Mechanism of Autoxidative Glycosylation: Identification of Glyoxal and Arabinose as Intermediates in the Autoxidative Modification of Proteins by Glucose†

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ABSTRACT: Glycation and oxidation reactions contribute to protein modification in aging and diabetes. Formation of dicarbonyl sugars during autoxidation of glucose is the hypothetical first step in the autoxidative glycosylation and subsequent browning of proteins by glucose [Wolff, S. P., & Dean, R. T. (1987) *Biochem. J. 245*, 243−250]. In order to identify the dicarbonyl sugar(s) formed during autoxidation of glucose under physiological conditions, glucose was incubated in phosphate buffer (pH 7.4) at 37 °C under air (oxidative conditions) or nitrogen with transition metal chelators (antioxidative conditions). Dicarbonyl compounds were analyzed spectrophotometrically and by HPLC after reaction with Girard-T reagent. Carbohydrates were analyzed by gas chromatography−mass spectrometry. Both dicarbonyl sugar and arabinose concentrations increased with time and glucose concentration in incubations conducted under oxidative conditions; only trace amounts of these products were detected in glucose incubated under antioxidative conditions. HPLC analysis of adducts formed with Girard-T reagent indicated that glyoxal was the only α-dicarbonyl sugar formed on autoxidation of glucose. Glyoxal and arabinose accounted for ≥50% of the glucose lost during a 21 day incubation. Neither glucosone nor its degradation product, ribulose, was detectable. Reaction of glyoxal with RNase yielded the glycoxidation product, N^{ϵ}-(carboxymethyl)lysine, while arabinose is a source of pentosidine. Our results implicate glyoxal and arabinose as intermediates in the browning and crosslinking of proteins by glucose under oxidative conditions. They also provide a mechanism by which antioxidants and dicarbonyl trapping reagents, such as aminoguanidine, limit glycoxidation reactions and support further evaluation of these types of compounds for inhibition of chemical modification and crosslinking of proteins during aging and diabetes.

The Maillard or browning reaction between reducing sugars and proteins is proposed to have a role in the pathophysiology of aging and diabetic complications (Brownlee, 1994; Vlassara *et al.*, 1994). This hypothesis is supported by the observation that advanced glycosylation end products (AGEs) and glycoxidation products increase in collagen and lens proteins with age (Sell & Monnier, 1989; Dunn *et al.*, 1991; Araki *et al.*, 1992; Nakayama *et al.*, 1993; Dyer *et al.*, 1993) and the accelerated accumulation of these products in tissues of diabetic patients, particularly of patients with complications (Sell *et al.*, 1992; McCance *et al.*, 1993; Beisswenger *et al.*, 1993). The two structurally characterized AGEs known to accumulate in long-lived proteins with age and in diabetes, N^{ϵ}-(carboxymethyl)lysine (CML)[1] and pentosidine, require oxygen at some stage in their formation from glucose (Fu *et al.*, 1992, 1994). Oxidative reactions also play a key role in formation of crosslinks and "Maillard-like" fluorescence during *in vitro* reactions of glucose with collagen (Chace *et al.*, 1991).

Wolff and Dean observed formation of ketoaldehydes (α-dicarbonyl compounds) and reactive oxygen species ($O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$) during incubations of glucose under pro-oxidant conditions in the presence or absence of protein (Wolff & Dean, 1987; Hunt *et al.*, 1988; Jiang *et al.*, 1990). Addition of diethylenetriaminepentaacetic acid (DTPA), a transition metal chelator, significantly reduced the yield of dicarbonyl sugar and the amount of carbohydrate bound to protein. On the basis of these experiments, Wolff and Dean (1987) proposed that metal-catalyzed oxidation of glucose and formation of reactive dicarbonyl sugars was a critical step in the chemical modification of protein by glucose, a process they termed *autoxidative glycosylation*. Despite their detection of dicarbonyl compounds in glucose reactions conducted under pro-oxidant conditions, specific dicarbonyl products have not yet been identified, although the scheme provided implicates glucosone as the relevant dicarbonyl sugar.

We report below the identification of glyoxal as the α-dicarbonyl sugar, and arabinose as the only pentose, formed on autoxidation of glucose. Glyoxal is recognized as a potent crosslinking agent, but incubation of glyoxal with protein also yields the glycoxidation product, CML, while arabinose yields pentosidine on reaction with protein (Sell & Monnier, 1989; Grandhee *et al.*, 1991). These results suggest that glyoxal and arabinose are intermediates in the

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[1] Abbreviations: AG, aminoguanidine; CML, N^{ϵ}-(carboxymethyl)-lysine; 3-DG, 3-deoxyglucosone; DTPA, diethylenetriaminepentaacetic acid; GC/MS, gas chromatography−mass spectrometry; HFBA, heptafluorobutyric acid; SIM-GC/MS, selected ion monitoring GC/MS; TNBS, trinitrobenzenesulfonic acid.

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autoxidative glycosylation and crosslinking of protein by glucose.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise indicated, reagents were of the highest quality obtainable from Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Glyoxal and methylglyoxal used in experiments were 40% aqueous monomer solutions from Aldrich. Bovine pancreatic RNase A (type XII-A) was obtained from Sigma. Glucosone was synthesized from the phenylosazone derivative (Richtmyer, 1969) as described by Bayne (1969).

Incubation Procedures. Glucose or glucosone solutions were freshly prepared in 0.2 M phosphate buffer, pH 7.4. Three milliliter aliquots were placed in individual 12 × 32 mm screw cap vials with open top caps containing 75 mil septa (National Scientific, Lawrenceville, GA), and one drop of toluene was added to prevent microbial growth. Oxidative reactions were conducted under an air atmosphere. Antioxidative reactions contained DTPA and phytic acid (1 mM each) to chelate trace amounts of transition metals in the phosphate buffer; these samples were evacuated and then purged for 30 s with N_2 which was scrubbed of trace contaminants of oxygen by bubbling through a 0.2 M solution of sodium dithionite. All samples were incubated in the dark at 37 °C; individual vials were removed at desired times and frozen at −20 °C until analyzed. For measurement of the kinetics of glyoxal disappearance, glyoxal was incubated in 0.2 M phosphate, pH 7.4, under oxidative or antioxidative conditions. Aliquots were removed at various times and stored frozen at −20 °C, then thawed, and analyzed as described below.

RNase (10 mg/mL) and glyoxal (5 mM) were incubated in 0.2 M phosphate, pH 7.4, under oxidative and antioxidative conditions at 37 °C as described above. Samples were dialyzed against deionized water to remove unreacted glyoxal and salts. The extent of modification of lysine residues was determined by the trinitrobenzenesulfonic acid assay (Spadaro *et al.*, 1979), using alanine as standard. Aliquots (~1 mg of protein) were reduced by addition of 5 μL of $NaBH_4$ solution (100 mg/mL stock solution in 0.1 M NaOH), incubated for 2 h at room temperature, treated with HCl to discharge excess $NaBH_4$, and then hydrolyzed in 6 N HCl for 24 h at 110 °C. CML was measured by selected ion monitoring gas chromatography−mass spectrometry (SIM-GC/MS) as the *N,O*-(trifluoroacetyl) trifluoroethyl ester derivative (Gieseg *et al.*, 1993) using CML-d_4 and Lys-d_8 as internal standards. Pentosidine was measured by reversed phase HPLC using fluorescence detection (Ex = 328 nm, Em = 378 nm) as previously described (Dyer *et al.*, 1991a).

Spectrophotometric and HPLC Analyses of Dicarbonyl Sugars. Dicarbonyl sugars were measured spectrophotometrically using the Girard-T assay of Mitchel and Birnboim (1977). This paper describes both acidic and basic pH conditions for analysis of dicarbonyl compounds by the Girard-T assay. We chose to perform the assay at pH 2.9, rather than pH 9.2, to limit base-catalyzed rearrangements and oxidation of glucose during the assay. Briefly, aliquots of glucose incubations (100 μL) or glyoxal were incubated with 50 μL of Girard-T stock solution (0.5 M) and 850 μL of sodium formate (0.5 M, pH 2.9) at room temperature for 1 h. Absorbance was measured at 294 nm. A calibration curve was prepared using glyoxal standards treated similarly.

Aliquots of glucose reactions (1 mL) were initially prepared for HPLC analysis by adding 300 μL of 2 M HCl (final pH ~3) and 50 μL of Girard-T stock solution and then incubating at room temperature for 24 h to allow sufficient time for product formation from slow reacting dicarbonyl compounds, such as glucosone. The sample was adjusted to 0.25% in heptafluorobutyric acid (HFBA) and then applied to a 1 mL C_{18} solid phase extraction column (Supelco, Bellefonte, PA) equilibrated in the same buffer, in order to remove glucose, phosphate, and salts. The column was washed with 2 mL of 0.25% HFBA, and adducts were eluted with 2 mL of 25% acetonitrile. The eluates were dried in a SpeedVac centrifugal evaporator (Savant, Farmingdale, NY) and then reconstituted in 500 μL of 0.25% HFBA. Aliquots, 50−100 μL, were analyzed by reversed phase HPLC, using a Zorbax SB-C18 column (MAC-MOD Analytical, Chadds Ford, PA) with absorbance detection at 280 nm. The buffer gradient used for elution was 12 to 14% buffer B in 40 min, then hold at 14% buffer B for 10 min; buffer A: 0.25% HFBA; buffer B: 100% CH_3CN containing 0.25% HFBA.

Carbohydrate Analysis. Carbohydrates in incubation mixtures were detected and quantitated, after addition of internal standards, by a GC/MS assay. The internal standards used were 2-deoxyribose for pentoses and *myo*-inositol for hexoses. Samples were reduced with a 25-fold molar excess of $NaBD_4$ (from a 100 mg/mL stock solution in 0.1 M NaOH) for 2 h at room temperature. Excess $NaBD_4$ was discharged by addition of 2 M acetic acid and the sample dried by centrifugal evaporation. Borate was removed as methyl borate by adding 2 mL of methanol/glacial acetic acid (10:1), heating at 65 °C for 30 s, and then drying under a stream of N_2 (N-Evap, Organomation, Berlin, MA); this step was repeated twice more to ensure complete removal of borate. Samples were acetylated by addition of 200 μL of acetic anhydride and 300 μL of pyridine, and by heating at 95 °C for 30 min. The samples were cooled to room temperature, 1 mL of $CHCl_3$ added, and the salts and pyridine extracted once with an equal volume of 0.1 N HCl and three times with deionized water. The $CHCl_3$ layer was dried under a stream of nitrogen, the sample redissolved in ethyl acetate, and 2 μL injected for GC/MS analysis. Analyses were conducted on a Hewlett-Packard Model 5890 gas chromatograph equipped with a Model 5970 mass selective detector, using a 30 m DB-5 capillary column (J & W Scientific, Folsom, CA). SIM-GC/MS was used to quantify glucose (*m/z* 361), glucosone (*m/z* 289), arabinose, and ribulose (*m/z* 145); the 145 and 210 ions were monitored for the internal standards, 2-deoxyribose and myoinositol, respectively. The temperature program used was 2 min at 120 °C, ramp to 240 °C at 4 °C/min, ramp to 290 °C at 25 °C/min, hold for 4 min at 290 °C.

Data Analysis. The rate constant for conversion of glucose to glyoxal (k_1) was estimated using a nonlinear curve fitting program MINSQ (MicroMath, Salt Lake City, UT) and the following equation:

$$\text{glucose} \xrightarrow{k_1} \text{glyoxal} \xrightarrow{k_2} ?$$

where k_2 was estimated from the slope of the line in Figure 4 (below).

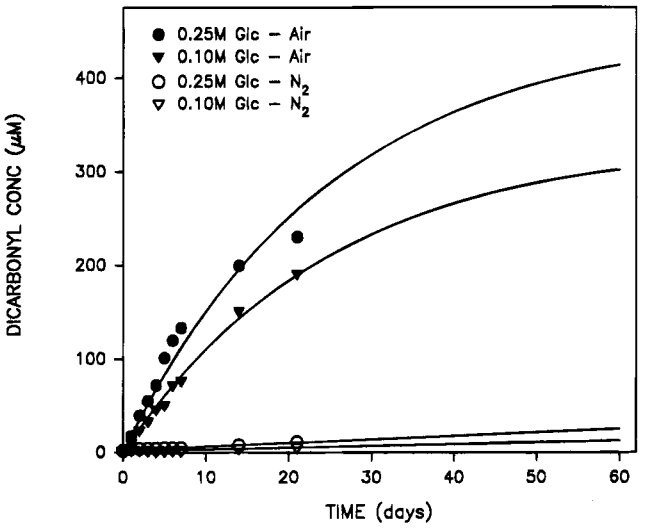

FIGURE 1: Kinetics of dicarbonyl sugar formation during autoxidation of glucose. Glucose, 100 mM (▽, ▼) and 250 mM (○, ●), was incubated in 0.2 M phosphate, pH 7.4, under oxidative (closed symbols) and antioxidative (open symbols) conditions. Aliquots were assayed for dicarbonyl formation at the indicated times, using the Girard-T assay described in Experimental Procedures. The lines drawn in the figure for reactions carried out under oxidative conditions were calculated from the kinetics of formation (Figure 1) and degradation (Figure 4) of glyoxal, using the MINSQ kinetic modeling program, as described in Experimental Procedures.

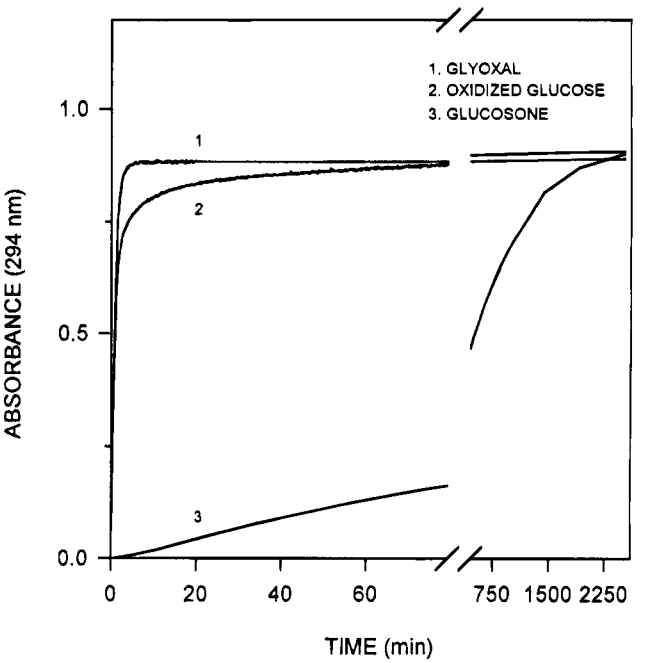

FIGURE 2: Kinetics of Girard-T adduct formation from various carbohydrates. Glyoxal (1), an aliquot of a 250 mM glucose solution (2) autoxidized for 3 weeks under air in 0.2 M phosphate buffer, pH 7.4, and glucosone (3) were analyzed using the Girard-T assay as described in Experimental Procedures. The glucosone and glyoxal samples contained 35 nmol of dicarbonyl sugar.

RESULTS

Kinetics of Dicarbonyl Sugar Formation. The rate of dicarbonyl sugar formation from glucose was measured as a function of both glucose concentration and oxidative conditions. As shown in Figure 1, at 100 and 250 mM glucose there was a time-dependent increase in dicarbonyl formation in reactions conducted under air, confirming earlier results of Wolff and Dean (1987). α-Dicarbonyls accumulated at an initial rate proportional to the starting glucose concentration; however, by 3 weeks, the rate of formation of dicarbonyls began to plateau, representing approximately 0.1% (200 μM) of the starting glucose concentration in the reaction at 250 mM glucose. Only small amounts of dicarbonyl compounds (<10 μM) were formed under antioxidative conditions, confirming the need for oxygen and traces of metal ions to form dicarbonyl sugars from glucose (Wolff & Dean, 1987).

Identification of Glyoxal in Glucose Incubations. The Girard-T reagent assay for dicarbonyl sugars was originally developed for measurement of glyoxal. Although the assay is widely applied for measurement of other dicarbonyl sugars, the rate of color formation varies significantly among substrates. For example, as shown in Figure 2, the reaction rate for glucosone is significantly slower than that for glyoxal, probably because glucosone exists largely in hemiacetal and hemiketal conformations in solution. The kinetics of color development in the Girard-T assay for equal amounts of glyoxal, glucosone, and the dicarbonyl sugar present in an aliquot of a 3-week glucose incubation under autoxidizing conditions are compared in Figure 2. The reaction of Girard-T reagent with glyoxal was rapid and complete after 15 min. In contrast, the reaction with glucosone was slow, requiring over 40 h to reach completion. Surprisingly, when an aliquot of the 3-week glucose incubation was assayed, the kinetics of color formation were similar to those observed with glyoxal. The rapid kinetics of color development in the Girard-T assay suggested that the dicarbonyl sugar(s) formed on autoxidation of glucose was small and existed to a greater extent in the acyclic form.

In order to identify the dicarbonyl sugar present in glucose incubations, a reversed phase HPLC assay was developed which would separate the Girard-T adducts of various dicarbonyl sugars. Figure 3A shows the chromatogram obtained for a mixture composed of glucosone, glyoxal, and methylglyoxal. When an aliquot from a 3-week glucose reaction was analyzed, only one peak was present in the chromatogram, which eluted at the same retention time as glyoxal (Figure 3B). A mixing experiment with authentic glyoxal confirmed the identity of this dicarbonyl sugar as glyoxal. HPLC analysis of aliquots of glucose incubations taken between 1 and 14 days revealed that glyoxal was the only dicarbonyl sugar formed; glyoxal or other dicarbonyl sugars were not detectable in incubations carried out under antioxidative conditions.

Kinetics of Glyoxal Formation. The decrease in the rate of glyoxal formation with time in glucose incubations suggested that both glyoxal production and degradation were occurring. To assess the stability of glyoxal in the reaction system, glyoxal was incubated in phosphate buffer under oxidative and antioxidative conditions. The graph in Figure 4 shows that the kinetics of degradation of glyoxal (0.5 mM) were similar under oxidative and antioxidative conditions ($t_{1/2}$ = 17.3 and 18.7 days, respectively), suggesting a nonoxidative route for disappearance of glyoxal at pH 7.4. Similar half-lives were also obtained at 5 and 10 mM glyoxal concentrations, 19.8 and 22.4 days, respectively (data not shown). The rate constant obtained for the degradation of glyoxal (Figure 4) and the rate of formation of dicarbonyl sugars from glucose (Figure 1) were analyzed mathematically, using the kinetic modeling program, MINSQ (see Experimental Procedures), and the rate constant for formation

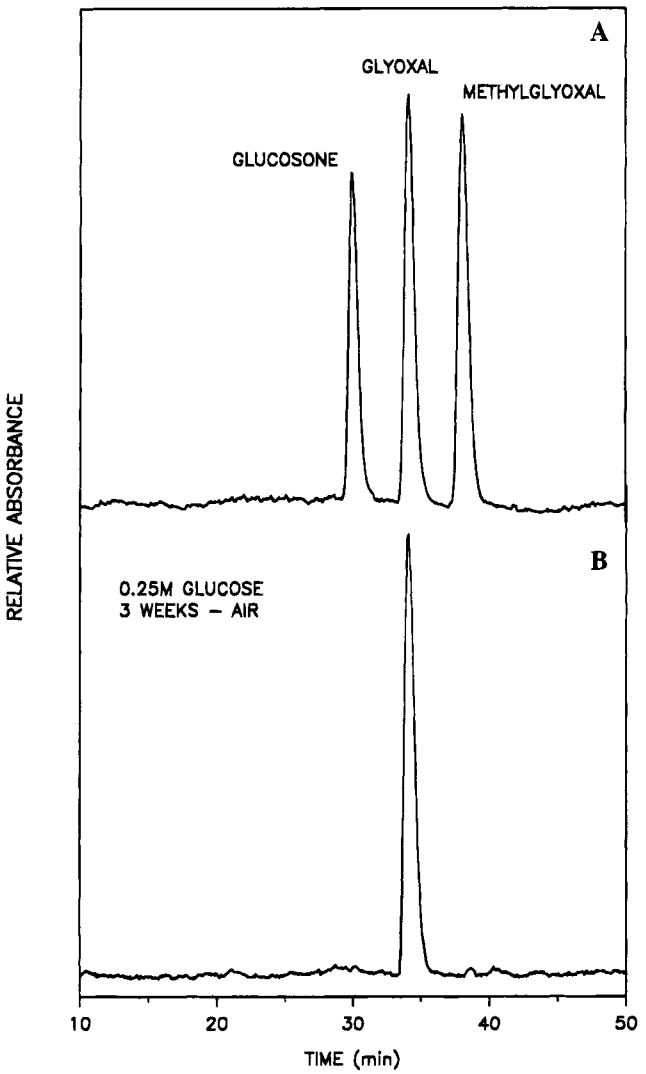

FIGURE 3: Identification of glyoxal as the dicarbonyl sugar formed during autoxidation of glucose. HPLC chromatograms of the Girard-T adduct of (A) dicarbonyl sugar standards glucosone, glyoxal, and methylglyoxal; and (B) a sample obtained from a 250 mM glucose incubation after autoxidation for 3 weeks under oxidative conditions in 0.2 M phosphate, pH 7.4.

of glyoxal from glucose was estimated to be $\sim 10^{-4}$ d^{-1} in 0.2 M phosphate buffer.

Formation of Arabinose during Autoxiation of Glucose. Glucose autoxidation reactions were analyzed for the formation of pentoses and tetroses by $NaBD_4$ reduction and analysis of alditol acetates. The total ion chromatograms, shown in Figure 5A,B, indicate the presence of only one new peak, corresponding to arabinitol. Trioses, tetroses, and their respective glyconic acids would also be observed as their alditol acetates by this techniques, but were not detected. The mass spectrum of $NaBD_4$-reduced aldoses yields two prominent fragments, differing by one mass unit, corresponding to elimination of the terminal carbon from opposite ends of the molecule (Lönngren & Svennson, 1974). Based on the presence of both the *m/z* 289 and 290 ions in the selected ion chromatogram shown in Figure 5C, and their relative peak areas compared to authentic arabinose, the arabinitol was derived from arabinose, which presumably arises from oxidative cleavage between C_1 and C_2 of glucose. The kinetics of arabinose formation during autoxidation of glucose (250 mM) were linear, and the arabinose concentration reached about 1 mM by 21 days (Figure 5D). As also

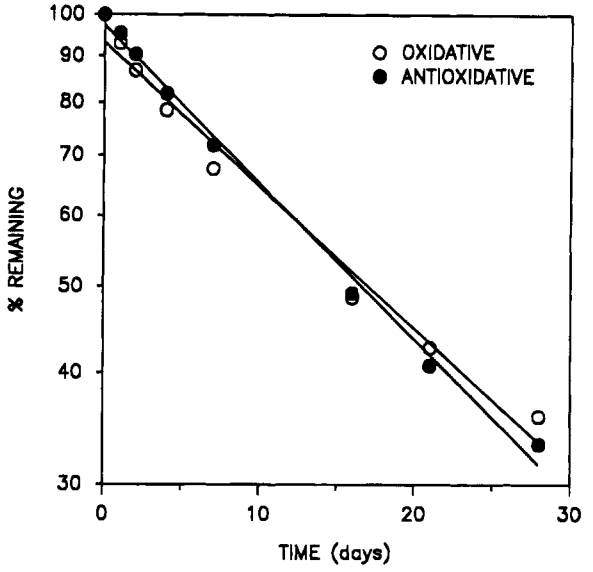

FIGURE 4: Kinetics of decomposition of glyoxal. Glyoxal (0.5 mM) was incubated under oxidative (O) and antioxidative (●) conditions in 0.2 M phosphate, pH 7.4. The slopes of the lines yielded estimated half-lives and rate constants for glyoxal degradation of 17.3 d (0.040 d^{-1}) and 18.7 d (0.037 d^{-1}) under oxidative and antioxidative conditions, respectively.

shown in Figure 5D, arabinose was formed at ~2.5 times the rate of glyoxal, their total yield accounting for ~0.7% of the starting glucose (1.2 mM arabinose + 0.5 mM glyoxal from 250 mM glucose) at 21 days. Estimates of the decrease in glucose concentration in these same experiments yielded 1.4 ± 0.7% (mean ± SD; $n = 5$). Because of the analytical limitations inherent in accurately quantitating the small decrease in glucose in these experiments, it is reasonable to conclude that formation of arabinose and glyoxal account for a significant fraction, if not all, of the major products formed by autoxidation of glucose in phosphate buffer. The split products formed in these reactions have not been identified.

Evidence against Formation of Glucosone during Autoxidation of Glucose. Failure to detect glucosone as a product of autoxidation of glucose (Figure 3) could be the result of its rapid degradation in the reaction mixtures. Indeed, glyoxal could be a product of decomposition of glucosone. To address this question, glucosone was incubated in phosphate buffer under oxidative and antioxidative conditions and analyzed for both the decrease in glucosone concentration and formation of degradation products as a function of time. The total ion chromatograms (*m/z* 50−550) obtained for glucosone incubations under air, shown in Figure 6A,B, revealed only two products, identified as ribitol and arabinitol (after $NaBD_4$ reduction). These products were formed in similar yields under both oxidative and antioxidative conditions. To determine if the ribitol and arabinitol were derived from $NaBD_4$ reduction of the aldoses, ribose and arabinose, or the ketose sugar, ribulose, the selected ion chromatograms for the *m/z* 289 and 290 ions were examined. After reduction with $NaBD_4$, ribose and arabinose should yield the 289 and 290 ions in comparable abundance (see above), while ribulose would show only the *m/z* 290 ion (Lönngren & Svennson, 1974). The absence of the 289 ion in the spectrum of the pentitols (Figure 6C) indicates that the alditols were derived exclusively from the ketose sugar, ribulose. The kinetics of glucosone degradation and ribulose formation over

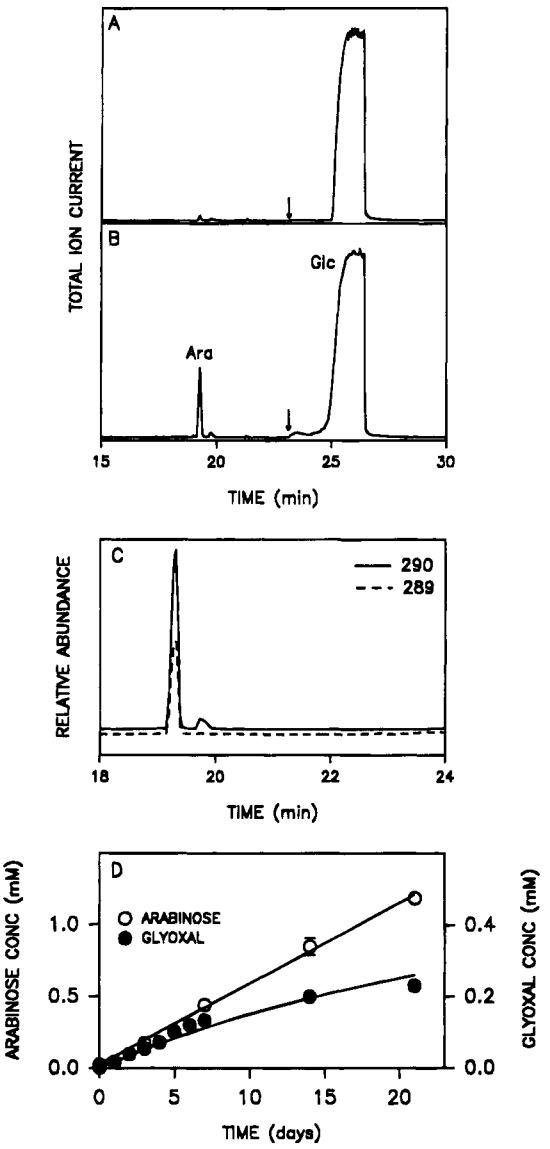

FIGURE 5: Arabinose is the only pentose sugar formed on autoxidation of glucose. Aliquots of 0.25 M glucose solutions (n = 3) were reduced with $NaBD_4$ and analyzed by SIM-GC/MS as described in Experimental Procedures. Profiles of (A) day 0 and (B) day 21 samples, showing formation of a single product. (C) Expanded view of the m/z 289 and 290 selected ion chromatograms demonstrating the presence of arabinose. (D) Kinetics of arabinose formation. For ease of comparison, the data for formation of glyoxal over 21 days, shown in Figure 1, are replotted in panel D.

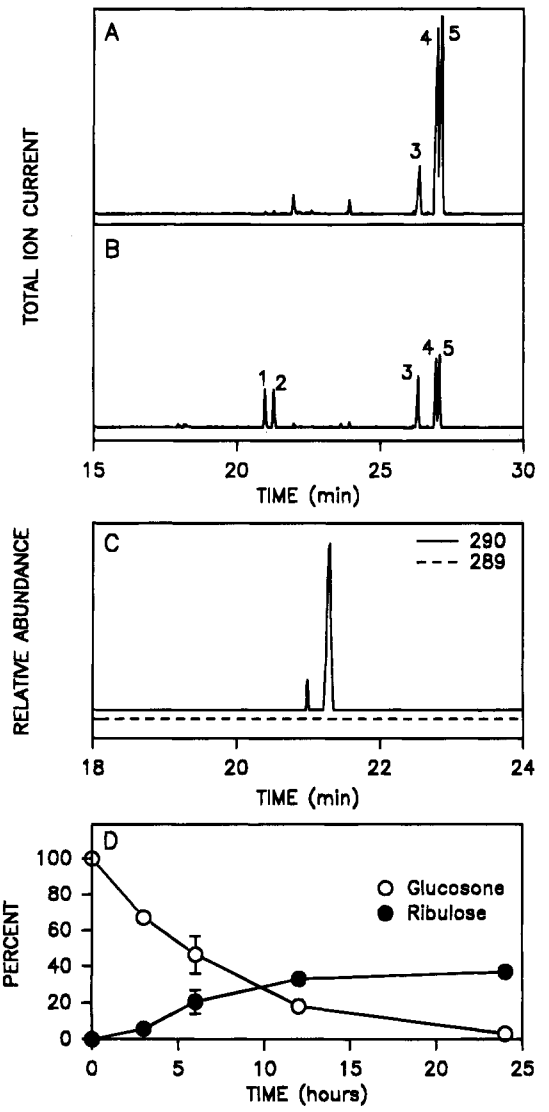

FIGURE 6: Analysis of kinetics and products of decomposition of glucosone. Aliquots of glucosone incubations were reduced with $NaBD_4$ and analyzed as alditol acetates by GC/MS as described in Experimental Procedures. (A and B) Total ion chromatograms (m/z 50−550) for 0 and 6 h incubations, respectively. Peak identifications were based on retention time and mass spectra of authentic standards: (1) ribitol, (2) arabinitol/lyxitol, (3) *myo*-inositol, (4) glucitol, and (5) mannitol. (C) Expanded view of the *m/z* 289 and 290 selected ion chromatograms demonstrating the formation of ribulose of the glucosone incubations (see text). (D) Kinetics of glucosone degradation (○) and ribulose formation (●). Data are expressed as percent of initial glucosone concentration (5 mM) and are the average of three experiments, with ranges shown when they exceeded the size of the symbol. The calculated half-life and rate constant for glucosone decomposition were estimated to be 4.9 h and 0.14 h^{-1}, respectively.

a 21 day period (Figure 6D) showed a rapid loss of glucosone ($t_{1/2}$ = 4.9 ± 0.1 h; n = 3) and a concurrent increase in ribulose formation. The yield of ribulose accounted for only about 20% of the glucosone degraded, suggesting that glucosone decomposed by alternative mechanisms to products not detectable in these assays. No other triose, tetrose, pentoses, or their alduloses or glyconic acids were detected, nor was glyoxal or any other dicarbonyl sugar detectable by HPLC analysis of Girard-T adducts from glucosone autoxidation reactions during the 21 day incubation. These experiments indicate that glucosone is not a precursor or intermediate in the formation of glyoxal during the autoxidation of glucose.

We considered the possibility that glucosone was, in fact, formed by autoxidation of glucose, but then decomposed to ribulose, which then also rapidly decomposed to other products, so that the formation of glucosone escaped detection. However, when authentic ribulose, representing 2% of the starting glucose concentration (the approximate amount of glucose autoxidized during the 21 day incubation in Figure 1), was added to a solution of 250 mM glucose and incubated under autoxidizing conditions, ribulose was readily detectable throughout the 21 day incubation period, with ~15% of the original ribulose remaining at 21 days. In addition, tetroses were also detected as degradation products of ribulose in these incubations, in contrast to their absence in autoxidized glucose (data not shown). In summary, we find no evidence for the formation of glucosone during autoxidation of glucose in phosphate buffer.

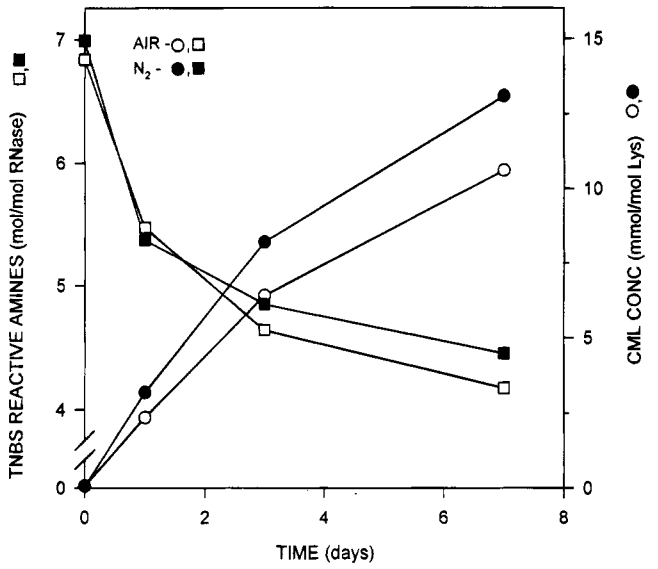

FIGURE 7: Kinetics of amine loss (□, ■) and CML formation (○, ●) during incubations of RNase with glyoxal. RNase (10 mg/mL) was incubated with glyoxal (5 mM) in 0.2 M phosphate, pH 7.4, under oxidative (open symbols) and antioxidative (closed symbols) conditions. Aliquots were assayed for amines using the trinitrobenzenesulfonic acid assay (TNBS) and CML, as described in Experimental Procedures.

Formation of CML by Reaction of Glyoxal with Protein. The reaction of glyoxal with RNase was used as a model system to determine the role of glyoxal in the formation of the known glycoxidation products, CML and pentosidine. During the course of the incubation, glyoxal reacted with RNase as evidenced by browning and loss of free amino groups (Figure 7). CML also increased with time under both oxidative and antioxidative conditions; however, the amount of CML formed after 7 days accounted for only about 3% of the lysines lost or 1.5% of the glyoxal added (Figure 7), indicating the formation of other products on reaction of glyoxal with protein. Glyoxal did not appear to be a precursor to pentosidine since only traces of pentosidine were detectable (data not shown). However, arabinose, which is also formed in the reaction mixture, is an efficient precursor to pentosidine (Grandhee *et al.*, 1991; Dyer *et al.*, 1991a). Reaction of arabinose and other pentoses with protein, including RNase and collagen, yielded pentosidine (data not shown), suggesting that arabinose is the probable precursor of pentosidine formed during browning of proteins by glucose under oxidative conditions.

DISCUSSION

Glucose Autoxidation. Wolff and Dean (1987) first demonstrated that dicarbonyl compounds derived from autoxidation of glucose contributed significantly to the chemical modifications of protein during the Maillard reaction. Although the identity of the dicarbonyl sugar was not determined, earlier work on autoxidation of glyceraldehyde (Thornalley *et al.*, 1984) suggested that glucosone would be the likely product formed on autoxidation of glucose. However, the experiments described here show that glyoxal is the sole dicarbonyl sugar formed on autoxidation of glucose (Figure 3). Failure to detect ribulose (Figure 5), a degradation product of glucosone (Figure 6), provides further evidence that glucosone is not formed. Finally, the failure of glucosone to yield glyoxal or arabinose on autoxidation excludes glucosone as a precursor of either of these compounds in the reaction mixture. Our observations are similar to those made in experiments on the radiolytic oxidation of glucose, in which arabinose and glyoxal were the major oxidation products detected (Phillips *et al.*, 1958). We have been unable to detect other 3- or 4-carbon split products of glyoxal formation from glucose, including aldoses, ketoses, α-ketoaldehydes, or glyconic acids. The lack of 4-carbon split products suggest an alternative pathway whereby autoxidation of glucose forms three 2-carbon products (two molecules of glyoxal and one molecule of glycoaldehyde), as reported for irradiation of glucose and mannose (Phillips, 1961, 1980). Arabinose is the only other carbohydrate detected on glucose autoxidation and presumably arises from cleavage of the C_1-C_2 bond of glucose.

Role of Glyoxal in the Maillard Reaction In Vitro. Formation of glyoxal during autoxidation of glucose and subsequent reaction with protein can explain many of the changes that occur in protein during the Maillard reaction. Glyoxal reacts readily with both lysine and arginine residues in proteins (Schauenstein *et al.*, 1977), and these are also the principle amino acids modified during reaction of glucose with protein under physiological conditions (Cho *et al.*, 1986; Fu *et al.*, 1994). CML was originally described as a product of cleavage of fructoselysine formed during glycation of protein under oxidative conditions (Ahmed *et al.*, 1986). However, the formation of CML from glyoxal generated directly by autoxidation of glucose provides an alternative route to formation of CML. These results are consistent with the earlier report of van Chuyen *et al.* (1973) that reaction of glyoxal with alanine results in carboxymethylation of the α-amino group. Similarly, the mechanism of carboxymethylation of lysine by glyoxal could involve the formation of a Schiff base adduct, followed by either classical enolization, dehydration, and elimination reactions (van Chuyen *et al.*, 1973) or a Cannizzaro-type rearrangement (Salomaa, 1956; Zyzak *et al.*, 1994). Protein browning and crosslinking are also characteristic features of the Maillard reaction in which glyoxal may play a role. Glyoxal forms stable inter- and intramolecular crosslinks during *in vitro* incubations with proteins, including collagen (Davis & Tabor, 1963; Bowes & Cater, 1968). Kato *et al.* (1986) also reported increased browning and polymerization of lysozyme by glyoxal, compared to xylose, accompanied by losses of lysine, tryptophan, and arginine. These observations suggest that the rate of autoxidation of sugars and their facility to form glyoxal may be a rate-limiting factor in the kinetics of browning reactions induced by some sugars, including glucose (Wolff & Dean, 1987; and present work), fructose (McPherson *et al.*, 1988; Suarez *et al.*, 1989), and ascorbate (Ortwerth *et al.*, 1988). However, classical nonoxidative pathways involving glycation and generation of deoxydicarbonyl sugars, i.e., 1-, 3- and 4-deoxyaldosuloses, from Amadori adducts (Ledl & Schleicher, 1990) also occur in parallel with autoxidation reactions and may be the primary route of the Maillard reaction for some sugars, even under autoxidative conditions.

Figure 8 presents a few of many possible products of reaction of glyoxal with protein. In addition to CML formed on reaction with lysine, cyclic imidazolidones may be formed on reaction of glyoxal with arginine residues (Bengelsdorf, 1953). Of particular interest is the nature of crosslinks formed in proteins. Namiki & Hayashi (1986) have proposed

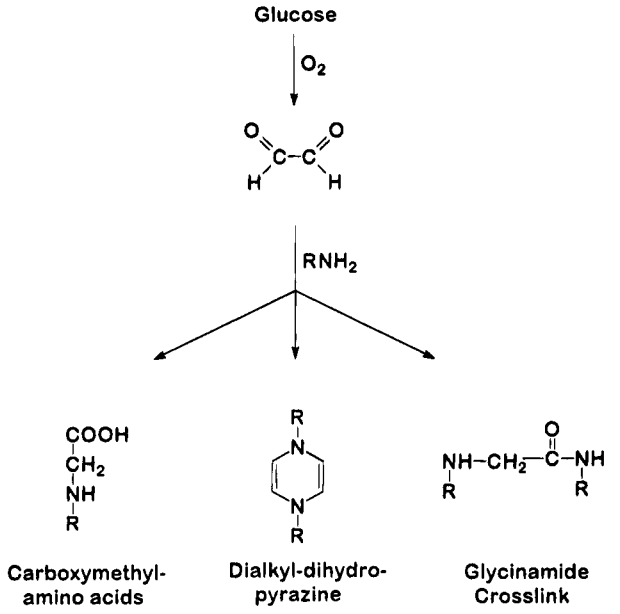

FIGURE 8: Proposed scheme for formation of glyoxal adducts and crosslinks in tissue proteins.

that glyoxal generates *N,N'*-dialkyldihydropyrazine crosslinks. On the basis of earlier work by Kliegman and Barnes (1970), we propose that glycinamide crosslinks (Figure 8) may also be formed following adduction of two lysines to glyoxal. Interestingly, this glycinamide crosslink would yield CML on hydrolysis of the protein, suggesting that CML may be an indicator of protein crosslinking during the Maillard reaction. The glycinamide crosslink would also be colorless and nonfluorescent, as we have previously proposed for the primary crosslinks formed in proteins during the Maillard reaction (Dyer *et al.*, 1991b). Related, substituted *N,N'*-dialkyldihydropyrazine and glycinamide structures might also be formed by similar mechanisms from other α-dicarbonyl sugars *in vivo*.

Role of Glyoxal in Accelerated Browning of Proteins in Diabetes. Increases in the level of glyoxal and other dicarbonyl sugars may play an important role in the cumulative modification of proteins in diabetes and in the development of diabetic complications. Aminoguanidine (AG) is known to inhibit the browning and crosslinking of collagen during Maillard reactions *in vitro* (Fu *et al.*, 1994) and *in vivo* (Brownlee *et al.*, 1986; Odetti *et al.*, 1990; Oxlund & Andreassen, 1992). In animal models of diabetes, AG also prevents development of complications, including nephropathy (Soulis-Liparota *et al.*, 1991; Itakura *et al.*, 1991), retinopathy (Hammes et al., 1991), neuropathy (Kihara *et al.*, 1991; Cameron *et al.*, 1992; Yagihashi *et al.*, 1992), and vascular disease (Kihara *et al.*, 1991; Corbett *et al.*, 1993). One hypothesis to explain AG inhibition of advanced stages of the Maillard reaction is its reaction with α-dicarbonyl compounds formed by oxidative (e.g., glyoxal) and nonoxidative (e.g., 3-deoxyglucosone) mechanisms, to form stable aminotriazine derivatives (Neunhoeffer, 1984; Hirsch *et al.*, 1991). We have detected 3-amino-1,2,4-triazine as a major product formed during autoxidation of glucose in the presence of AG (unpublished), consistent with the proposed role of glyoxal as a key intermediate in autoxidative glycosylation of protein during the Maillard reaction *in vitro*. We suggest that if glucose autoxidation and autoxidative glycosylation of protein contribute to the accelerated browning of proteins in diabetes, then there should be a corresponding increase in the steady state concentration of glyoxal in plasma and tissues in diabetes and 3-amino-1,2,4-triazine should be detectable among the products formed during inhibition of the Maillard reaction by AG *in vivo*.

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