Nonenzymatic protein glycation (Maillard reaction) leads to heterogeneous, toxic, and antigenic advanced glycation end products (“AGEs”) and reactive precursors that have been implicated in the pathogenesis of diabetes, Alzheimer’s disease, and normal aging. In vitro inhibition studies of AGE formation in the presence of high sugar concentrations are difficult to interpret, since AGE-forming intermediates may oxidatively arise from free sugar or from Schiff base condensation products with protein amino groups, rather than from just their classical Amadori rearrangement products. We recently succeeded in isolating an Amadori intermediate in the reaction of ribonuclease A (RNase) with ribose (Khalifah, R. G., Todd, P., Booth, A. A., Yang, S. X., Mott, J. D., and Hudson, B. G. (1996) Biochemistry 35, 4645–4654) for rapid studies of post-Amadori AGE formation in absence of free sugar or reversibly formed Schiff base precursors to Amadori products. This provides a new strategy for a better understanding of the mechanism of AGE inhibition by established inhibitors, such as aminoguanidine, and for searching for novel inhibitors specifically acting on post-Amadori pathways of AGE formation. Aminoguanidine shows little inhibition of post-Amadori AGE formation in RNase and bovine serum albumin, in contrast to its apparently effective inhibition of initial (although not late) stages of glycation in the presence of high concentrations of sugar. Of several derivatives of vitaminoles and B6, only recently studied for possible AGE inhibition in the presence of glucose (Booth, A. A., Khalifah, R. G., and Hudson, B. G. (1996) Biochem. Biophys. Res. Commun. 220, 113–119), pyridoxamine and, to a lesser extent, thiamine pyrophosphate proved to be novel and effective post-Amadori inhibitors that decrease the final levels of AGEs formed. Our mechanism-based approach to the study of AGE inhibition appears promising for the design and discovery of novel post-Amadori AGE inhibitors of therapeutic potential that may complement others, such as aminoguanidine, known to either prevent initial sugar attachment or to scavenge highly reactive dicarbonyl intermediates.

Nonenzymatic protein glycation (glucosylation or glycosylation) by glucose is a complex cascade of condensations, rearrangements, fragmentations, and oxidative modifications that lead to poorly characterized heterogeneous products often collectively termed Advanced Glycation End Products or AGEs.

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The abbreviations used are: AGE, advanced glycation end products; BSA, bovine serum albumin; RNase, bovine pancreatic ribonuclease A; ELISA, enzyme-linked immunosorbent assay; CmL, N-carboxymethyllysine.
bose, a more reactive analogue of glucose (40, 41). Those studies led to the unique preparation, kinetic characterization, and stabilization of a reactive glycation intermediate in ribonuclease A. After removing excess and reversibly bound ribose from this presumed Amadori intermediate (Scheme 1), it rapidly forms AGEs with a half-time for the exponential kinetics of about 10 h (39). This "interrupted glycation" method provides a new mechanism-based strategy for a better understanding of AGE inhibition by established inhibitors, such as aminoguanidine, through isolating post-Amadori pathways of AGE formation (cf. Scheme 1) and thus removing effects arising from glycoxidation of free sugar or Schiff base (Namiki pathway) (35). More importantly, it opens the way to searching for novel inhibitors that specifically act on post-Amadori pathways of AGE formation. Baynes and co-workers (38) have recently emphasized the importance of Amadori products for the in vivo formation of AGEs. We note that exogenously administered Amadori and AGE proteins have been shown to produce diabetic-like glomerular sclerosis, basement membrane thickening, and albuminuria (42, 43).

We now report the successful use of the above approach to elucidate important aspects of the inhibition by aminoguanidine and to discover novel post-Amadori inhibitors of AGE formation. In particular, we examined several derivatives of vitamins B1 and B6 (Scheme 2) that were recently screened for possible AGE inhibition in the presence of high glucose (44). This "interrupted glycation" method provides a new mechanism-based strategy for a better understanding of AGE inhibition by established inhibitors, such as aminoguanidine, through isolating post-Amadori pathways of AGE formation (cf. Scheme 1) and thus removing effects arising from glycoxidation of free sugar or Schiff base (Namiki pathway) (35). More importantly, it opens the way to searching for novel inhibitors that specifically act on post-Amadori pathways of AGE formation. Baynes and co-workers (38) have recently emphasized the importance of Amadori products for the in vivo formation of AGEs. We note that exogenously administered Amadori and AGE proteins have been shown to produce diabetic-like glomerular sclerosis, basement membrane thickening, and albuminuria (42, 43).

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**—Bovine pancreatic ribonuclease A was obtained from Worthington as a chromatographically pure and aggregate-free protein. Bovine serum albumin (fraction V, fatty acid free), human methemoglobin, d-ribose, pyridoxine, pyridoxal, pyridoxal 5'-phosphate, pyridoxamine, thiamine, thiamine monophosphate, thiamine pyrophosphate, and goat alkaline phosphatase-conjugated antirabbit IgG were all from Sigma. Aminoguanidine hydrochloride was purchased from Aldrich.

**Preparation of Polyclonal Antibodies to AGE Proteins**—Immunogen preparation was carried out primarily following earlier protocols (46–48). We prepared polyclonal antibodies against glucose-modified AGE-BSA antigen (designated R479) for experiments on glycation of RNase A and polyclonal antibodies against glucose-modified AGE-RNase antigen (designated R618) for experiments on glycation of serum albumin. Glycated antigens were prepared by incubating the proteins at 1.6 g in 15 ml of 1.5 M glucose in 0.4 M phosphate containing 0.05% sodium azide at pH 7.4 and 37 °C for 90 days. New Zealand White male rabbits of 8–12 weeks were immunized by subcutaneous administration of a 1-ml solution containing 1 mg/ml AGE-protein antigen 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, and the serum was collected by centrifugation of clotted whole blood.

**ELISA Detection of AGE Products**—ELISA was performed according to methods described by Engvall (49), essentially as described earlier (39). Glycated protein samples were diluted to approximately 1.5 μg/ml in 0.1 M sodium carbonate buffer of pH 9.5–9.7. The protein was coated overnight at room temperature onto a 38-well polystyrene plates by pipetting 200 μl of the protein solution in each well (0.3 μg/well). After coating, the protein was washed from the wells with a saline solution containing 0.05% Tween 20. The wells were then blocked with 200 μl of 1% casein in carbonate buffer for 1 h at 37 °C, followed by washing. As noted above, antibodies (R479) used to detect AGE in glycated RNase were raised against glycated BSA, and antibodies (R618) used to detect AGE in glycated BSA and glycated human methemoglobin were raised against glycated RNase. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of
1:2000 and incubated for 1 h at 37 °C, followed by washing. The p-nitrophenyl phosphate substrate solution was then added (200 μM/well) to the plates, with the absorbance of the released p-nitrophenolate being monitored at 410 nm with a Dynatech MR4000 microplate reader. The ELISA reader absorbances were monitored to ensure that readings were within the linear range of the instrument. Typically, readings of plates were taken at various time intervals after substrate was added in order to find the highest acceptable absorbances that provide the best dynamic range for each kinetic series. Absolute values of ELISA readings are thus not valid for comparison of different plates. Conversely, different series that need to be compared are plated and read together (same developing time).

Uninterrupted Ribose Glycation Assays—Bovine serum albumin, ribonuclease A, and human methemoglobin were incubated with ribose at 37 °C in 0.4 M sodium phosphate buffer of pH 7.5 containing 0.02% sodium azide. The protein (10.0 or 1.0 mg/ml), 0.05 mM ribose, and prospective inhibitors at 0.5, 3, 15, or 50 mM were introduced into the incubation mixture simultaneously. Solutions were kept in the dark in capped tubes. Aliquots were taken and immediately frozen until analyzed by ELISA at the conclusion of the reaction. The incubations were for 3 (human methemoglobin) or 6 weeks (RNase, BSA). Glycation reaction mixtures were carefully monitored to make sure constant pH conditions were maintained throughout the duration of the experiments.

Interrupted (Post-Amadori) Ribose Glycation Experiments—Glycation was carried out by first incubating the protein (10 mg/ml) with ribose (0.5 mM) at 37 °C in 0.4 M phosphate buffer of pH 7.5 containing 0.02% sodium azide for 24 h in the absence of inhibitors. Glycation was then interrupted to remove excess and reversibly bound sugar by extensive dialysis against frequent cold buffer changes at 4 °C. The glycated BSA or RNase intermediate containing maximal amount of Amadori product and little AGE (depending on the protein) was then quickly warmed to 37 °C without re-addition of any ribose. This initiated conversion of Amadori intermediates to AGE products in the absence or presence of various concentrations (typically 3, 15, and 50 mM) of prospective inhibitors. Aliquots were taken at various intervals and frozen for later ELISA assays at the end of the reaction. Solutions were kept in the dark in capped tubes. The procedure has been fully described (39).

Numerical Analysis of Kinetics Data—We routinely fit kinetics data (time progress curves) to mono- or bi-exponential functions using nonlinear least squares methods utilizing either SCIENTIST 2.0 (MicroMath, Inc.) or ORIGIN (Microcal, Inc.) software that permit user-defined functions and control of parameters to iterate. Standard deviations of the parameters of the fitted functions (initial and final ordinate values and rate constants) were returned as measures of the precision of the fits. Apparent half-times for bi-exponential kinetics fits were determined with the “solve” function of MathCad software (MathSoft, Inc.).

RESULTS

Before presenting and then discussing the results, it may be useful to emphasize that there is no standard method of defining or following AGE formation and its inhibition. Quantitation of initial Schiff base condensation of labeled reducing sugars with protein amino groups provides little information on post-Amadori steps of AGE formation, since such steps may not lead to changes in the extent of labeling. Many studies of AGE formation have monitored the increase in blue fluorescence arising from “browning” glycation products. Such fluorescent changes can be produced by dicarbonyl or glyoxidation products that arise from free sugar, from the initial Schiff bases, and from Amadori and other intermediates (50–52). Carboxymethyllysine (CmL), a notable AGE, can also arise from a variety of glyoxidation intermediates besides the Amadori product (35, 36, 53). The acid-stable fluorescent AGE pentosidine (54–56) can be utilized in principle, but the chemical work-up, protein hydrolysis, and high performance liquid chromatography separation required at each time point makes its use inconvenient for large sets of kinetics. In this work we chose to use sensitive ELISA techniques (46–48, 57, 58) that utilize anti-AGE polyclonal antibodies developed against proteins typically glycated for 60–90 days with glucose. This approach recently proved highly suitable for detailed kinetics studies of the formation of antigenic AGE products and its inhibition (39, 44).

The results in this section are presented as two series of inhibition experiments. In the first series, the indicated proteins are mixed with ribose to initiate glycation in the presence and absence of the inhibitors, and the formation of AGEs was monitored by ELISA. This assay is referred to as “overall glycation kinetics” or as “uninterrupted glycation.” In the second series of experiments, an interrupted glycation method was used to follow “post-Amadori kinetics” of AGE formation in absence of ribose. The proteins were first incubated at 37 °C with ribose for 24 h during which Amadori intermediates accumulated (39). The excess and reversibly bound sugar was then removed by dialysis at 4 °C, and AGE formation was initiated (the zero time) in the presence and absence of inhibitors by warming the solutions back to 37 °C.

Inhibition by Vitamin B₆ Derivatives of the Overall Kinetics of AGE Formation—The inhibitory effects of the B₁ and B₆ vitamins on the kinetics of antigenic AGE formation were evaluated by polyclonal antibodies specific for AGEs. Our initial inhibition studies were carried out on the glycation of bovine ribonuclease A (RNase) in the continuous presence of 0.05 M ribose. It has been shown elsewhere (39) that the rate of AGE formation is near-maximal at this concentration. Fig. 1 (control curves, filled rectangles) demonstrates that the formation of antigenic AGEs on RNase when incubated with 0.05 M ribose is relatively rapid, with a half-time of approximately 6 days under these temperature and buffer conditions. Pyridoxal 5’-phosphate (Fig. 1B) and pyridoxal (Fig. 1C) significantly inhibited the rate of AGE formation on RNase at concentrations of 50 and 15 mM. Surprisingly, pyridoxine, the alcohol form of vitamin B₆ (Scheme 2), also moderately inhibited AGE formation on RNase (Fig. 1D). Of the B₆ derivatives examined above, 3 Polyclonal anti-AGE antibodies have proven to be a sensitive analytical tool for the study of AGE formation in vitro and in vivo, although the nature of the dominant antigenic AGE epitope of hapten remains in doubt. Baynes and co-workers (71) recently demonstrated that the protein glyoxidation product carboxymethyllysine (CmL) is a dominant antigen of their antibodies, and they were able to rationalize why earlier studies had failed to reveal strong ELISA reactivity with model carboxymethyllysine compounds (48). Our preliminary characterization of our polyclonal antibodies reveals a strong, although variable, CmL reactivity.5

![FIG. 1. Effect of vitamin B₆ derivatives on AGE formation during uninterrupted glycation of ribonuclease A by ribose. RNase (1 mg/ml) was incubated with 0.05 M ribose in the presence and absence of the various indicated derivatives in 0.4 M sodium phosphate buffer of pH 7.5 at 37 °C for 6 weeks. Aliquots were assayed by ELISA using R479 anti-AGE antibodies. Concentrations of the inhibitors were 0.5, 3.0, 15, and 50 mM. A, pyridoxamine (PM); B, pyridoxal-5-phosphate (PLP); C, pyridoxal (PL); D, pyridoxine (PN).](image-url)
pyridoxamine at 50 mM provided the best inhibition of “final” levels of AGE formation on RNase over the 6-week period monitored (Fig. 1A).

Inhibition by Vitamin B₃ Derivatives of the Overall Kinetics of AGE Formation—All of the B₃ vitamers inhibited antigenic AGE formation on RNase at high concentrations, but the inhibition appeared more complex than for the B₆ derivatives (Fig. 2, A–C). In the case of thiamine pyrophosphate (Fig. 2A), both the rate of AGE formation and the final levels of AGE produced at the plateau appeared diminished by the compound. In the case of thiamine phosphate (Fig. 2B) and thiamine (Fig. 2C), there appeared little effect on the rate of AGE formation, but a substantial decrease in the final level of AGE formed in the presence of the highest concentration of inhibitor. In general, thiamine pyrophosphate demonstrated greater inhibition than the other two compounds at the lower concentrations examined.

Inhibition by Aminoguanidine of the Overall Kinetics of AGE Formation—Inhibition of AGE formation by aminoguanidine (Fig. 2D) was distinctly different from that seen by the B₃ and B₆ derivatives above. Increasing concentrations of aminoguanidine decreased the rate of AGE formation on RNase but did not reduce the final levels of AGE formed, so that the level of AGEs produced by the 6th week was almost identical to that of the control.

Inhibition of the Overall Kinetics of AGE Formation in Serum Albumin and Hemoglobin—Comparative studies were carried out with bovine serum albumin and human methemoglobin to determine whether the observed inhibition was protein-specific. The different derivatives of vitamin B₆ (Fig. 3, A–D) and vitamin B₃ (Fig. 4, A–C) exhibited similar inhibition trends when incubated with bovine serum albumin, with pyridoxamine and thiamine pyrophosphate being the most effective inhibitors in each of the respective families. Pyridoxine failed to inhibit AGE formation on BSA (Fig. 3D). Pyridoxal phosphate and pyridoxal (Fig. 3, B–C) mostly inhibited the rate of AGE formation but not the final levels. Pyridoxamine (Fig. 3A) again exhibited some inhibition at lower concentrations and at the highest concentration appeared to inhibit the final levels of AGE formation more effectively than the other B₆ vitamers. In the case of the B₃ vitamers, the overall extent of inhibition on BSA (Fig. 4, A–C) was less than that observed with RNase (Fig. 2, A–C). Higher concentrations of thiamine and thiamine monophosphate only slightly inhibited AGE formation on BSA (Fig. 4, B and C), whereas thiamine pyrophosphate inhibited the final levels of AGE formation without greatly affecting the rate of formation (Fig. 4A). Aminoguanidine again displayed the inhibition effects with BSA that it did with RNase (Fig. 4D), appearing to only slow the rate of AGE formation with lesser effects on decreasing the final levels of AGE.

The kinetics of AGE formation were also examined with human methemoglobin in the presence of the B₆ and B₃ vitamers and aminoguanidine. The absolute rates of AGE formation appeared higher with human methemoglobin than with the other two proteins, but the compounds revealed largely similar inhibition trends (data not shown). Of the vitamin B₆ derivatives, pyridoxamine showed the greatest inhibition at concentrations of 3 mM and above when compared with pyridoxal phosphate, pyridoxal, and pyridoxine. In the case of the
Inhibition by Vitamin B<sub>1</sub> Derivatives of the Kinetics of Post-Amadori Ribose AGE Formation—In the interrupted glycation assays for following post-Amadori AGE formation, kinetics were followed by incubating isolated Amadori intermediates of either RNase or BSA at 37 °C in absence of free or reversibly bound ribose. Ribose sugar that was initially used to prepare the intermediates was removed by cold dialysis after an initial glycation period of 24 h. Following this interruption, AGE formation resumes and the kinetics of this post-Amadori AGE formation is quite rapid (half-times of about 10 h) in absence of inhibitors (39). Fig. 5 shows the effects of pyridoxamine (Fig. 5A), pyridoxal phosphate (Fig. 5B), and pyridoxal (Fig. 5C) on the post-Amadori kinetics of BSA. Pyridoxine did not produce any inhibition (data not shown). Similar experiments were carried out on RNase. Pyridoxamine showed nearly complete inhibition at 15 and 50 mM concentrations of the indicated inhibitors. Aliquots were assayed by ELISA using R479 anti-AGE antibodies for RNase and R618 antibodies for BSA, A, pyridoxamine (PM); B, pyridoxal phosphate (PLP); C, pyridoxal (PL); D, pyridoxamine (PM).

B<sub>1</sub> compounds, the inhibitory effects were more similar to the BSA inhibition trends than to RNase. The inhibition was modest at the highest concentrations tested (50 mM), being nearly 30–50% for all three vitamers (data not shown). It was primarily manifest as a decrease in the final levels of AGE.

Inhibition by Vitamin B<sub>6</sub> Derivatives of the Kinetics of Post-Amadori Ribose AGE Formation—In the interrupted glycation assays for following post-Amadori AGE formation, kinetics were followed by incubating isolated Amadori intermediates of either RNase or BSA at 37 °C in absence of free or reversibly bound ribose. Ribose sugar that was initially used to prepare the intermediates was removed by cold dialysis after an initial glycation period of 24 h. Following this interruption, AGE formation resumes and the kinetics of this post-Amadori AGE formation is quite rapid (half-times of about 10 h) in absence of inhibitors (39). Fig. 5 shows the effects of pyridoxamine (Fig. 5A), pyridoxal phosphate (Fig. 5B), and pyridoxal (Fig. 5C) on the post-Amadori kinetics of BSA. Pyridoxine did not produce any inhibition (data not shown). Similar experiments were carried out on RNase. Pyridoxamine showed nearly complete inhibition at 15 and 50 mM concentrations of the indicated inhibitors. Aliquots were assayed by ELISA using R479 anti-AGE antibodies for RNase and R618 antibodies for BSA.

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Inhibition by Vitamin B<sub>6</sub> Derivatives of the Kinetics of Post-Amadori Ribose AGE Formation—In the interrupted glycation assays for following post-Amadori AGE formation, kinetics were followed by incubating isolated Amadori intermediates of either RNase or BSA at 37 °C in absence of free or reversibly bound ribose. Ribose sugar that was initially used to prepare the intermediates was removed by cold dialysis after an initial glycation period of 24 h. Following this interruption, AGE formation resumes and the kinetics of this post-Amadori AGE formation is quite rapid (half-times of about 10 h) in absence of inhibitors (39). Fig. 5 shows the effects of pyridoxamine (Fig. 5A), pyridoxal phosphate (Fig. 5B), and pyridoxal (Fig. 5C) on the post-Amadori kinetics of BSA. Pyridoxine did not produce any inhibition (data not shown). Similar experiments were carried out on RNase. Pyridoxamine showed nearly complete inhibition at 15 and 50 mM concentrations of the indicated inhibitors. Aliquots were assayed by ELISA using R479 anti-AGE antibodies for RNase and R618 antibodies for BSA.

DISCUSSION

Two different strategies have been utilized in the present work in order to better elucidate the kinetics and mechanism of AGE inhibition by aminoguanidine and the other compounds. In the first, we examined the much more rapid glycation by ribose, where the time course could be completely observed and quantitated (39), and we compared that with inhibition of glycation by glucose (44). In the second approach, we have utilized our unique ability to prepare ribose Amadori intermediates that are free of AGEs (39) to follow the extremely rapid kinetics of post-Amadori antigenic AGE formation on RNase and BSA in absence of free and reversibly bound ribose. Our initial attempts to isolate an Amadori-rich, AGE-free intermediate from glucose capable of producing significant amounts of anti-
Post-Amadori Inhibition of AGE Formation

FIG. 7. Effect of aminoguanidine on post-Amadori AGE formation after interrupted glycation by ribose. BSA and RNase were incubated for 24 h with 0.5% ribose for 24 h at 37°C followed by extensive dialysis for 24 h at 4°C to remove excess and reversibly bound ribose. AGE formation was then initiated (zero time) by incubating the Amadori-rich proteins (RNase in A and BSA in B) at 0.1 mg/ml in 0.4 M phosphate buffer of pH 7.5 at 37°C in the absence and presence of 3, 15, and 50 mM concentrations of aminoguanidine. Aliquots were assayed by ELISA using R479 anti-AGE antibodies for RNase and R618 antibodies for BSA.

genic AGES have not been successful using a 3-day interruption⁴ (39).

Numerous studies have demonstrated that aminoguanidine is an inhibitor of many manifestations of nonenzymatic glycation (28, 45, 60, 61), and it recently entered second phase III clinical trials for ameliorating the complications of diabetes (11, 61). Our recent kinetic studies confirmed that aminoguanidine is a potent inhibitor of antigenic AGE formation induced by high glucose concentrations (44). However, the inhibition unexpectedly appeared to diminish at later stages of the kinetics (cf. Fig. 2D and Fig. 4D of Ref. 44). Due to the slowness of the glycation with glucose, this surprising observation could not be completely established. Furthermore, the basis for this diminished inhibition is open to question due to the possible long term instability of aminoguanidine and its potential to produce hydrogen peroxide (62). The present, more complete kinetics of aminoguanidine inhibition of ribose glycation (Fig. 2D and Fig. 4D) appear fully consistent with the earlier glucose findings. The ribose glycation results clearly demonstrate that aminoguanidine slows the rate of antigenic AGE formation in the presence of sugar but has little effect on the final amount of AGE formed. As a consequence, observations limited to the initial stages of either the glucose or the ribose glycation will show remarkable apparent inhibitor efficacy that may be misleading.

Mechanistically, these kinetics results indicate that aminoguanidine most likely inhibits in our assay by interacting with rate-determining initial reactants (protein or sugar). In the case of glycation by ribose, the overall rate of AGE formation has been shown to be strongly dependent on ribose concentration (39) but independent of protein over a 1000-fold concentration range.⁵ In terms of the classical pathway of glycation (Scheme 3), aminoguanidine could decrease the rate of initial Schiff base formation by complexing the acyclic free aldehyde forms of the sugars.⁶ Inhibition through formation of such an aminoguanidine-sugar interaction was first advanced by Khatami et al. (32), and recently a glucose-aminoguanidine adduct was produced, isolated, and structurally characterized by Hirsch et al. (33).

The hypothesis that aminoguanidine does not significantly react with the Amadori or later intermediates on the path of formation of AGEs could be directly tested by observing its possible effects on the isolated post-Amadori kinetic, i.e. starting with protein Amadori intermediates. Preliminary studies had indicated low effects of aminoguanidine on the rate of post-Amadori AGE formation in RNase (39). Since ribose is closely related to the enzymatic substrate of RNase and Lys-41 of the active site most easily forms Amadori products (63), it was important to verify this for other proteins. Fig. 7B demonstrates weak or negligible effects of aminoguanidine on the post-Amadori formation of AGEs in serum albumin at inhibitor concentrations up to 50 mM. The findings on RNase were also independently re-determined over the same concentration range (Fig. 7A). These results are especially noteworthy, as aminoguanidine was originally proposed by its discoverers to be a nucleophilic blocker of Amadori glycation intermediates (45, 64).

Studies from several laboratories have also not supported this originally proposed mechanism. Instead, aminoguanidine, through its guanidinium functionality, was found to inhibit another AGE formation pathway by scavenging reactive dicarbonyl intermediates (Scheme 1) that arise from glycoxidation during glycation (65–68). Dicarbonyls can include glyoxal and glycoaldehyde that arise from free sugar or from Schiff bases (via the Namiki pathway) and "glucosones" (deoxydiketoses or deoxyaldoketoses) which can arise from Amadori intermediates (35, 36, 38, 53). Hirsch et al. (66) found very rapid irreversible formation of 5- and 6-substituted triazines from reaction of aminoguanidine with model dicarbonyls, while Chen and Cermi (68) have reported that reaction of a model Amadori compound with aminoguanidine only leads to formation of triazine bis-hydrazone products of dicarbonyl fragments derived from the Amadori compound. Edelstein and Brownlee (67) also reported that no aminoguanidine adducts are formed with a model glycated peptide, concluding that it only acts on Amadori-derived fragments. Since reactive dicarbonyls can also lead to the formation of the AGE carboxymethyllysylmine (CmL), it is significant that Glomb and Monnier (35) demonstrated that aminoguanidine at 5 mM is entirely ineffective in preventing the formation CmL when starting from a model lysine Amadori product and that it has an effect only when glycation is started in the presence of free sugar.

Our recent studies on AGE inhibition in the presence of high glucose concentrations (44) revealed substantial inhibition by some derivatives of vitamins B₁ and B₆, notably pyridoxamine, pyridoxal phosphate, and thiamine pyrophosphate. As with aminoguanidine, the inability to observe the full course of the glucose glycation kinetics with RNase and BSA left unresolved the important question of whether the apparent decreased rate of AGE formation was also accompanied by a decrease in the final levels of AGEs formed. In the present ribose study, where we followed both the uninterrupted (Figs. 1–4) and the post-

⁴ The apparently lower Amadori build-up with glucose could be due to differences in rate constant ratios (\( k_3 \) versus \( k_4 + k_5 \) in Scheme 3) rather than to differences in glycation mechanism between the two sugars.

⁵ R. G. Khalilah, manuscript in preparation.

⁶ Although at equilibrium the acyclic forms constitute about 0.002 and 0.05% for glucose and ribose, respectively (41, 76), these are the species that amino groups react with to initiate glycation.
Amadori “interrupted” (Figs. 5–7) glycation kinetics, the inhibition patterns shown by some of the derivatives of vitamins B$_1$ and B$_6$ differed substantially from those of aminoguanidine. For example, pyridoxamine displayed both a decrease in rate of AGE formation and a decrease in apparent final AGE levels. The latter effect was more prominent for BSA (Fig. 3A) than for RNase (Fig. 1A), and it was quite dramatic for hemoglobin (data not shown). The other derivatives such as pyridoxal and pyridoxal phosphate predominantly showed inhibition of the rate of formation of AGEs in RNase and BSA with some inhibition of final AGE levels at the highest concentrations with hemoglobin. In the case of the vitamin B$_1$ derivatives, more consistent decreases in final AGE levels than rates were observed with RNase (Fig. 2), with more modest effects being seen with BSA (Fig. 4).

The observed decreases in rate of formation of AGEs in the ribose glycation for some of these inhibitors suggests, as in the case of aminoguanidine, that they exert some of their effects through interactions with the open chain aldehyde forms of the reducing sugars or with the protein sites of glycation. Little is known about the former possibility for these compounds. However, pyridoxal and pyridoxal phosphate, as aldehydes, are known to compete with sugars for Schiff base formation with protein amino groups and have, indeed, been proposed as inhibitors of glycation through this “competitive” mechanism (32, 69, 70). In the particular case of RNase, pyridoxal phosphate has been shown to act as an affinity label for the active site by interacting with critical Lys-41 and Lys-7 (59).

The suggestive decreases in final levels of AGE formed by some of these derivatives in the presence of ribose imply that they may exert inhibitory effects through an additional mechanism, namely at the Amadori product level within the framework of the Scheme 3. Our attempt to verify this hypothesis through observation of the post-Amadori kinetics of AGE formation has led to the most striking result of this work, namely the discovery of the first examples of what may be termed a novel class of “post-Amadori AGE inhibitors.” Pyridoxamine proved unique among the vitamin B$_6$ derivatives in showing striking inhibition with both BSA (Fig. 5A) and RNase (Fig. 5D) at concentrations as low as 15 mM. Similarly, thiamine pyrophosphate among the vitamin B$_1$ compounds showed a dose-dependent decrease in final AGE levels formed with both proteins (Fig. 6, A–B), although its effects were less than those of pyridoxamine. A fixed time (6–7 d) comparison of the inhibition by the three inhibitors is given in Fig. 8 for both BSA and RNase. While they earlier displayed nearly equal inhibition by the three inhibitors at the completion of the experiments shown in Figs. 5–7 (RNase, 7 days; BSA, 6 days).

In conclusion, our mechanism-based approach has led to the identification of pyridoxamine and thiamine pyrophosphate as novel inhibitors of AGE formation and first examples of what may be termed post-Amadori inhibitors. Amadori pathways of formation of AGEs may be of particular importance in vivo (38). Although these inhibitors belong to the B$_1$ and B$_6$ vitamins series, there is no evidence at present to suggest that they function as endogenous AGE inhibitors, since the levels needed for effective in vitro inhibition are far greater than their in vivo occurrence. Both contain an amino group, but a simple amino functionality is insufficient to inhibit post-Amadori formation of AGEs, since N$\text{-}$acetyl-lysine is not inhibitory. Furthermore, aminoguanidine, a strongly nucleophilic hydrazine capable of interacting with carbonyl groups, shows little inhibition (Fig. 7). Their interesting and rich chemical roles as coenzymes for $\alpha$-carbonyl reactions in carbohydrate metabolism (cf. 73, 74) suggest that they may provide valuable leads for the design and discovery of potent post-Amadori inhibitors that could have therapeutic potential. By effectively targeting a different step of the glycation pathway, post-Amadori inhibitors may complement established inhibitors such as aminoguanidine that either prevent initial sugar attachment to proteins or that scavenge highly reactive dicarbonyl intermediates that may arise from glycoxidative pathways.

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