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Kinetics of Nonenzymatic Glycation of Ribonuclease A Leading to Advanced Glycation End Products. Paradoxical Inhibition by Ribose Leads to Facile Isolation of Protein Intermediate for Rapid Post-Amadori Studies†

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ABSTRACT: Nonenzymatic glycation (Maillard reaction) of long-lived proteins is a major contributor to the pathology of diabetes and possibly aging and Alzheimer's disease. We report here kinetic studies of the glycation of the model protein ribonuclease A by glucose and ribose leading to the formation of antigenic advanced glycation end products ("AGEs"), detectable by AGE-specific polyclonal antibodies, and pentosidine, an acid-stable fluorescent AGE. As anticipated, the kinetics of glycation by ribose were considerably faster than by glucose, and the rate of AGE formation initially increased with increasing sugar concentrations. However, ribose above 0.15 M appeared to paradoxically slow the kinetics of AGE formation, suggesting ribose inhibits the conversion of "early" Amadori rearrangement products to "late" AGEs and thus favors the accumulation of reactive Amadori intermediates. The facile isolation of such protein intermediates was achieved by an "interrupted glycation" protocol in which free and reversibly bound (Schiff base) ribose was removed following a short (24 h) initial incubation with 0.5 M ribose at 37 °C. The kinetics of buildup of the Amadori intermediates and the kinetics of their post-Amadori conversion to antigenic AGEs were then independently studied. A rapid and reversible inhibition of the post-Amadori kinetics by free ribose was verified by direct re-addition of ribose to the isolated, sugar-free intermediate. The pH dependence of the kinetics of antigenic AGE formation from such intermediates was measured and exhibited an unusual bell-shaped profile over the pH range of 5.0−9.5 with a maximum near pH 8.0. Aminoguanidine, a pharmacological AGE inhibitor, was found to moderately or weakly inhibit antigenic AGE formation in such post-Amadori steps. The isolation of the glycated ribonuclease intermediate thus simplifies kinetic and mechanistic studies of AGE formation, permits AGE studies in the absence of complications arising from free or Schiff base bound sugar, and provides a novel methodology for evaluating the mechanism and efficacy of therapeutic agents that may inhibit AGE formation.

The elucidation of the complex kinetics and chemistry of nonenzymatic protein glycation reactions is essential not only to understanding the progression of important pathologies, such as diabetes (Brownlee & Cerami, 1981; Nathan, 1993; McCance et al., 1993; Vlassara et al., 1994; Reiser, 1991; Reiser et al., 1992; Stevens et al., 1978; Ruderman et al., 1992), neurodegenerative diseases (Vitek et al., 1994; Smith et al., 1994; Colaco & Harrington, 1994; Harrington & Colaco, 1994), and aging (Monnier & Cerami, 1981;

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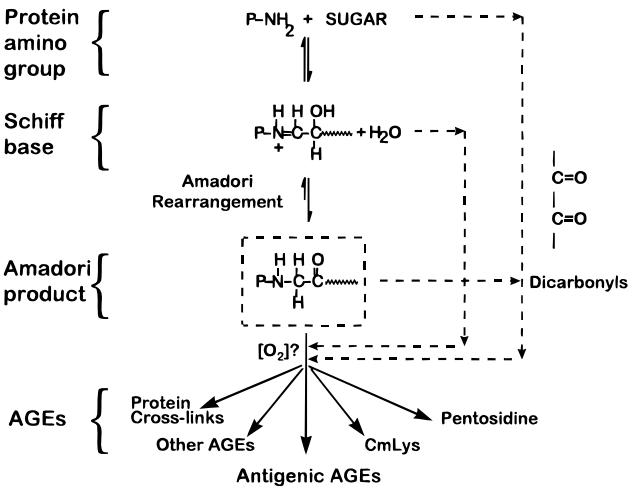
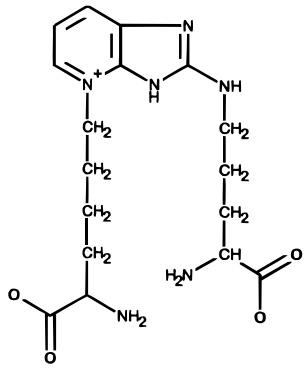
Scheme 1



Monnier, 1989; Monnier et al., 1990; Dyer et al., 1993), but also to the design of potentially therapeutic chemical agents that can inhibit and interfere with deleterious glycation reactions. Glycated proteins have also been shown to be toxic, antigenic, and capable of triggering cellular injury responses after uptake by specific cellular receptors (Vlassara et al., 1992, 1994; Daniels & Hauser, 1992; Brownlee, 1994; Cohen et al., 1994; Brett et al., 1993; Yan et al., 1994). In an effort to shed light on the mechanism of formation and inhibition of advanced glycation end products (Maillard products), commonly referred to as AGEs,[1] we report studies of the kinetics of the glycation reaction of the model protein ribonuclease A (RNase) with the pentose ribose. This is a much more reactive analogue of glucose due to the higher equilibrium concentration of the linear, open-chain aldehyde form that reacts with amines (Angyal, 1984, 1991; Bunn & Higgins, 1981; Means & Chang, 1982). Our studies have led to the unique isolation and kinetic characterization of a reactive protein intermediate competent in forming AGEs, thus opening the way for rapid quantitative kinetic studies of "late" stages of the protein glycation reaction.

In contrast to "late" AGE formation, the "early" steps of the glycation reaction have been relatively well characterized and identified for several proteins (cf. Harding, 1985; Baynes et al., 1989; Monnier & Baynes, 1990; Finot et al., 1990; Vlassara et al., 1994). Typically, sugars initially react in their open-chain (not the predominant pyranose and furanose structures) aldehydo or keto forms with lysine side-chain ϵ-amino and terminal α-amino groups through reversible Schiff base condensation (Scheme 1). The resulting aldimine or ketimine products then undergo Amadori rearrangements to give ketoamine Amadori products, i.e., formally 1-amino-1-deoxyketoses in the case of reaction with aldoses (Means & Chang, 1982; Harding, 1985; Baynes et al., 1989). The latter then undergo over periods of weeks and months slow and irreversible Maillard "browning" reactions, forming fluorescent and other products via rearrangement, dehydration, oxidative fragmentation, and cross-linking reactions. As with Amadori and other glycation intermediates (Baynes, 1991; Smith & Thornalley, 1992; Glomb & Monnier, 1995; Reddy et al., 1995), *free glucose* itself can undergo oxidative reactions (Thornalley et al., 1984; Wolff & Dean, 1987; Hunt et al., 1988; Jiang et al., 1990; Wells-Knecht et al., 1995) that lead to the production of peroxide and highly reactive fragments like the dicarbonyls glyoxal and glycoaldehyde. The elucidation of the mechanism of formation of a variety of AGEs thus becomes particularly complex, since most *in vitro* studies are carried out at extremely high sugar concentrations.

Scheme 2



Pentosidine

The "late" Maillard products that result are complex and poorly characterized (Reynolds, 1963; Njoroge & Monnier, 1989; Ledl, 1990; Ledl and Schleicher, 1990; Fu et al., 1992), with little kinetic information on the rates and mechanisms of their formation. Some notable AGEs are pentosidine (Scheme 2), an acid-stable fluorescent cross-link of lysine and arginine (Sell & Monnier, 1989; Grandhee & Monnier, 1991; Dyer et al., 1991), and N^{ϵ}-(carboxymethyl)lysine (Ahmed et al., 1986; Dunn et al., 1989, 1990, 1991; Fu et al., 1992, 1994; Slight et al., 1992). The former can arise from a variety of sugars, and the latter can be formed from dicarbonyls arising from free sugar (Glomb & Monnier, 1995), from Schiff base adducts (via the Namiki pathway), and from degradation products (glucosones) of Amadori intermediates (Reddy et al., 1995). Our present studies have monitored AGE formation through sensitive ELISA detection of antigenic AGEs using polyclonal AGE-specific antibodies (Nakayama et al., 1989, 1991; Horiuchi et al., 1991; Araki et al., 1992; Makita et al., 1992) and, to a lesser extent, pentosidine through its fluorescence. Although the dominant antigenic AGE epitopes have generally not been identified, there have been recent studies suggesting that (carboxymethyl)lysine may be an important antigenic component (Reddy et al., 1995).

The foundation of our present report is the novel observation that ribose at high concentrations is paradoxically *inhibitory* to AGE formation in ribonuclease by acting on the post-Amadori ("late") stages of the glycation reaction. This unexpected inhibition suggests that "early" reactive intermediates, presumably Amadori products, can be accumulated with little formation of late AGE products. The investigation of this phenomenon has enabled us to (1) define conditions for the kinetic isolation of an apparent Amadori (or post-Amadori) glycated intermediate(s), (2) study the fast kinetics of buildup of such an intermediate, (3) study the surprisingly *rapid* kinetics of conversion of such intermediates to AGE products *in the absence of free or reversibly bound sugar*, and (4) utilize these intermediates to study the

[1] Abbreviations: AGE, advanced glycation end product (Maillard product); BSA, bovine serum albumin; GlcAGE-BSA, BSA glycated with 1.5 M glucose for 90 days; RNase, bovine pancreatic ribonuclease A; ELISA, enzyme-linked immunosorbant assay.

inhibition of *post-Amadori* steps of AGE formation, thus providing a novel system to investigate the mechanism of action and efficacy of potential agents that could block AGE formation. These findings open the way to structural studies using approaches such as ^{13}C NMR to study the reactive intermediates and monitor their conversion to various candidate AGEs.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. Ribonuclease A was obtained from Worthington Biochemicals as a chromatographically pure and aggregate-free protein. Bovine serum albumin, D-ribose, and goat alkaline phosphatase-conjugated anti-rabbit IgG were from Sigma Chemicals. Aminoguanidine hydrochloride was purchased from Aldrich Chemicals.

Amino Acid Analysis. Amino acid analyses were carried out at the Biotechnology Support Facility of the Kansas University Medical Center. They were performed after hydrolysis of glycated protein (reduced with sodium cyanoborohydride) with 6 N HCl at 110 °C for 18−24 h. Phenyl isothiocyanate was used for derivatization, and PTH derivatives were analyzed by reverse-phase HPLC on an Applied Biosystems amino acid analyzer (420A derivatizer, 130A separation system, 920A data analysis system).

Preparation of Polyclonal Antibodies to Glycated BSA (AGE-BSA). Antibodies used to detect AGE-RNase were raised against AGE-BSA. Immunogen preparation generally followed earlier protocols (cf. Nakayama et al., 1989; Horiuchi et al., 1991; Makita et al., 1992; Papanastasiou et al., 1994). For most of the experiments, we utilized antibodies (designated R479) against an antigen (GlcAGE-BSA) prepared by glycation of BSA (1.6 g in 15 mL) for 90 days with 1.5 M glucose in 0.4 M phosphate containing 0.05% sodium azide at pH 7.4 and 37 °C. New Zealand white rabbit males of 8−12 weeks were used to produce the antibody response. They were immunized with subcutaneous administration of a 1 mL solution containing 1 mg/mL GlcAGE-BSA in Freund's adjuvant. The primary injection used the complete adjuvant, and subsequently three boosters were made at 3 week intervals with Freund's incomplete adjuvant. The rabbits were bled 3 weeks after the last booster. The serum was collected by centrifugation of clotted whole blood and was kept frozen for use in the determination of AGE formation in RNase. In agreement with previous studies by others, it was verified that these antibodies do not recognize Amadori products on proteins, since reduction of glycated protein with sodium borohydride does not diminish their recognition by the antibodies.

ELISA Detection of AGE Products. The general method of Engvall (1980) was used to perform ELISA assays. Protein samples, modified to varying extents with ribose, were diluted to approximately 1.5 μg/mL in 0.05 M carbonate buffer of pH 9.5−9.7. Then typically 0.3 μg of protein was coated[2] onto polystyrene wells overnight at room temperature. After coating, the protein was washed from the well with a saline solution containing 0.05% Tween-20. The wells were then blocked with 1% casein in carbonate buffer for 1 h, followed by washing. Rabbit anti-AGE antibodies were then added at a suitable titer (1:300 to 1:1000), and the primary antibody was incubated for 1 h at 37 °C followed by washing and pounding dry. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of about 1:2500 and incubated for 1 h, followed by washing and pounding dry. The *p*-nitrophenyl phosphate substrate was then added to the wells and incubated at 37 °C, with the absorbance of the released *p*-nitrophenolate being monitored at 410 nm until a suitable reading was obtained. Controls containing unmodified protein were routinely included, and their readings were subtracted, the corrections usually being negligible.

Pentosidine Reverse-Phase HPLC Analysis. Pentosidine production in RNase was quantitated by HPLC (Sell & Monnier, 1989; Odetti et al., 1992). Ribose-modified protein samples were hydrolyzed in 6 N HCl for 18 h at 100 °C and then dried in a Speed Vac. They were then redissolved, and aliquots were taken into 0.1% trifluoroacetic acid and analyzed by HPLC on a Shimadzu system using a Vydac C-18 column equilibrated with 0.1% TFA. A gradient of 0−6% acetonitrile (0.1% in TFA) was run in 30 min at a flow rate of about 1 mL/min. Pentosidine was detected by 335 nm excitation/385 nm emission fluorescence, and its elution time was determined by running a synthesized standard. Due to the extremely small levels of pentosidine expected (Grandhee & Monnier, 1991; Dyer et al., 1991), no attempt was made to quantitate the absolute concentrations. Only relative concentrations were determined from peak areas.

Glycation Modifications. Modification with ribose or glucose was generally done at 37 °C in 0.4 M phosphate buffer of pH 7.5 containing 0.02% sodium azide. The high buffer concentration was always used with 0.5 M ribose modifications. The solutions were kept in capped tubes and opened only to remove timed aliquots that were immediately frozen for later carrying out the various analyses. "Interrupted glycation" experiments were carried out by first incubating protein with the ribose at 37 °C for 8 or 24 h, followed by immediate and extensive dialysis against frequent cold buffer changes at 4 °C. The samples were then reincubated by quickly warming to 37 °C in the absence of external ribose. Aliquots were taken and frozen at various intervals for later analysis. Due to the low molecular weight of RNase, protein concentrations were remeasured after dialysis even when low molecular weight cut-off dialysis tubing was used. An alternative procedure was also devised (see below) in which interruption was achieved by simple 100-fold dilution from reaction mixtures containing 0.5 M ribose. Protein concentrations were estimated from UV spectra. The difference in molar extinction between the peak (278 nm) and trough (250 nm) was used for RNase concentration determinations in order to compensate for the general increase in UV absorbance that accompanies glycation. Time-dependent UV-difference spectral studies were carried out to characterize the glycation contributions to the UV spectrum.

Data Analysis and Numerical Simulations of Kinetics. Kinetic data were routinely fit to monoexponential or biexponential functions using nonlinear least-squares methods. The kinetic mechanisms of Schemes 3−4 have been examined by numerical simulations of the differential equations of the reaction. Both simulations and fitting to observed

[2] The validity of the use of the ELISA method in quantitatively studying kinetics of AGE formation depends on the linearity of the assay (Kemeny & Challacombe, 1988). We have determined by control experiments that the linear range for RNase is below a coating concentration of about 0.2−0.3 mg/well.

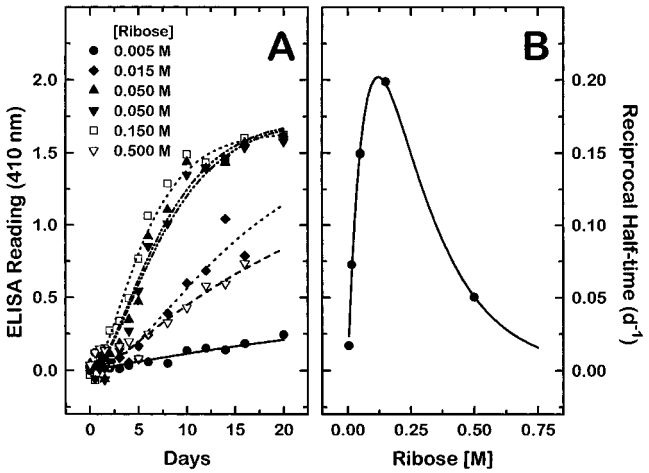


FIGURE 1: (A) Kinetics of glycation of RNase A (10 mg/mL) by ribose in 0.4 M phosphate buffer at 37 °C as monitored by reactivity against antibodies (R479) to glucose-modified BSA using ELISA. (B) Dependence of reciprocal half-times on ribose concentration at pH 7.5.

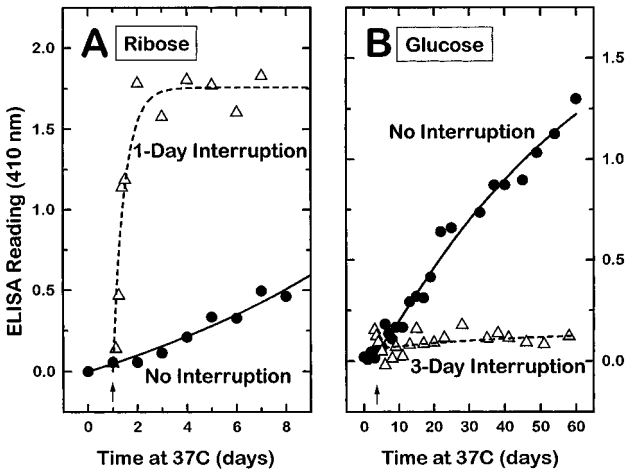


FIGURE 2: Comparison of uninterrupted and interrupted glycation of RNase by glucose (0−60 days) and ribose (0−8 days) at 37 °C in 0.4 M phosphate buffer of pH 7.5. Glycation was detected with R479 antibodies to glucose-modified BSA using ELISA. Interruption was carried out by cooling samples after the indicated times (3 days for glucose, 24 h for ribose) at 37 °C and then extensively dialyzing in the cold for 24 h, followed by rewarming to 37 °C. Time (abscissa) indicates the total 37 °C incubation time, i.e., excluding the cold dialysis time. (A) Solid line and solid circles, uninterrupted reaction with 0.5 M ribose; dashed line and open triangles, reaction with 0.5 M ribose interrupted at 24 h (arrow). (B) Solid line and solid circles: uninterrupted reaction with 1.0 M glucose; dashed line and open triangles, glucose was dialyzed out after 3 days of reaction (arrow). Note the different time ranges in (A) and (B) and that absolute ELISA readings in (A) and (B) cannot be compared.

kinetics data were carried out with the SCIENTIST 2.0 software package (Micromath, Inc.). Determination of apparent half-times (Figure 1B) from kinetic data fit to two-exponential functions (Figure 1A) was carried out with the "solve" function of MathCAD 4.0 software (MathSoft, Inc).

RESULTS

Comparison of Glycation by Glucose and Ribose. The reaction of RNase A with ribose and glucose has been followed primarily with ELISA assays, using R479 rabbit AGE-specific antibodies developed against glucose-modified BSA. To a lesser extent, we also followed the production of pentosidine, the only known acid-stable fluorescent AGE, which was quantitated by HPLC following acid hydrolysis. Preliminary studies using 0.05 M ribose at 37 °C showed that the rate of antigenic AGE formation appears to be modestly increased (roughly 2−3-fold as measured by the apparent half-time) as the pH is increased from 5.0 to 7.5, with an apparent small *induction period* at the beginning of the kinetics in all cases. The glycation of RNase with 0.05 M ribose at pH 7.5 (half-time near 6.5 days) appears to be almost an order of magnitude faster than that of glycation with 1.0 M glucose (half-time in excess of 30 days; cf. Figure 2B below, solid line). The latter kinetics also displayed a small induction period but incomplete leveling off even after 60 days, making it difficult to estimate a true half-time. When the dependence of the kinetics on ribose concentration was examined at pH 7.5, a most unexpected result was obtained. The rate of reaction initially increased with increasing ribose concentration, but *at concentrations above about 0.15 M the rate of reaction leveled off and then significantly decreased* (Figure 1A). A plot of the dependence of the reciprocal half-time on the concentration of ribose (Figure 1B) shows that high ribose concentrations are paradoxically *inhibitory* to antigenic AGE formation. This unusual but consistent effect was found to be independent of changes in the concentration of either buffer (2-fold) or RNase (10-fold), and it was not changed by affinity purification of the R479 antibody on a column of immobilized AGE-RNase. It is also not due to effects of ribose on the ELISA assay itself.[3]

Kinetics of Formation of Antigenic AGEs by "Interrupted Glycation". In view of the small induction period seen, an attempt was made to determine whether there was some accumulation during the reaction of an early precursor, such as an Amadori intermediate, capable of producing the ELISA-detectable AGE antigens. RNase was glycated at pH 7.5 and 37 °C with a high ribose concentration of 0.5 M, and the reaction was interrupted after 24 h by immediate cooling to 4 °C and dialysis against several changes of cold buffer over a period of 24 h to remove free and reversibly bound (Schiff base) ribose. Such a ribose-free sample was then rapidly warmed to 37 °C *without re-adding of any ribose*, and was sampled for AGE formation over several days. The AGE antigen production of this 24 h "interrupted glycation" sample is shown by the dashed line and open triangles in Figure 2A, the time spent in cold dialysis not being included. An uninterrupted control (solid line and filled circles) is also shown for comparison. Dramatically different kinetics of antigenic AGE formation are evident in the two samples. The kinetics of AGE antigen production of the ribose-free interrupted sample now show (1) *mono-exponential* kinetics with no induction period, (2) a *greatly enhanced rate* of antigenic AGE formation, with remarkable half-times of the order of 10 h, and (3) production of levels of antigen comparable to those seen in long incubations in the continued presence of ribose (cf. Figure 1A). Equally significant, the data also demonstrate that negligible AGE antigen was formed during the cold dialysis period, as shown

[3] The inhibitory effect by ribose on AGE formation is extremely unlikely to be due to ribose interference with antibody recognition of antigenic sites on protein in the ELISA assay. Prior to the first contact with the primary anti-AGE antibody on the ELISA plates, glycated protein has been diluted over 1000-fold, washed extensively with Tween-20 after adsorption, and blocked with a 1% casein followed by further washing with Tween-20.

by the small difference between the open triangle and filled circle points at time 1 day in Figure 2A. These observations suggest that *a fully competent isolatable intermediate or precursor to antigen AGE has been generated during the 24 h contact with ribose prior to the removal of the free and reversibly bound sugar*.

Samples interrupted after only 8 h produced a final amount of AGE antigen that was about 72% of the 24 h interrupted sample. Samples of RNase glycated with only 0.05 M ribose and interrupted at 8 h by cold dialysis and reincubation at 37 °C revealed less than 5% production of ELISA-reactive antigen after 9 days. Interruption at 24 h, however, produced a rapid rise of ELISA antigen (similar to Figure 2A) to a level of roughly 50% of that produced in the uninterrupted presence of 0.05 M ribose. The general interruption effects reported above were not unique to the reaction of RNase. We have also examined the interrupted glycation of BSA and hemoglobin (data not shown). Except for somewhat different absolute values of the rate constants and the amount of antigenic AGEs formed during the 24 h 0.5 M ribose incubation, the same dramatic increase in the rate of AGE antigen formation was observed after removal of 0.5 M ribose.

Glycation is much slower with *glucose* than with ribose (note the difference in time scales between Figure 2A and Figure 2B). However, unlike the case with ribose, interruption after 3 days of glycation by 1.0 M glucose produced negligible buildup of precursor to ELISA-reactive AGE antigens (Figure 2B, dashed curve).

Kinetics of Pentosidine Formation. The content of pentosidine, the acid-stable AGE, was measured for the same RNase samples previously analyzed for antibody reactivity by ELISA. Glycation by ribose in 0.4 M phosphate buffer of pH 7.5 produced pentosidine in RNase A that was quantitated by its fluorescence after acid hydrolysis. Figure 3A shows that under uninterrupted conditions, 0.05 M ribose produces a progressive increase in pentosidine. When glycation is carried out under interruption conditions with 0.5 M ribose, a dramatic increase in the rate of pentosidine formation is seen immediately after removal of excess ribose (Figure 3B), which is similar to but a little more rapid than the kinetics of the appearance of the ELISA-reactive AGE antigens (Figure 2A). A greater amount of pentosidine was also produced with 24 h interruption as compared to 8 h. Reproducible differences between the kinetics of formation of antigenic AGEs and pentosidine can also be noted. A significant amount of the AGE pentosidine is formed during the 24 h incubation and also during the cold dialysis, resulting in the jump of the dashed vertical line in Figure 3B. Our observations thus demonstrate that a pentosidine precursor accumulates during ribose glycation that can rapidly produce pentosidine after ribose removal (cf. Odetti et al., 1992).

Rate of Buildup of the Reactive Intermediate(s). The "interrupted glycation" experiments described above demonstrate that a precursor or precursors to both antigenic AGEs and pentosidine can be accumulated during glycation with ribose. The kinetics of formation of this intermediate can be independently followed and quantitated by a variation of the experiments described above. The amount of intermediate generated in RNase at different contact times with ribose can be assayed by the maximal extent to which it can produce antigenic AGE after interruption. At variable times after initiating glycation, the free and reversibly-bound ribose is

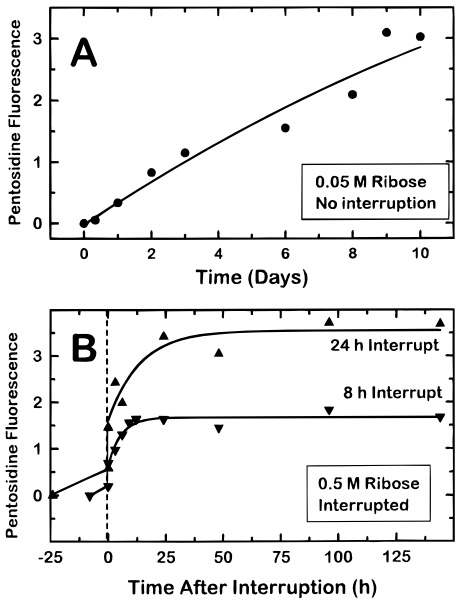


FIGURE 3: Kinetics of pentosidine fluorescence (arbitrary units) increase during uninterrupted and interrupted ribose glycation of RNase (10 mg/mL) in 0.4 M phosphate buffer of pH 7.5. Fluorescence was measured on identical protein aliquots after acid hydrolysis as described under Experimental Procedures. (A) Uninterrupted glycation in the presence of 0.05 M ribose. (B) Interrupted glycation after 8 and 24 h of incubation with 0.5 M ribose at 37 °C. Ribose was then removed by cold dialysis (zero time indicated by vertical dashed line), and samples were reincubated at 37 °C. Note the small jump in pentosidine that occurs at zero time during the dialysis.

removed by dialysis in the cold or by rapid dilution (see below). Sufficient time (5 days, which represents several half-lives according to Figure 2A) is then allowed after warming to 37 °C for *maximal* development of antigenic AGEs. The ELISA readings 5 days after each interruption point, representing maximal AGE development, would then be proportional to the intermediate concentration present at the time of interruption.

Figure 4 shows such an experiment where the kinetics of intermediate buildup are measured for RNase A in the presence of 0.5 M ribose (solid symbols and curve). For comparison, the amount of AGE present before ribose removal at each interruption point is also shown (open symbols and dashed lines). As expected (cf. Figure 2A), little AGE is formed prior to removal (or dilution) of ribose, so that ELISA readings after the 5 day secondary incubation periods are mostly a measure of AGE formed *after* ribose removal. The results in Figure 4 show that the rate of build up of intermediate in 0.5 M ribose is exponential and very fast, with a half-time of about 3.3 h. This is about 3-fold more rapid than the observed rate of conversion of the intermediate to antigenic AGEs after interruption (open symbols and dashed curve in Figure 2A).

It should be noted that in these experiments the removal of ribose at each interruption time was achieved not by dialysis but by a simple 100-fold dilution of the solution that reduced the ribose concentration to 0.005 M. We independently determined (Figure 1A) that little AGE is produced in this time scale with the residual 5 mM ribose. This dilution approach was primarily dictated by the need for quantitative point-to-point accuracy. Such accuracy would not have been achieved by the dialysis procedure that would be independently carried out for the samples at each

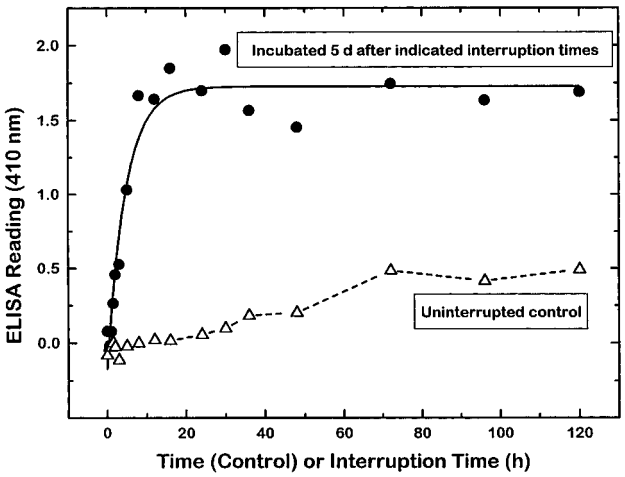


FIGURE 4: Kinetics of reactive intermediate buildup in 0.5 M ribose. RNase (10 mg/mL) was mixed with 0.5 M ribose at 37 °C in 0.4 M phosphate buffer of pH 7.5. At different times as indicated on the abscissa, aliquots were diluted 100-fold with buffer to interrupt the glycation and were measured by ELISA immediately after dilution (open triangles, dashed curve) and after 5 days of incubation at 37 °C (filled circles, solid line). The difference between the two curves (vertical distance at any given time) represents the amount of AGE that developed in the 5 days of incubation following the dilution interruption.

time point. A separate control experiment (cf. Figure 5 below) demonstrated that the instantaneous 100-fold dilution gave nearly identical results to the dialysis procedure. These control experiments demonstrate that the reversible ribose−protein binding (Schiff base) equilibrium is quite rapid on this time scale. This is consistent with the data of Bunn and Higgins (1981) that indicated that the half-time for Schiff base formation with 0.5 M ribose should be of the order of a few minutes. The 100-fold rapid dilution method to reduce ribose thus appears valid and has been successfully used in our other experiments (see below) where quantitative accuracy is essential and cannot be achieved by multiple dialyses of many samples.

Direct Inhibition of Post-Amadori AGE Formation from the Intermediate by Ribose and Glucose. The increase in the rate of AGE formation after interruption and sugar dilution suggests, but does not prove, that high concentrations of ribose are inhibiting the reaction at or beyond the first "stable" intermediate, presumably the Amadori derivative (boxed in Scheme 1). A test of this was then carried out by studying the effect of directly adding ribose on the post-Amadori reaction. RNase was first incubated for 24 h in 0.5 M ribose in order to prepare the intermediate. Two protocols were then carried out to measure possible inhibition of the post-Amadori formation of antigenic AGEs by different concentrations of ribose. In the first experiment, the 24 h ribated sample was simply diluted 100-fold into solutions containing varying final concentrations of ribose ranging from 0.005 to 0.505 M (Figure 5A). The rate and extent of AGE formation are clearly seen to be diminished by increasing ribose concentrations. Significantly, up to the highest concentration of 0.5 M ribose, the kinetics appear exponential and do not show the induction period that occurs with uninterrupted glycation (Figures 1A and 2A) in high ribose concentrations.

A second experiment (Figure 5B) was also conducted in which the 24 h interrupted sample was extensively dialyzed in the cold to release free and reversibly bound ribose as

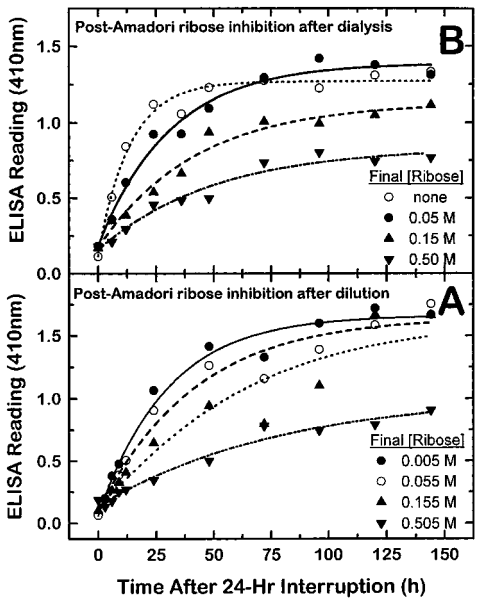


FIGURE 5: Post-Amadori inhibition of AGE formation by ribose. RNase (10 mg/mL) was incubated with 0.5 M ribose in 0.4 M phosphate buffer of pH 7.5 at 37 °C for 24 h. (A) At that time (time 0 on the abscissa), aliquots were diluted into buffers containing the indicated final concentrations of fresh ribose, and the solutions were incubated at 37 °C. Samples were then removed at various times for assay by ELISA. (B) At 24 h interruption, the sample was extensively dialyzed in the cold to remove excess and reversibly bound (Schiff base) ribose. It was then diluted into buffers containing the indicated concentrations of fresh ribose as in (A).

well as any inhibitory products that may have formed during the 24 h incubation with ribose. Following this, aliquots were diluted 100-fold into varying concentrations of freshly made ribose, and the formation of antigenic AGE products was monitored as above. There results were nearly identical to the experiment of Figure 5A where the dialysis step was omitted. In both cases, the rate and extent of AGE formation were diminished by increasing concentrations of ribose, and the kinetics appeared exponential with no induction period.

The question naturally arises as to whether glucose or other sugars can also inhibit the formation of AGEs from the reactive intermediate obtained by interrupted glycation in 0.5 M ribose. The effects of glucose at concentrations of 1.0−2.0 M were tested (data not shown). Glucose was clearly not as inhibitory as ribose. When the 24 h ribose interrupted sample was diluted 100-fold into these glucose solutions, the amount of antigenic AGE formed was diminished by about 30%, but there was little decrease in the apparent rate constant. Again, the kinetics appeared exponential.

Effect of pH on Post-Amadori Kinetics of AGE Formation. We have used the above interruption method to investigate the pH dependence of the post-Amadori kinetics of AGE formation from the reactive intermediate. In these experiments, RNase A was first reacted for 24 h with 0.5 M ribose at pH 7.5 to generate the reactive intermediate. The kinetics of the decay of the intermediate to AGEs were then measured by 100-fold dilution into buffers of variable pH, and the amount of AGEs formed at various times was measured by ELISA. Figure 6 shows that an extremely wide pH range of 5.0−9.5 was achievable when the kinetics were measured by initial rates. A remarkable bell-shaped dependence was observed, showing that the kinetics of antigenic AGEs formation are decreased at both acidic and alkaline pH ranges, with an optimum near pH 8.

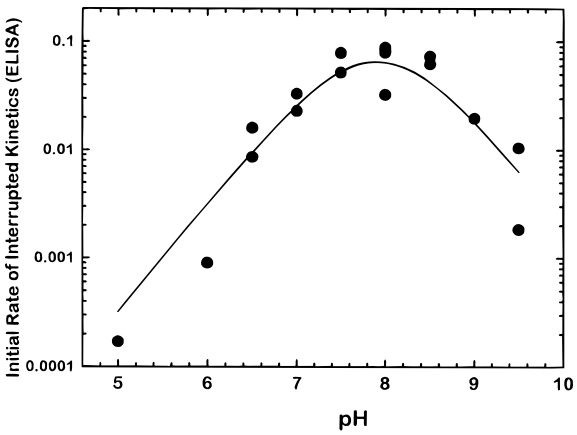


FIGURE 6: Dependence of the initial rate of formation of antigenic AGE on pH following interruption of glycation. RNase (10 mg/mL) was reacted for 24 h with 0.5 M ribose at 37 °C in 0.4 M phosphate buffer of pH 7.5. Reaction was then interrupted by 100-fold dilution of aliquots into 0.4 M phosphate buffers of different pH covering the range of 5.0−9.5. Each pH solution was then assayed for AGE by ELISA as a function of time, and the initial rates were determined by curve-fitting of the kinetics. In the case of the very slow reaction below pH 6, linear determinations were utilized, whereas for the other data initial slopes were estimated after exponential nonlinear least-squares curve-fitting.

A single "pH jump" experiment was also carried out on the pH 5.0 sample studies above which had the slowest rate of antigenic AGE formation. After 12 days at 37 °C in pH 5.0 buffer, the pH was adjusted quickly to 7.5, and antigenic AGE formation was monitored by ELISA. Within experimental error, the sample showed identical kinetics (same first-order rate constant) of AGE formation to interrupted glycation samples that had been studied directly at pH 7.5. In this experiment, the relative amounts of antigenic AGE could not be directly compared on the same ELISA plate, but the pH-jumped sample appeared to have formed substantial though somehow diminished levels of antigenic AGE.

Inhibition of Post-Amadori AGE Formation by Aminoguanidine. We have tested the efficacy of aminoguanidine by our interrupted glycation method, i.e., by testing its effect on the post-Amadori formation of antigenic AGEs after removal of excess and reversibly bound ribose. Figure 7 demonstrates that aminoguanidine has modest effects on blocking the formation of antigenic AGEs in RNase under these conditions, with little inhibition below 50 mM. Approximately 50% inhibition is achieved only at or above 100−250 mM. Note again that in these experiments, the protein was exposed to aminoguanidine only after interruption and removal of free and reversibly bound ribose. Comparable results were also obtained with the interrupted glycation of BSA and hemoglobin (data not shown).

Amino Acid Analysis of Interrupted Glycation Samples. Amino acid analysis was carried out on RNase after 24 h contact with 0.5 M ribose (undialyzed), after extensive dialysis of the 24 h glycated sample, and after 5 days of incubation of the latter sample at 37 °C. These determinations were made after sodium cyanoborohydride reduction, which reduces any Schiff base present on lysines or the terminal amino group. All 3 samples, normalized to alanine (12 residues), showed the same residual lysine content (4.0 ± 0.5 out of the original 10 in RNase). This indicates that after 24 h contact with 0.5 M ribose, most of the formed Schiff base adducts had been converted to Amadori or subsequent products. No arginine or histidine residues were lost by modification.

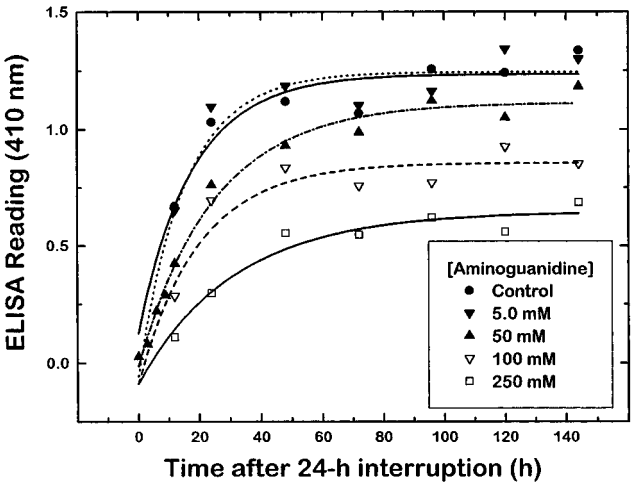


FIGURE 7: Inhibition by aminoguanidine hydrochloride of AGE formation after interruption of glycation. RNase (10 mg/mL) was incubated with 0.5 M ribose at 37 °C in 0.4 M phosphate buffer for 24 h. At that time (time 0 on the abscissa), samples were 100-fold diluted into buffers containing the indicated concentrations of aminoguanidine hydrochloride, and the solutions were incubated at 37 °C, with aliquots being taken at different times for ELISA.

DISCUSSION

The use of the rapidly reacting ribose and the discovery of its reversible inhibition of post-Amadori steps have permitted us to dissect and determine the kinetics of different steps of protein glycation in RNase. Most previous kinetic studies of protein "glycation" have actually been restricted to the "early" steps of Schiff base formation and subsequent Amadori rearrangement. Some kinetic studies have been carried out starting with synthesized fructosylamines, i.e., small model Amadori compounds of glucose [cf. Smith and Thornalley (1992) and references cited therein], but such studies, with few exceptions,[4] have hitherto not been possible with proteins. The greater reactivity of ribose has also proven a distinct advantage in quantitatively defining the time course of AGE formation. We note that glucose and ribose are both capable of producing similar AGE products, such as pentosidine (Grandhee & Monnier, 1991; Dyer et al., 1991), and some ^{13}C NMR model compound work has been done with ADP-ribose (Cervantes-Laurean et al., 1993). Our present work shows that antigenic AGE products of ribose fully cross-react with anti-AGE antibodies directed against glucose-modified proteins, suggesting that ribose and glucose produce similar antigenic AGEs. The primary kinetic differences observed between these two sugars are probably due to relative differences in the rate constants of steps leading to AGE formation, rather than in the mechanism.

The results presented in this study reveal a marked and paradoxical inhibition of overall AGE formation by high concentrations of ribose (Figure 1) that has not been

[4] Although earlier attempts have shown that proteins incubated with glucose for weeks are still capable of undergoing some cross-linking and fluorescence generation when the free sugar is removed (Eble et al., 1983; Suarez et al., 1991; McPherson et al., 1988), the reactive intermediates and possible AGE products could not be identified or quantitated. One notable exception is the demonstration by Monnier (Odetti et al., 1992) that BSA partially glycated with ribose can rapidly produce pentosidine after ribose removal.

anticipated by earlier studies. This inhibition is rapidly reversible in the sense that it is removed by dialysis of initially modified protein (Figure 2A) or by simple 100-fold dilution (as used in Figure 6). The experiments of Figure 5 demonstrate that it is not due to the accumulation of dialyzable inhibitory intermediates during the initial glycation, since dialysis of 24 h modified protein followed by addition of different concentrations of fresh ribose induces the same inhibition. The data of Figure 5A,B show that the inhibition occurs by reversible and rapid interaction of ribose with a protein intermediate containing reactive Amadori products. The inhibition is unlikely to apply to the early step of formation of Amadori product due the rapid rate of formation of the presumed Amadori intermediate that we determined in the experiment of Figure 4. The identification of the reactive intermediate as an Amadori product is well supported by the amino acid analysis carried out (after sodium cyanoborohydride reduction) before and after dialysis at the 24 h interruption point. The unchanged residual lysine content indicates that any dischargeable Schiff bases have already been irreversibly converted (presumably by Amadori rearrangement) by the 24 h time.

The secondary ribose suppression of "late" but not "early" glycation steps significantly enhances the accumulation of a fully-competent reactive Amadori intermediate containing little AGE. Its isolation by the interruption procedure is of importance for kinetic and structural studies, since it allows one to make studies in the absence of free or Schiff base bound sugar and their attendant reactions and complications. For example, we have measured the post-Amadori conversion rates to antigenic AGE and pentosidine AGE products (Figure 2A, open symbols, and Figure 3B) and demonstrated that they are much faster ($t_{1/2} \leq 10$ h) than reflected in the overall kinetics under uninterrupted conditions (Figure 1A and Figure 3A). The rapid formation of pentosidine that we measured appears consistent with an earlier interrupted ribose experiment on BSA by Odetti et al. (1992).[4] Since ribose and derivatives such as ADP-ribose are normal metabolites, the very high rates of AGE formation seen here suggest that they be considered more seriously as sources of potential glycation in various cellular compartments (Cervantes-Laurean et al., 1993), even though their concentrations are well below those of the less reactive glucose.

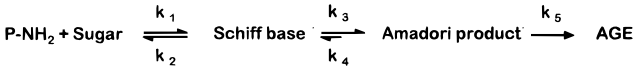
Another new application of the isolation of the intermediate is in studying the pH dependence of this complex reaction. The unusual bell-shaped pH profile seen for the post-Amadori AGE formation (Figure 6) is in striking contrast to the mild pH dependence of the overall reaction. The latter kinetics reflect a composite effect of pH on all steps in the reaction, including Schiff base and Amadori product formation, each of which may have unique pH dependencies. This complexity is especially well illustrated in studies of hemoglobin glycation (Lowrey et al., 1985). The bell-shaped pH profile suggests, but does not prove, the involvement of two ionizing groups. If true, the data may imply the participation of a second amino group, such as from a neighboring lysine, in the formation of dominant antigenic AGEs. The observed pH profile and the pH-jump observations we described under Results both suggest that a useful route to isolating and maintaining the reactive intermediate would be by the rapid lowering of the pH to near 5 after 24 h interruption.

Our kinetic studies also provide new insights into the mechanisms of action of aminoguanidine (guanylhydrazine), an AGE inhibitor proposed by Cerami and co-workers to combine with Amadori intermediates (Brownlee et al., 1986). This proposed pharmacological agent (Brownlee et al., 1986) is now in Phase III clinical trials for possible therapeutic effects in diabetes (Vlassara et al., 1994). However, its mechanism of AGE inhibition is likely to be quite complex, since it is multifunctional (cf. Smith and Thornalley, 1992). As a nucleophilic hydrazine, it can reversibly add to active carbonyls, including aldehydo carbonyls of open-chain glucose and ribose (Khatami et al., 1988; Hirsch et al., 1995), as well as keto carbonyls of Amadori compounds. It is also a guanidinium compound that can scavenge highly reactive dicarbonyl glycation intermediates such as glyoxal and glucosones (Chen & Cerami, 1993; Hirsch et al., 1992; Ou & Wolff, 1993). The interrupted glycation method allowed us to examine its potential efficacy on only post-Amadori steps of AGE formation. Equally important, it allowed studies in the absence of free sugar or dicarbonyl-reactive fragments from free sugar (Wolff & Dean, 1987; Wells-Knecht et al., 1995) that can combine with aminoguanidine. The results of Figure 7 demonstrate that aminoguanidine has, at best, only a modest effect on post-Amadori AGE formation reactions, achieving 50% inhibition at concentrations above 100−250 mM. Its efficacy may thus predominantly arise either from inhibiting the early steps of glycation (Schiff base formation) or from scavenging highly reactive dicarbonyls generated during glycation.

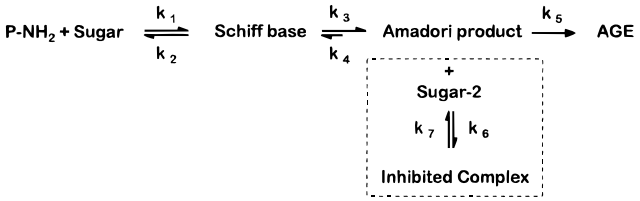
The use of interrupted glycation is not limited for kinetic studies. We believe it has clear potential for also simplifying structural studies of glycated proteins and identifying unknown AGEs using techniques such as ^{13}C NMR that has been used to detect Amadori adducts of RNase (Neglia et al., 1983, 1985). The combined use of structural and kinetic approaches should also be of special interest. For example, although the identity of the dominant antigenic AGEs reacting with our polyclonal antibodies remains uncertain, candidate antigenic AGEs, such as the recently proposed (carboxymethyl)lysine (Reddy et al., 1995; cf. Makita et al., 1992), should display the same kinetics of formation from the reactive intermediate that we have observed. The availability of the interrupted kinetics approach we described should also help determine the importance of the Amadori pathway to the formation of this AGE (cf. Glomb and Monnier, 1995). Similarly, monitoring of the interrupted glycation reaction by techniques such as ^{13}C NMR (Neglia et al., 1983, 1985; Cervantes-Laurean et al., 1993) should identify resonances of other candidate antigenic AGEs as being those displaying similar kinetics of appearance. In all cases, the ability to remove excess free and Schiff base sugars through interrupted glycation should considerably simplify isolation, identification, and structural characterization.

Finally, it should be emphasized that the chemical nature of the secondary inhibition of AGE formation by ribose that we have described remains to be elucidated. The results of this study only demonstrate that it is reversible, rapid, and involves reactions steps subsequent to Amadori formation. Kinetically, the inhibition is not simply the consumption of free lysines that are sites for initiation of glycation. Numerical simulations show that the simplest kinetic mechanism, shown in Scheme 3, will *not* lead to a decrease in rate

Scheme 3



Scheme 4



of AGE formation with increasing sugar concentrations. Interestingly, however, Scheme 3 does predict that the rate of AGE formation will reach a maximum in the limit of high sugar concentration, i.e., when the rate of Schiff base formation will increase so as to no longer be rate-limiting. To simulate the observed kinetics of AGE formation in RNase, where ribose inhibition of the kinetics was found (Figure 1 and Figure 5), an additional step involving inhibition is required such as that shown boxed in Scheme 4. There we add a second sugar molecule that rapidly and reversibly combines with an Amadori intermediate to form a complex that either does not convert to AGE products or does so much more slowly. Scheme 4 can qualitatively and quantitatively reproduce all the kinetic features seen under both uninterrupted (Figure 1A) and interrupted (Figure 2A, Figure 5) ribose glycation of RNase.

The molecular basis for such inhibition is unknown, but it can arise in several ways. Ribose may inhibit in a protein-specific manner by binding noncovalently at sites of RNase close to glycation sites, thus interfering with conversion to AGEs. It can also arise from sugar molecules forming a reversible Schiff base with available amino groups in the modified (Amadori) protein intermediate. This could occur either with an unmodified lysine amino group adjacent to a glycated site or with the secondary amine of an Amadori product itself to give a mixed Amadori−Schiff base compound. Secondary amines are known to react with carbonyl compounds, such as in the reductive di-alkylation of lysines with formaldehyde (Reynolds, 1963; Jentoft & Dearborn, 1979; Jentoft et al., 1979, 1981; Means & Feeney, 1971) or with reducing disaccharides (Schwartz & Gray, 1977). It has also long been known that two Schiff bases (Linek et al., 1993) or two Amadori products can form on a single primary amino group (Reynolds, 1963). The latter is illustrated by the isolation of di-fructose-glycine and related compounds (Anet, 1959a,b; 1960). A most unusual structure of such a di-Amadori compound has recently been determined (Mossine et al., 1995). Obviously, much further work is needed to distinguish between such possibilities before the basis for the observed ribose inhibition is elucidated. There is no obvious reason why glucose, as we reported, and other sugars cannot be inhibitory by the same processes, and it would be anticipated that such inhibition would be dependent on sugar structure and on protein.

In conclusion, the observation of ribose inhibition of AGE formation and its subsequent use in interrupted glycation has provided novel opportunities to study protein glycation. It has opened the way to more searching kinetic, mechanistic, and structural studies of glycation end products. The use of interrupted ribose glycation conditions that we have described not only should facilitate the elucidation of steps leading to the formation of antigenic AGEs but also should provide a novel system for the *in vitro*, and possibly *in vivo*, evaluation of the efficacy of therapeutic or pharmacological agents that may inhibit AGE formation. Current studies are in progress to investigate these applications.

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