end-products will be presented first, along with a description of the factors that determine the extent of nonenzymatic glycosylation. With this information as background, major physiologic processes altered by non-

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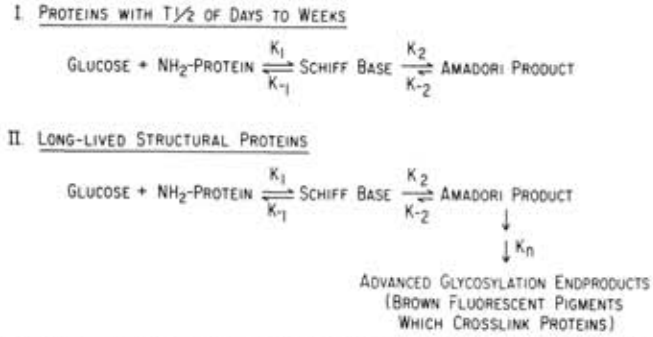

Figure 1. Formation of early reversible and advanced irreversible nonenzymatic glycosylation products. Steady-state levels of the reversible Schiff base and Amadori product are reached within hours and weeks, respectively. In contrast, irreversible advanced glycosylation end-products continue to accumulate over long periods of time.

enzymatic glycosylation will be discussed, and the possible relationship of these to diabetic complications will be considered. The central concept that emerges is that excessive nonenzymatic glycosylation appears to be the common biochemical link between chronic hyperglycemia and a number of pathophysiologic processes potentially involved in the development of long-term diabetic complications.

Types of Nonenzymatic Glycosylation

PROTEINS WITH HALF-LIVES OF DAYS TO WEEKS

Nonenzymatic glycosylation begins in all cases with glucose attachment to protein amino groups via nucleophilic addition with formation of a Schiff base (Figure 1). The labile Schiff base rapidly reaches an equilibrium level in vivo reflecting ambient glucose concentration. The rate of Schiff base formation (k_1) is approximately equal to the rate of dissociation (k_{-1}). Over a period of weeks, a slow chemical rearrangement of the Schiff base occurs (k_2 is approximately equal to 1.6% of k_1), which results in the accumulation of a stable but chemically reversible sugar-protein adduct, the Amadori product (11). Accumulation of Amadori products does not continue indefinitely, however, even on long-lived proteins, because equilibrium is reached over a period of several weeks. After equilibrium is attained, measured levels of Amadori products reach a constant steady-state value that does not increase as a function of time beyond that point. The chemistry of Amadori nonenzymatic glycosylation products formed in vivo has been extensively studied using human hemoglobin as a model protein (10, 11).

LONG-LIVED STRUCTURAL PROTEINS

In contrast to proteins whose turnover time is equal to or less than the time required to reach equilibrium amounts of the Amadori product, proteins that turn over at a much slower rate such as crystallins, collagen, elastin, and myelin accumulate post-Amadori nonenzymatic glycosylation products (10). These advanced glycosylation end-products form very slowly from Amadori products, through a series of further reactions, rearrangements, and dehydrations. Advanced glycosylation end-products are qualitatively identified by their characteristic brown pigment, fluorescence, and participation in protein-protein crosslinking (12, 13). Structure has been determined for only one advanced glycosylation end-product compound formed under physiologic conditions [2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole]. Although the rate of formation of these products under physiologic conditions is extremely slow, advanced glycosylation end-product derivatives, unlike the Amadori products, are chemically irreversible once formed. As a result, the quantity of the advanced glycosylation end-product continues to accumulate for the life of the protein at a rate proportional to the equilibrium concentration of the reversible Amadori product.

In vitro and in vivo, nonenzymatic glycosylation has been shown in a large number of biologically relevant proteins (Table 1) (9, 14-35). Methodologic difficulties limited earlier studies to isolated proteins such as hemoglobin, the lens protein crystallins, and albumin. Subsequently developed techniques made it possible to accurately quantitate nonenzymatic glycosylation in complex biological samples such as tissue homogenates, using either affinity chromatography or high pressure liquid chromatography (36-38). Only Amadori products are detected by current methods used to quantitate the extent of nonenzymatic glycosylation, however. The effect of diabetes-induced hyperglycemia on levels of Amadori products in various tissue proteins is remarkably similar to its effect on levels of Amadori products formed on hemoglobin. A twofold to threefold increase is consistently seen when diabetic tissue proteins are compared with

Table 1. Nonenzymatic Glycosylation of Proteins

Protein	Physiologic Function
Hemoglobin	Oxygen exchange
Red cell membrane	Deformability in microvasculature
Antithrombin III*	Inhibition of excessive coagulation
Fibrinogen	Plasma viscosity and clot formation
Fibrin*	Clot maintenance
Endothelial cell membrane*	Maintenance of vascular integrity
Lens crystallins†	Transmission of light to retina
Lens capsule	Focusing of light on retina
Myelin†	Nerve impulse conduction
Tubulin	Axonal transport
Glomerular basement membrane	Renal filtration barrier
Collagen†	Tissue structural properties; scar and plaque formation
Coronary artery proteins	Vessel integrity for myocardial perfusion
Low density lipoprotein	Lipid transport and metabolism
High density lipoprotein*	Lipid transport and metabolism
Albumin	Osmotic regulation; transport of metabolites
Cathepsin B*	Intracellular protein degradation
Beta-NAc-D-glucosaminidase*	Glycoprotein sugar removal
Pancreatic RNase*	Hydrolysis of RNA
Ferritin*	Iron storage

* Not yet evaluated in vivo.
† Presence of advanced glycosylation end-products documented.

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normal proteins despite different lengths of exposure to hyperglycemia ranging from 18 weeks in experimental animals to years in human autopsy specimens (28, 37). These values represent equilibrium levels of Amadori products reached relatively quickly on all proteins whose survival time is not shorter than the time required for equilibrium to be achieved.

Detection of accumulated advanced glycosylation end-products in biological macromolecules can be accomplished using spectroscopic and fluorescence techniques, but direct quantitation of specific, chemically defined products has not been possible. This inability to obtain information about the level of nonenzymatic glycosylation products beyond the Amadori product has deprived both clinical investigation and clinical practice of a means for assessing time-averaged exposure of patients' long-lived proteins to glucose over extended periods (months to years). As a result, clinical studies correlating the extent of total nonenzymatic protein glycosylation with degree of complications have not yet been feasible. Recently, determination of exact chemical structure has been accomplished for the first successfully isolated advanced glycosylation end-product protein hydrolysis product. Development of an advanced glycosylation end-product analytic method based on this work should greatly expand the scope of future research.

Factors Determining the Extent of Nonenzymatic Glycosylation

For a given protein, the extent of nonenzymatic glycosylation is determined by the sum of effects of a number of independently acting variables (10) (Table 2). The first four of these variables are fixed and constant in living systems. In vitro, pH has a profound influence. Experimentally, significant formation of the Amadori product is not seen below pH 7.0. Increasing pH over the range of 7.0 to 9.0 results in a concomitant increase in the concentration of the Amadori product reached at equilibrium. This observation is consistent with the chemical prediction that only uncharged amino groups on the protein can participate in this type of addition reaction with glucose. Increasing temperature produces a proportional acceleration of Amadori product formation rate, similar to that seen in other nonenzymatic chemical reactions. Two other factors constant in vivo are protein concentration and amino group microenvironment. With higher protein concentration, the absolute number of amino groups potentially available to react with glucose increases, whereas the protein's local environment in the areas immediately surrounding each amino group has a direct effect on that amino group's reactivity with glucose. These last factors may explain the non-random pattern of amino group glycosylation seen within a given protein, as well as differences in susceptibility to nonenzymatic glycosylation seen between different proteins (39, 40).

Glucose concentration and incubation time are the most clinically relevant variables affecting the extent of nonenzymatic glycosylation, because only these two variables have counterparts that also differ in vivo. Increasing glucose concentrations (via mass action) cause the level of accumulated Amadori products on proteins to rise in a parallel fashion. Length of incubation time is also critical, for two reasons. The first reason is that Amadori products continue to accumulate as a function of time until equilibrium is reached. The degree of pre-equilibrium or equilibrium excessive glycosylation of amino groups caused by exposure to hyperglycemia (either Schiff base or Amadori product) may be sufficient to significantly impair important functional properties of several critical proteins. The second reason is that even though steady-state levels of Amadori products do not increase beyond the time required for equilibrium to occur at a particular mean blood glucose level, advanced glycosylation end-products continue to accumulate over the entire lifetime of proteins that turn over slowly or not at all. Significant clinical consequences of continuous net advanced glycosylation end-product accumulation would be expected to arise from altered physiologic processes related to increased crosslinking within and between protein molecules, as well as from other glycosylation-associated structural changes. Accumulation of advanced glycosylation end-products on proteins such as collagen appears to occur at a rather slow rate in patients with normal glucose tolerance, and at a significantly faster rate in patients with juvenile onset diabetes (27). This information is consistent with the clinically recognized time scales over which both age-associated degenerative vascular disease in nondiabetics and hyperglycemia-accelerated atherosclerosis in diabetics develop.

Table 2. In-vitro Variables That Determine the Extent of Nonenzymatic Glycoslation and Their In-vivo Counterparts

In Vitro	In Vivo
pH	Constant
Temperature	Constant
Protein concentration	Constant
NH_2 microenvironment	Constant
Glucose concentration	Mean blood glucose level
Incubation time	Duration of hyperglycemia and protein half-life

Physiologic Processes Altered by Nonenzymatic Glycosylation

The studies shown in Table 1 demonstrate that nonenzymatic glycosylation occurs on many proteins throughout the body. These studies also confirm that the hyperglycemia of diabetes produces elevated levels of the Amadori glycosylation product on these proteins, as it does with hemoglobin. Relative increases in advanced glycosylation end-products have been seen on several diabetic long-lived structural proteins as well. The functional consequences of such excessive nonenzymatic glycosylation were essentially unknown until quite recently, however. Only now has information about the biological effects of excessive protein glycosylation accumulated to a point where a conceptual schema organizing this material can be constructed, as shown in Table 3.

ENZYME ACTIVITY

From the discussion in previous sections, it may seem

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Table 3. Physiologic Processes Altered by Nonenzymatic Glycosylation

Enzyme activity
Binding of regulatory molecules
Crosslinking of proteins
Susceptibility to proteolysis
Function of nucleic acids
Macromolecular recognition and endocytosis
Immunogenicity

that nonenzymatic glycosylation would not occur to an appreciable extent on enzymes, because most of these proteins have relatively short half-lives. However, reversible glucose-protein Schiff base compounds form quite rapidly under physiologic conditions (Figure 1), and an increased concentration of these compounds in vivo could significantly alter the catalytic properties of certain types of enzymes. The most likely inactivation mechanism would involve glucose attachment to a lysine epsilon-amino group essential for normal function at the active site. In ribonuclease A, for example, loss of a single lysine at position 41 is known to cause total loss of enzyme activity. Incubation of this enzyme with glucose for 24 hours results in a 50% loss of initial enzymatic activity, associated with nonenzymatic glycosylation of two lysine residues per molecule (34). Similar results would be predicted for a number of decarboxylases and aldolases, because their mechanism of action requires formation of reversible covalent intermediates involving unsubstituted active-site lysine amino groups (41).

A second type of enzyme whose activity appears to be influenced by nonenzymatic glycosylation is the so-called sulfhydryl protease. Enzymes of this type, such as cathepsin B from animal cells and papain from plants, are proteases that resemble in their reaction mechanism the serine proteases trypsin, thrombin, and plasmin. These enzymes differ, however, in having a cysteine rather than a serine at the active site to form covalent intermediates with substrates (41). The activity of cathepsin B isolated from human liver cells is completely abolished after incubation for 2 weeks with a glucose concentration of 300 mg/dL (32). Similarly, papain activity is reduced 70% to 90% after nonenzymatic glycosylation. In contrast, the serine proteases trypsin and chymotrypsin retain full activity after identical glucose incubations (42). The presence of critical lysine residues only in the extended active site areas of sulfhydryl proteases may explain this differential effect of glycosylation on enzyme activity.

Studies of the glycoconjugate-degrading enzyme beta-*N*-acetyl-D-glucosaminidase from kidney provide evidence that loss of activity associated with nonenzymatic glycosylation may also result from conformational changes in the molecule (33). Loss of enzyme activity was shown to progress as a function of both incubation time and glucose concentration. After 15 days incubation in 44.4 mmol glucose, activity of isoenzyme A decreased to 20% of its initial activity. This glucose-induced decrease in enzyme activity was accompanied by an increase in apparent molecular weight of the enzyme from 130 000 to 250 000, suggesting that glucose-induced changes in protein aggregation and crosslinking may contribute to loss of enzyme activity in some cases.

BINDING OF REGULATORY MOLECULES

Steady-state levels of metabolites are maintained in vivo by constant modulation of protein functional activity. One general mechanism by which maintenance is accomplished involves reversible interaction with metabolites or cofactors that are not involved in the primary reaction. Binding of these regulatory molecules alters the equilibrium between different conformational states of the protein, each of which is associated with a defined level of function. In cases where the binding of regulatory molecules requires unsubstituted N-terminal or lysine epsilon-amino groups, nonenzymatic glycosylation would be expected to inhibit effector-molecule binding.

The reversible binding of 2,3-diphosphoglycerate to hemoglobin is a well-studied model of regulatory molecule binding. In the erythrocyte, where 2,3-diphosphoglycerate and hemoglobin are found in equimolar concentrations, this negatively charged organic phosphate binds to properly oriented positively charged amino groups on tetrameric hemoglobin contributed by the N-terminal valine, lysine 82, and histidine 143 of the beta chains. Binding of 2,3-diphosphoglycerate to hemoglobin stabilizes the deoxy form and decreases the affinity of hemoglobin for oxygen. Conversely, hemoglobin structural changes induced by oxygen binding reduce the affinity of hemoglobin for 2,3-diphosphoglycerate. An equilibrium exists, therefore, between hemoglobin, 2,3-diphosphoglycerate, and oxygen, which serves as a sensitive control of oxygen exchange (43).

The functional properties of nonenzymatically glycosylated hemoglobins differ significantly from those described above for normal studied hemoglobin A_o. The first glycosylated hemoglobins to be recognized and studied were minor hemolysate components that appeared to be more negatively charged than the single major hemoglobin, Hb A_o. The functional properties of four of these components, designated Hb A_{1a1}, Hb A_{1a2}, Hb A_{1b}, and Hb A_{1c}, have been evaluated (44). Compared to Hb A_o, Hb A_{1a1} and Hb A_{1a2} have low oxygen affinities, Hb A_{1b} has high affinity, and Hb A_{1c} has moderately high affinity. Removal of organic phosphate increases the oxygen affinities of Hb A_{1b} and Hb A_{1c}, whereas the affinities of Hb A_{1a1} and Hb A_{1a2} remain low. Addition of organic phosphates substantially decreases the oxygen affinity of Hb A_{1b} and Hb A_{1c}, although to a much smaller degree than that of Hb A_o. Hemoglobin A_{1a1} and Hb A_{1a2} are essentially unaffected by addition of organic phosphate. In the presence of high concentrations of organic phosphate, all of the glycohemoglobins are still 50% saturated with oxygen at partial pressures where Hb A_o has given up most of its oxygen. Although compensatory mechanisms in vivo essentially restore oxygen affinity of diabetic whole blood to normal (45), these studies are important because they show that nonenzymatic glycosylation of proteins can significantly interfere with the binding of critical regulatory molecules.

Another protein whose regulatory activity is modified

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by nonenzymatic glycosylation is antithrombin III. This factor, after binding catalytic amounts of heparin to critical lysine residues, functions as the major inhibitor of activated serine-protease coagulation factors in plasma (46). Binding of heparin to lysine amino groups in the antithrombin III molecule is the initial and rate-determining step of the antithrombin III and thrombin reaction. This heparin-binding step, which is independent of thrombin, enhances the rate of inhibition of thrombin by antithrombin III nearly 1000-fold (47).

Nonenzymatic glycosylation of antithrombin III produces a significant decrease in thrombin-inhibiting activity in vitro (15). The degree of antithrombin III activity loss correlates with both factors determing the extent of nonenzymatic glycosylation, glucose concentration, and incubation time. Nonenzymatic glycosylation-induced inhibition of heparin-catalyzed human antithrombin III activity can be completely overcome, however, by addition of a large molar excess of heparin to the glycosylated antithrombin III before assay. This observation suggests that the antithrombin III molecule contains a lower-affinity binding site for heparin after nonenzymatic glycosylation. Nonenzymatic attachment of glucose to antithrombin III thus reduces the affinity of antithrombin III for heparin, but does not eliminate its heparin-binding capacity. Inhibition of heparin binding to human antithrombin III in hyperglycemic patients would result in a transient functional deficiency of this protein. This finding could explain the significant in-vivo inhibition of biological function of human antithrombin III that occurs in both type II and type I diabetic patients (48, 49). In type I patients the degree of reduction in antithrombin III activity was directly related to the level of both Hb A_{1c} and fasting blood glucose. Glycosylation-induced interference with antithrombin III activity could explain the accelerated disappearance rate of fibrinogen that is normalized by improving glucose control in hyperglycemic diabetic patients (50). A specific effect of nonenzymatic glycosylation on antithrombin III heparin binding affinity would also explain why infused heparin alone corrected the accelerated disappearance of fibrinogen in poorly controlled diabetics.

In vivo, the glucose-induced defect in heparin binding to antithrombin III may well be accentuated by a markedly reduced heparan sulfate content in several diabetic tissues (51). This inhibition of heparin-catalyzed antithrombin III activity by nonenzymatic glycosylation, in conjunction with glycosylation-induced changes in susceptibility of fibrin to degradation, could play a role in the abnormal accumulation of fibrin reported to occur in several diabetic tissues affected by long-term complications (52-55).

CROSSLINKING OF PROTEINS

Extensive early studies of nonenzymatic glycosylation showed that this process ultimately gave rise to pigmented, fluorescent, glucose-derived protein crosslinks (12, 13). The incubation conditions used in these studies were nonphysiologic, however, and a degree of uncertainty existed concerning the formation of glucose-derived crosslinks under diabetic conditions. The ability of hyperglycemia to aggregate and crosslink proteins under physiologic conditions in vitro and in vivo was first shown using the lens protein crystallins (56). Incubation of lens proteins with glucose or glucose-6-phosphate resulted in opacification of clear protein solutions as nonenzymatic glycosylation of lysine epsilon-amino groups increased. This opacification was shown to be a consequence of high molecular weight, light-scattering protein aggregate formation. Addition of disulfide-bond reducing agents such as dithiothreitol substantially reduced the degree of glucose-induced protein aggregation. Similar data were obtained from in-vivo studies with diabetic animals (19). These observations suggest that protein conformational changes induced by nonenzymatic glycosylation render some previously unexposed sulfhydryl groups susceptible to oxidation. In vivo, lens proteins are normally protected against oxidation-induced crosslink formation by intracellular reduced glutathione. In the diabetic lens, decreased levels of reduced glutathione resulting from increased polyol pathway activity may act synergistically with nonenzymatic glycosylation to cause accelerated formation of disulfide bonds (57).

Lens proteins examined after longer-term incubation under physiologic conditions showed the presence of covalent crosslinks that were not disulfide in nature (20). Concomitantly, these glycosylated protein solutions developed pigment and showed spectroscopic properties similar to those of lens proteins from brunescent human cataracts. More rapid accumulation of these glucose-derived crosslinks could contribute to accelerated senile cataract formation in diabetic patients.

Extensive glucose-derived crosslink formation may also contribute to the reversible defects in axoplasmic transport seen in experimental diabetic neuropathy (58). In vitro, rapid formation of non-reducible, high molecular weight protein aggregates occurs during nonenzymatic glycosylation of the neuronal microtubular protein, tubulin (23). In vivo, tubulin prepared from diabetic rats shows an increased quantity of nonreducible, high molecular weight material, corresponding to a dramatic increase in the amount of nonenzymatic glycosylation. Despite this nonenzymatic glycosylation-induced increase in tubulin aggregate formation, however, guanosine triophosphate-dependent tubulin polymerization is profoundly inhibited, because normal polymerization of monomers into functional microtubules requires unsubstituted lysine residues.

Glucose-derived crosslinks also form when reactive groups generated by nonenzymatic glycosylation of long-lived structural proteins trap potentially damaging non-glycosylated soluble proteins. Experimentally, addition of serum albumin or IgG to nonenzymatically glycosylated collagen washed free of glucose resulted in binding of both these proteins to the collagen (59). Furthermore, the albumin and anti-bovine serum albumin IgG that bound to nonenzymatically glycosylated collagen retained their ability to form immune complexes in situ with the corresponding free antibody and antigen (Figure 2). These observations provide a biochemical expla-

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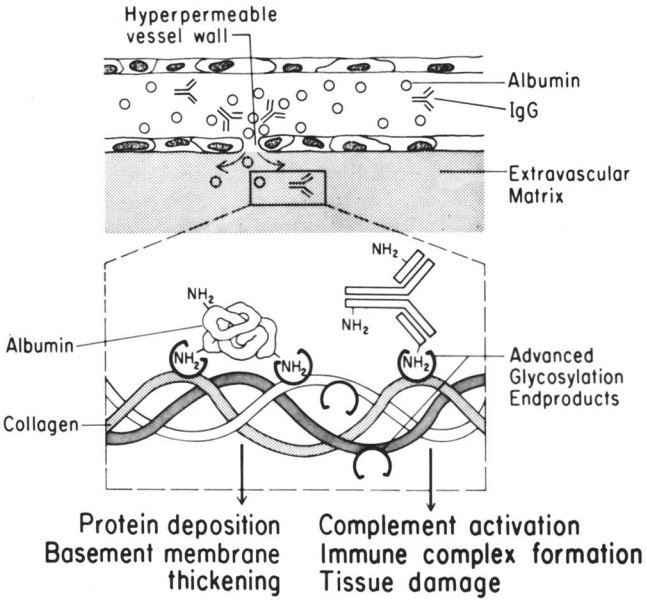

Figure 2. Covalent trapping of potentially damaging nonglycosylated plasma proteins by advanced glycosylation end-products on collagen. Increased diabetic vascular permeability would accelerate this process.

nation for the intense linear immunofluorescent staining for albumin and IgG characteristically seen in diabetic extravascular membranes (60). Binding of these serum proteins to diabetic kidney has been shown to be essentially undissociable. Persistent accumulation of circulating proteins such as albumin may contribute to the characteristic thickening of diabetic basement membranes, and trapping of IgG may be responsible for the activated-complement (poly C9) membrane-attack complex deposition that occurs in diabetic kidneys (61). In addition, covalent attachment of soluble proteins by nonenzymatically glycosylated collagen may be the first step in a process of in situ formation of immune complexes in some diabetic tissues, where discontinuous granular ("lumpy-bumpy") deposits of immunoglobulins have been identified as immune complexes by electron microscopy (62). The reported production of pseudodiabetic glomerular basement membrane thickening in normal mice after repeated injections of glucosylated proteins may involve glucose-derived crosslinks with free amino groups in the glomerular extracellular matrix (63), but direct evidence for this has not been obtained. Binding of plasma protein constituents to nonenzymatically glycosylated perivascular structural proteins may be enhanced in vivo by the well-described increase in vascular permeability associated with diabetes in both animals and humans.

The ability of nonenzymatically glycosylated collagen to trap soluble proteins after being washed free of glucose suggests that glucose itself may not be a required participant in protein crosslinking. The clinical implications of this possiblity are sobering. If glucose-derived protein crosslink formation can progress in the absence of glucose, then perfect correction of hyperglycemia in diabetic patients in the future may not prevent the continued progression of diabetic complications. In-vitro evidence has recently come from a model system using the protein RNase A (34). After extensive nonenzymatic glycosylation of this protein, the rate of continuing crosslink formation was determined in both the absence and presence of glucose. The observed rates of continued protein crosslinking were essentially identical. If continuing crosslink formation occurs in vivo, then additional pharmacologic approaches may ultimately be required to directly interfere with these processes.

To develop such pharmacologic approaches, more complete chemical information will be needed about glucose-derived protein crosslinks formed under physiologic conditions. A recent advance towards this end has been the isolation of an advanced glycosylation end-product-protein hydrolysis product involved in crosslinking, followed by determination and confirmation of its exact chemical structure (64). The isolated product has spectral properties identical to those that characterize intact advanced glycosylation end-product proteins. From nuclear magnetic resonance, mass spectroscopy, and chemical derivatization studies, this compound has been assigned the novel structure 2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole (FFI). This compound is a condensation product of two glucose molecules and two lysine-derived amino groups into a conjugated system of three aromatic heterocycles (Figure 3). Formation of FFI could occur through interaction of two Amadori products, but this would not explain the previously discussed ability of advanced glycosylation end-product proteins to trap other unglycosylated proteins. The FFI crosslinks between glycosylated and nonglycosylated protein amino groups could form, however, if nonglycosylated protein amino groups reacted with either a monoglycosylated Amadori product, followed by addition of a second glucose molecule, or with a diglycosylated Amadori product alone.

SUSCEPTIBILITY TO PROTEOLYSIS

Accumulation of collagen-related proteins in the glomerular extravascular matrix is the central pathologic alteration that characterizes diabetic nephropathy. Continual accumulation over many years ultimately results in progressive renal failure due to progressive glomerular capillary occlusion. In experimental animals, some of the morphologic features of diabetic renal disease are reversible by islet transplantation. Diabetes-induced glomerular basement membrane thickening does not regress, however, suggesting that its susceptibility to physiologic degradative mechanisms may be abnormally low (65, 66). In vitro, susceptibility of nonenzymatically glycosylated glomerular basement membrane preparations to digestion by nonspecific general proteases such as pepsin, papain, and trypsin, is considerably reduced (67). Similarly, susceptibility of tendon collagen from diabetic patients to digestion by nonspecific protease activities such as bacterial collagenase and pepsin is also significantly reduced (68). The collagen cleavage products released by pepsin have more high molecular weight components, and the pepsin-resistant portion more associated Amadori glycosylation products, than the corresponding fractions from normal subjects.

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In collagen from normal subjects, an age-related increase in diabetic-like changes is seen. Both basement membrane and the structurally related collagens undergo nonenzymatic glycosylation in vitro and in vivo (21, 24-26). Collagen from nondiabetics shows an age-related linear increase in advanced glycosylation end-product pigment accumulation, and associated age-related changes in collagen mechanical properties consistent with increased crosslink formation (27, 69, 70). The acceleration of both of these processes by diabetes could result in reduced susceptibility of diabetic glycosylated basement membrane and collagen to in-vivo proteolysis. Further studies using the appropriate mammalian enzymes will be necessary to demonstrate this finding, however. Abnormal accumulation of several other proteins in particular diabetic tissues may reflect the same general pathophysiologic mechanism.

One such protein appears to be fibrin. Excessive nonenzymatic glycosylation of circulating fibrinogen has been shown in diabetic patients, and after fibrin deposition, the degree of glycosylation could increase even further (16). This nonenzymatic glycosylation of fibrin would be expected to reduce susceptibility to degradation by the specific fibrinolytic enzyme plasmin, because this protease cleaves only at substrate arginine and lysine peptide bonds. Using both a fibrin plate assay and a fluorogenic synthetic plasmin substrate assay, it was found that glucose blocking of the epsilon-amino group of lysines in the fibrinogen and fibrin molecule interferes with the specific fibrinolytic enzyme-substrate interaction (17). Acetylation and carbamylation had qualitatively similar effects, showing that chemical modification of lysine amino groups, rather than crosslink formation, is the underlying phenomenon responsible for the degradative defect produced by glucose. Experimental conditions that increased the rate of nonenzymatic protein glycosylation were associated with correspondingly greater degrees of resistance to degradation by plasmin. Analogous reductions in susceptibility to degradation would be expected consequences of excessive nonenzymatic glycosylation in other proteins cleaved preferentially at substrate lysine residues. Extensive glucose-derived crosslinking of long-lived structural proteins discussed above may also reduce susceptibility to degradation by proteases cleaving at non-lysine residues.

Defective fibrin degradation induced by excessive nonenzymatic glycosylation in vivo could lead to the fibrin accumulation seen in various diabetic tissues. In the diabetic kidney, immunohistochemical studies have shown the presence of fibrin in glomerular capillary basement membrane (52). Local responses to mesangial and endothelial fibrin trapping may represent the initial phase of Kimmelsteil-Wilson nodule development (71), and persistence of this fibrin may contribute to the capillary occlusion and progressive glomerular drop-out of long-term diabetes. Fibrin deposition has also been reported in diabetic retinal capillaries (53), and in small epineurial arterioles from patients with long-standing diabetic neuropathy (54). In the arterial wall, fibrin appears to enhance the proliferation of arterial smooth muscle cells, whereas

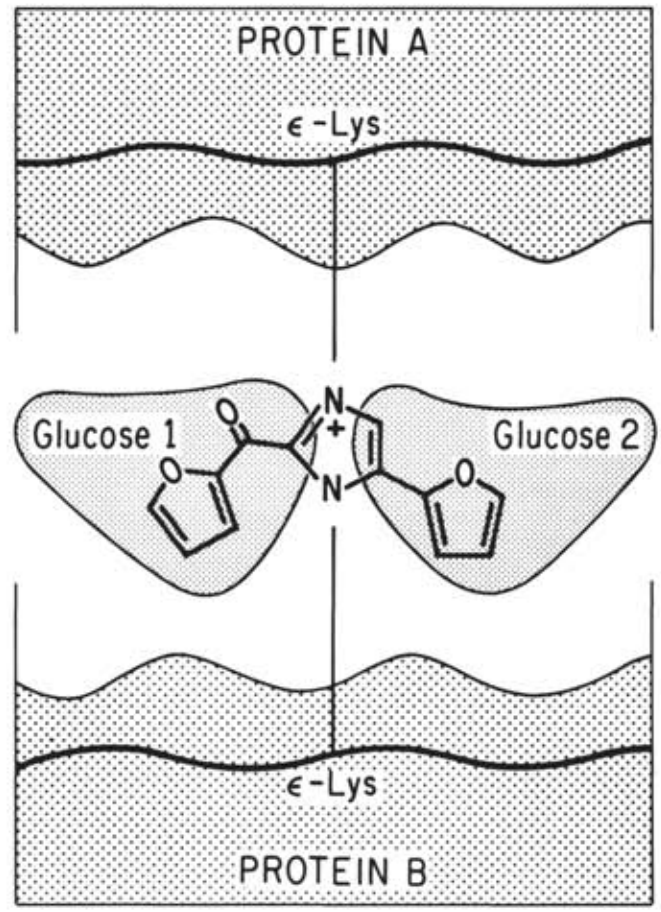

Figure 3. Glucose-derived protein crosslink formed under physiologic conditions. This compound, 2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole, is a condensation product of two glucose molecules and two lysine-derived amino groups.

fibrin degradation products (fragments D and E) inhibit the proliferation of smooth muscle cells (72). Defective degradation of nonenzymatically glycosylated fibrin could thus play an important role in the development of several major diabetic complications.

FUNCTION OF NUCLEIC ACIDS

Although the primary amino groups of nucleotides are chemically less reactive toward reducing sugars than epsilon-amino groups of lysine, nonenzymatic glycosylation of nucleic acid bases can occur, with resultant abnormalities of DNA template function (73). The spectral and fluorescent properties of DNA advanced glycosylation end-products were similar to those of advanced glycosylation end-product proteins. Such nonenzymatic glycosylation reduced the ability of viral DNA fl to transfect *Escherichia coli* at a rate proportional to both incubation time and sugar concentration. Loss of transfection potential was related primarily to DNA glycosylation itself, rather than to a subsequently occurring strand scission reaction. Reducing sugars also have been found to act as mutagens for DNA. Incubation of glucose-6-phosphate with the plasmid pBR322 containing ampicillin- and tetracycline-resistance genes resulted in the occurrence of

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mutants that were antibiotic sensitive (BUCALA R, MODEL P, CERAMI A. Unpublished observations.).

Although DNA, like protein, reacts with glucose-6-phosphate at a much faster rate than with glucose, it was found that the DNA-glucose reaction could be greatly accelerated by the presence of lysine (73). After an initial lag period, the rate of DNA inactivation increased to that seen with glucose-6-phosphate. This finding implies that a common intermediate is formed initially between the lysine and the glucose or glucose-6-phosphate that can react subsequently with DNA. Such glucose-derived crosslinking of amino acids to nucleic acids may be the mechanism by which increasing amounts of proteins become covalently attached to DNA as persons age (74).

Because nucleic acids are long-lived molecules in the resting cell, in-vivo advanced glycosylation end-products would progressively accumulate on DNA over time. Such accumulation may be responsible for age-dependent changes in the genetic material that include chromosomal aberrations, DNA strand breaks, and a decline in DNA repair, replication, and transcription (75-79). Acceleration of this process by diabetic hyperglycemia would result in an earlier onset of cellular senescence. The decrease in diabetic fibroblast replicative capacity resembling that associated with normal aging may be one example (80, 81). Nonenzymatic glycosylation of nucleic acids may also be responsible for the increased frequency of congenital abnormalities in children of diabetic mothers. The exposure of the early embryo to high glucose concentrations could lead to an increased reaction of glucose or a glucose metabolite with DNA at critical developmental stages, causing chromosomal breaks and mutagenesis. Fetal abnormalities similar to those produced by diabetic pregnancy have been reported after exposure to mutagens.

MACROMOLECULAR RECOGNITION AND ENDOCYTOSIS

Most mammalian cells have various unique surface structures that recognize particular chemical signals such as hormones and neurotransmitters. High-affinity binding of such chemical signals to their specific cell-surface receptors initiates a response within the cell that contributes to the maintenance of organismal homeostasis. In a few cell types, such as capillary endothelial cells and monocyte plus macrophages, binding of certain ligands to sites on the cell membrane stimulates rapid interiorization and intracellular processing. Consequences of ligand-induced endocytosis seen experimentally include unidirectional protein transport, alterations in intracellular enzyme activities, and extracellular secretion of neutral proteases (82). Although macromolecular recognition is not well understood at the biochemical level, carbohydrate structure and protein conformation have been identified as two potentially critical factors in this process. Modification of proteins by nonenzymatic glycosylation may therefore be expected to alter macromolecular recognition and endocytosis significantly.

The effects of nonenzymatic glycosylation on endocytic ingestion were first studied using serum albumin and isolated microvessels (18). Unmodified albumin was excluded from ingestion by microvessel endothelial cells, whereas glycosylated albumin was rapidly taken up by endocytosis. Concentrations of glycosylated albumin similar to those found in diabetic plasma appeared to stimulate the ingestion of unmodified albumin as well. Similar recognition and endocytosis of nonenzymatically glycosylated albumin by capillary endothelial cells may be involved in the blood-retinal barrier dysfunction which occurs soon after the onset of diabetes.

In nondiabetic patients, endocytosis of cholesterol-rich low-density lipoprotein (LDL) is thought to influence the development of atherosclerosis by affecting both the rate of accumulation and the rate of removal of tissue cholesterol deposits (83, 84). The existence of high-affinity LDL receptors on fibroblasts allows these and many other nonhepatic cells to be supplied with adequate amounts of cholesterol (required for membrane synthesis) while maintaining the lowest possible level of plasma LDL. Low plasma LDL is critical, because the rate of cholesterol deposition in tissues increases linearly with plasma LDL concentration. When normal LDL receptor function is impaired, compensatory increases in plasma LDL concentrations lead to accelerated atherogenesis. Removal of deposited lipoprotein cholesterol appears to involve tissue macrophages that recognize and ingest both beta-very-low-density lipoproteins, and LDL that has been modified by endothelial cells (85). In vitro, the modified LDL receptor also interacts with lipoproteins whose lysine residues have been modified using formaldehyde, gluteraldehyde, malondialdehyde, acetylation, acetoacetylation, carbamylation, and maleylation (84).

Nonenzymatic glycosylation of LDL from diabetic patients exceeds that of normal persons (29). The absolute amount of glycosylation is relatively small, however, because the apolipoproteins have short half-lives. More extensive nonenzymatic glycosylation of LDL can be readily accomplished in vitro. This hyperglycosylated LDL is internalized and degraded by cultured human fibroblasts significantly less than control LDL, and the fractional catabolic rate of this material is reduced in guinea pigs (86, 87). These studies confirm earlier work showing that extensive chemical modification of LDL lysine residues interferes with recognition and uptake by fibroblasts (88). The extent to which similar changes occur in vivo is unclear, however, because the abnormally high plasma LDL levels associated with genetic disorders of LDL-receptor function are not a characteristic feature of diabetes (89). Although degradation of glycosylated LDL by mouse peritoneal macrophages did not occur via the acetyl-LDL mechanism, this may indicate only that the required threshold level of recognizable modifications had not been reached (90). In vivo, advanced glycosylation end-products would continue to accumulate on LDL trapped in the extracellular matrix, and recognition of these by macrophages may contribute to the pathogenesis of atherosclerosis.

In contrast to the plasma proteins discussed above, structural proteins with long half-lives accumulate advanced glycosylation end-products. Recently, these advanced glycosylation end-products have been shown to

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mediate specific macrophage recognition and uptake of proteins glycosylated both in vitro and in vivo (91). Long-term exposure of peripheral nerve myelin proteins to glucose markedly altered the way in which myelin interacts with elicited macrophages. Myelin that had been incubated with glucose in vitro for 8 weeks reached a steady-state accumulation within thioglycolate-elicited macrophages that was five times greater than myelin incubated without glucose (Figure 4a). Similarly, myelin isolated from rats having diabetes for 1.5 to 2.0 years had a steady-state level that was nine times greater than that of myelin from young rats, and 3.5 times greater than myelin from age-matched controls (Figure 4c). In contrast, myelin isolated from rats having diabetes for 4 to 6 weeks had the same degree of accumulation as did myelin from age-matched normal rats (Figure 4b). These data suggest that the amount of increased nonenzymatic glycosylation seen in the myelin of short-term diabetic rats had not yet resulted in the significant accumulation of advanced glycosylation end-product myelin present both in vitro and in the long-term diabetic rats. The addition of increasing amounts of unlabeled normal unmodified myelin failed to compete with ^{125}I-advanced glycosylation end-product myelin accumulation, whereas the addition of unlabeled advanced glycosylation end-product myelin competed effectively.

Formation of irreversible advanced glycosylation end-product adducts on myelin appears to promote recognition and uptake by macrophages. This interaction between advanced glycosylation end-product myelin and macrophages could initiate or contribute to the segmental demyelination associated with diabetes and the normal aging of peripheral nerve by mechanisms that remain to be elucidated. Demyelination may result in part from augmented secretion of proteolytic enzymes triggered by interaction of advanced glycosylation end-product myelin with its receptor. Macrophage secretion of several neutral proteases has already been reported in response to maleylated albumin binding (92).

IMMUNOGENICITY

Covalent attachment of small molecules to autologous proteins can produce conjugates that are capable of inducing an immune response in the host. Formation of specific glucose-derived adducts on body proteins could function in a similar fashion, leading to potentially damaging autoantibody formation in diabetic patients. The Amadori product of nonenzymatic glycosylation has been shown experimentally to be a poor immunogen, however. The LDL-Amadori products, for example, induce low titers of low affinity antibodies in guinea pigs, even when given with complete Freund's adjuvant. In contrast, when similar immunization is done using lipoproteins whose Amadori products have been chemically converted in vitro to a hexitolamino derivative, high titers of region-specific antibodies are generated that do not cross-react with any of the Amadori product proteins (93). Qualitatively similar results have been obtained in preliminary studies using the chemically produced hexitolamino derivative of collagen Amadori products (94).

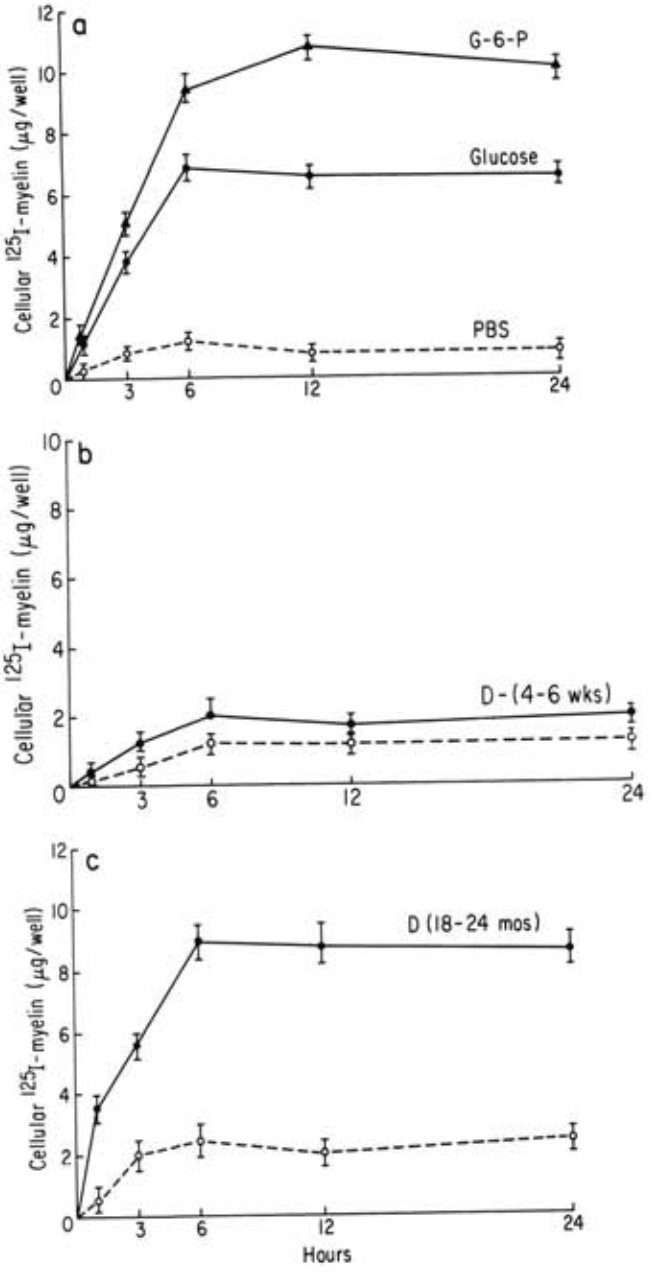

Figure 4. Macrophage recognition and uptake of peripheral nerve myelin proteins glycosylated both in vitro and in vivo. **Figure 4a.** Proteins from 8-week incubations in 50 mmol/L glucose (*closed circle*), 50 mmol/L glucose-6-phosphate (*triangle*), or phosphate-buffered saline (*open circle*). **Figure 4b.** Proteins from rats having diabetes for 4 to 6 weeks (*closed circle*) and from age-matched normal rats (*open circle*). **Figure 4c.** Proteins from rats having diabetes for 1.5 to 2.0 years (*closed circle*) and from age-matched normal rats (*open circle*).

These data suggest that in vivo, a structural analog of the open-chain hexitolamino Amadori product derivative would have greater immunogenic potenial than the Amadori product itself. Such glucose-derived open-chain structures probably occur in vivo during formation of the advanced glycosylation end-product FFI. Direct experimental evidence for the immunogenicity of advanced glycosylation end-products on long-lived structural proteins has not yet been provided, however.

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Potential Clinical Applications of Research on Nonenzymatic Glycosylation

New knowledge about nonenzymatic glycosylation may lead to significant advances in both diagnosis and therapy. Diagnostically, the isolation and identification of FFI now make possible the development of an analytical method for measuring accumulated advanced glycosylation end-products in clinical samples. This information about patients' long-term, time-averaged exposure to glucose may help identify subsets of clinically indistinguishable diabetic patients who differ in inherent susceptibility to hyperglycemia-induced tissue damage. Epidemiologic evidence suggests that for any given level of sustained hyperglycemia, some patients appear to be at greater risk. Although the presence of HLA-DR4 may be associated with this additional risk (1, 95), there is at present no laboratory procedure that can be used to determine prognosis.

Therapeutically, new agents may be required to directly interfere with the self-perpetuating component of glycosylation-mediated tissue damage, if perfect correction of hyperglycemia in some diabetic patients will not prevent the continued progression of diabetic complications. These new pharmacologic developments must be based on increased understanding of the biochemistry of advanced glycosylation end-product formation on proteins of diabetic tissues, because attempts at preventing Amadori product formation by nonspecific chemical modification of protein amino groups would result in pathophysiologic changes similar to those produced by diabetes itself (96).

ACKNOWLEDGMENTS: Grant support: in part by a grant from the American Diabetes Association-New York Diabetes Affiliate, and grant R01-AM19655 from the National Institutes of Health. Dr. Vlassara is the recipient of a Research Scientist Development Award (1-K01-AG00148) from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, and the National Institute of Aging.

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