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Nonenzymatic Glucosylation and Glucose-dependent Cross-linking of Protein*

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A model system using RNase A has been established for studying the nonenzymatic glucosylation and glucose-dependent cross-linking of protein (Maillard reaction) under physiological conditions in vitro. The rate of glucosylation of RNase was first order in glucose. Glucosylation was accompanied by a comparable decrease in primary amino groups in the protein and lysine recoverable by amino acid analysis. Analysis of glucosylation reaction mixtures by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of mercaptoethanol revealed the time-dependent formation of RNase dimer and trimer. The polymerization reaction was mixed order with respect to glucose concentration, but was approximately first order with respect to protein concentration. When glucosylated protein was separated from glucose, the protein continued to polymerize even in the absence of glucose. Under these conditions, the primary cross-linking reaction occurred by condensation of a glucosylated amino acid on one RNase molecule with a free amino group on another. Lysine efficiently inhibited cross-linking between glucosylated and native RNase in the absence of glucose. An attempt to model the cross-linking reaction was made by studying the incorporation of [3H]lysine and N'-formyl-[3H]lysine into glucosylated RNase. Both were incorporated covalently into glucosylated but not native protein. However, free lysine was the major product recovered following NaBH4 reduction and amino acid analysis of the lysine derivative of glucosylated protein. The data are discussed in terms of the mechanism of protein cross-linking by glucose and the relevance of this reaction to the pathophysiology of diabetes.

Nonenzymatic glucosylation of protein occurs by direct chemical reaction between reducing sugars and primary amino groups in protein. The reaction proceeds through a Schiff base adduct, followed by an Amadori rearrangement, to yield a stable, ketoamine derivative of the protein (1, 2). Nonenzymatic glucosylation is a common post-translational modification of body protein, and occurs primarily at intrachain lysine residues. The extent of the reaction is increased during diabetic hyperglycemia; however, to date no direct pathological sequelae of the reaction have been demonstrated. Thus, although nonenzymatic glucosylation alters hemoglobin's oxygen affinity, diabetes is not characterized by hematological disease. Similarly, we and others have been unable to detect any effects of nonenzymatic glucosylation (1 mol of glucose/mol of protein) on the biological properties of albumin, including its circulating half-life or ligand-binding capacity (3, 4). However, the extent of nonenzymatic glucosylation of many proteins in the body does correlate with the severity of hyperglycemia, and chronic hyperglycemia is implicated in the complications of diabetes (1, 2).

From extensive studies in vitro, it is recognized that nonenzymatic glucosylation is only the first step in a complex series of Maillard or "browning" reactions of protein in the presence of reducing sugars (5, 6). The in vitro products are not well characterized, but include denatured and cross-linked protein polymers and protein-bound, fluorescent pigments. It is not known whether or to what extent the secondary cross-linking and polymerization reactions which occur in vitro also occur in living systems. An answer to this question is essential for understanding the consequences of the enhanced nonenzymatic glucosylation of protein observed in diabetes (1, 2). Thus, glucosylation and subsequent Maillard reactions of protein may contribute to the development of the pathophysiology of diabetes through effects on the structure, function, and metabolism of protein.

The purpose of the present work is to develop a simple model system for studying nonenzymatic glucosylation and glucose-dependent cross-linking of protein in vitro, with the goal of identifying secondary products and cross-link structures formed in the in vitro system. Eventually, the analysis of normal and pathological tissues for these same products should be useful for assessing the significance of the Maillard reaction in vivo. We describe here our initial observations on the glucosylation, cross-linking, and polymerization of RNase A by glucose.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreatic RNase A (type XII-B), l-lysine monohydrochloride, TNBS, and sodium borohydride were obtained from Sigma Chemical Co. $\beta$-Glucose was purchased from P-L Biochemicals, and 1,3,4,6-tetrachloro-3,6-dinitrobenzo-2,1,3-benzoxadiazol (DN) was from Pierce Chemical Co. 1-[4,5-3H]lysine, [3H]sodium borohydride, and d-[6-3H]glucose were obtained from New England Nuclear and carrier-free Na2341 was from Amersham Corp. N'-Formyl-[3H]lysine was prepared from radioactive lysine as described by Hofmann et al. (7). Fructose-lysine was prepared as described by Finot and Mauron (8), and hexitol-lysine by reduction of FL with NaBH4 (see below).

Purification of Radioactive Tracers—Trueb et al. (9) showed that

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The abbreviations used are: RNase A, bovine pancreatic ribonuclease A; TNBS, trinitrobenzenesulfonyl acid; FL, fructose-lysine; 1-(N'-lysine)-1-deoxy-D-fructose; HL, hexitol-lysine; HL', acid rearrangement product of HL; Glc and GlaRNase. RNase substituted with 3 and 8 mol of glucose, respectively, per mol of protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
commercial preparations of radioactive glucose contain contaminants which covalently modify proteins at a rate many times faster than glucose itself. In order to remove these contaminants, D-[^3H]glucose (1 mCi) was incubated with 10 mg of RNase A in 1.0 ml of 0.2 M sodium phosphate, pH 8.0, for 2 days at 4 °C. Free [^3H]glucose was separated from protein by chromatography on Sephadex G-25 (Pharmacia). Peak fractions containing free [^3H]glucose were pooled and concentrated to dryness on a rotary evaporator. The recovered [^3H]glucose was dissolved in 1 ml of H2O, stored at -20 °C, and used within 7 days after purification. A similar procedure was used for purification of radioactive lysine.

Glucosylation of RNase A—The concentration of native RNase in solution was determined by measuring absorbance at 280 nm (ε1% = 7.1 (10)). During the glucosylation reaction, there was a gradual browning of the protein which interfered with the determination of protein concentration at 280 nm. In these cases, the concentration of glucosylated protein was determined by the method of Lowry et al. (11) using RNase A as a standard or by amino acid analysis. Unless otherwise indicated, all dialyses and glucosylation reactions were conducted in 0.2 M sodium phosphate, pH 7.4. The pH of glucosylation reactions was monitored using narrow range pH paper, and adjusted when necessary with small additions of 1 N NaOH to maintain pH 7.4-7.8. Glucosylation reactions containing RNase and 1 M glucose were made 0.01% (w/v) in gentamicin sulfate (Grand Island Biological Co.) to prevent bacterial contamination; reaction mixtures with lower glucose concentrations were further sterilized by filtration through a 0.2-μ filter (Gelman). Aliquots (50-100 μl) were removed from glucosylation reactions at desired times and frozen at -20 °C until assayed. In experiments in which the incorporation of [3H]glucose into protein was measured, sufficient radioactive glucose was added to the incubation mixture to obtain the desired specific activity. To determine the amount of radioactivity incorporated into protein, RNase was precipitated with an equal volume of 1 N NaOH and reprecipitating with acid. Final protein pellets were dissolved in 200 μl of 1 N NaOH, neutralized with an equal volume of 1 N NaCl, and counted for radioactivity in 10 ml of Beckman Ready-solvEP. Primary amino groups in protein aliquots from glucosylation experiments were measured directly by the TNBS reaction of Spadaro et al. (12) using norleucine as a standard.

RNase substituted with 3 mol of glucose/mol of protein was prepared by incubating RNase (20 mg/ml) in 500 mM glucose at 37 °C for 7 days. To prepare [3H]-GlcRNase, the incubation mixture also contained 2 μCi RNase (see below). RNase substituted with 8 mol of glucose/mol of protein was prepared by incubating RNase (20 mg/ml) in 1 M glucose at 44 °C for 15 days. Both the Glc and GlcRNase preparations were dialyzed against 0.2 M phosphate, pH 7.4, at 4 °C to remove free glucose. The degree of glucosylation of these RNase preparations was verified by amino acid analysis (see below) which indicated loss of 3 and 8 of a total of 10 lysine residues from the protein, respectively.

Quantitation of 1-(N'-Lysine)-1-deoxy-D-fructose in Protein—The major product of nonenzymatic glucosylation of proteins is the N'- (1-deoxyfructosyl) derivative of lysine, commonly called fructose-lysine (1-4). This adduct can be isolated following NaBH4 reduction and acid hydrolysis of protein as a mixture of hexitol-lysines, consisting of the C-2 epimers, mannitol-lysine, and glucitol-lysine. These epimers are not normally resolved by conventional buffer systems for amino acid analysis, and co-chromatograph as HL and its rearrangement product, and the reaction was terminated by slow addition of 1 N HCl to destroy excess borohydride. Reductions with NaB3H4 were performed in essentially the same manner as for nonradioactive NaBH4 reductions except that the molar ratio of NaB3H4 to protein was 100-500:1. After reduction, protein samples were dialyzed as above, and hydrolyzed at ~1 mg/ml in 6 N HCl at 110 °C for 24 h and analyzed on a Beckman model 119C amino acid analyzer. Amino acids and derivatives were quantitated either by the automated ninhydrin reaction or by collecting the column effluent and assaying fractions for radioactivity.

Iodination of RNase—Iodinated RNase was prepared using iodogen as oxidizing agent. RNase (~200 μg) was incubated in 100 μl of Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 0.8 mM KH2PO4, 125 mM NaHCO3) at pH 7.1 (10). During the incubation reaction, there was a gradual browning of the protein which interfered with the determination of protein concentration at 280 nm. Thus, the concentration of iodinated RNase was determined by the method of Lowry et al. (13) using RNase A as a standard.

Reduction of Protein with NaBH4 and Amino Acid Analyses—Prior to reduction, aliquots from glucosylation experiments were diluted to 0.5 mg/ml in 0.2 M sodium phosphate, pH 8.0, followed by dialysis against 4000 volumes of the same buffer at 4 °C overnight to remove free glucose. The dialyzed protein was reduced for 4 h at room temperature by addition of a 500-1000-fold molar excess of NaBH4, and the reaction was terminated by slow addition of 1 N HCl to destroy excess borohydride. Reductions with NaB3H4 were performed in essentially the same manner as for nonradioactive NaBH4 reductions except that the molar ratio of NaB3H4 to protein was 100-500:1. A similar procedure was used for glucosylation experiments in which the incorporation of [3H]glucose into protein was measured, sufficient radioactive glucose was added to the incubation mixture to obtain the desired specific activity.
RESULTS

Kinetics and Products of Nonenzymatic Glucosylation of RNase—The kinetics of the reaction between glucose and RNase was determined by measuring the rate of incorporation of purified [6-3H]glucose into acid-precipitable protein (Fig. 2). The initial rates of the reactions at 25 mM (data not shown) and 1 M glucose concentrations were essentially identical, i.e. 0.0025 ± 0.0005 mol of glucose bound mol−1 of RNase mM−1 glucose day−1 (average ± range). Eight glucose residues had been incorporated into RNase after 2 weeks in 1 M glucose, but the reaction rate constant decreased gradually during the incubation. There are 11 primary amino groups potentially available for derivatization in RNase, 2 from the NH2-terminal, and 9 from intrachain, lysine residues. The reaction rate constant, calculated per amino group, had declined to ~20% of its initial value by 1 week, indicating a range of reactivity of amino groups with glucose.

The kinetics of RNase modification by glucose was also followed by quantitating the disappearance of lysine residues by amino acid analysis, and by loss of primary amino groups using the TNBS reagent. These data for the reaction in 1 M glucose are also shown in Fig. 2, and indicate that the rate and extent of glucosylation determined by incorporation of [6-3H]glucose agrees closely with the kinetics of disappearance of lysine and primary amino groups from the protein. Comparison of amino acid analyses of NaBH4-reduced, glucosylated RNase at each of the times indicated in Fig. 2 revealed that lysine was the major amino acid lost; the only other detectable change was the loss of 0.4 of 3 mol of histidine after 16 days.

Quantitation of HL + HL′ yield by amino acid analysis was also used as an indicator of the progress of the glucosylation reaction. As shown in Fig. 2, a significantly lower extent of protein modification was indicated from the yield of HL + HL′ compared to the other parameters examined. The discrepancy between the amounts of lysine lost and HL + HL′ recovered was apparent even at early times during the glucosylation reaction. It was observed regardless of reduction conditions such as time (up to 24 h), pH (7.5–12), or NaBH4 concentration (up to 1 M NaBH4, >10,000-fold molar excess). Similarly, when [6-3H]glucose-labeled RNase (16-day incubation) was reduced with NaBH4, and acid hydrolyzed, the yield of radioactivity in the HL + HL′ peaks was only 51% of the total expected based on glucose incorporation (data not shown). The remainder of the radioactivity was recovered in unidentified peaks on the chromatogram. Thus, either by quantitation of HL′ with the ninhydrin reaction or measurement of H-labeled HL + HL′, a substantial fraction of glucose bound to protein was not recoverable as HL or HL′.

In control experiments, we have established that greater than 90% of chemically synthesized HL can be recovered as HL + HL′ after hydrolysis in the presence of added protein. These observations indicate that although lysine residues are the primary sites of RNase modification by glucose, adducts other than FL are being formed. In addition to HL and HL′, several unidentified ninhydrin-reactive peaks were also observed on the amino acid chromatogram of glucosylated RNase (see Fig. 1). Studies on the structures of these compounds are in progress.

Since there are lysine residues in the active site of RNase, the effect of glucosylation on RNase enzymatic activity was also studied. There was a significant decrease in RNase enzymatic activity during the course of the glucosylation reaction described in Fig. 2, compared to less than 5% loss in enzymatic activity in sorbitol2 or buffer controls. However, the relationship between the extent of RNase modification by glucose and the loss of enzymatic activity was not linear. Thus, ~50 and 75% of initial enzymatic activity were lost on modification of 2 (20%) and 4 (40%) lysine residues, respectively. Details of the kinetics of RNase inactivation by glucose and the location of modified lysine residues in the polypeptide chain will be presented elsewhere.

Cross-linking of RNase by Glucose—To further characterize the protein products of the glucosylation reaction, aliquots of the incubation mixtures were analyzed by SDS-PAGE in the presence of β-mercaptoethanol. As shown in Fig. 3, the glucosylation of RNase leads to a gradual decrease in its electrophoretic mobility as a function of time, and a broadening of the protein-staining band. In addition, a dimer band of protein, visible on the gel by 1 day of incubation, increased gradually during the course of the reaction. Because of the difficulty in quantitating the small percentage of dimer in these gels by densitometry, separate glucosylation experiments were conducted using 125I-RNase. The reaction aliquots were analyzed by SDS-PAGE, and radioactivity was then measured in gel slices to determine the kinetics of RNase polymerization. The total radioactivity profiles of gel lanes at 0 and 10 days of incubation are shown in Fig. 4, A and B. In addition to detecting 125I-RNase dimer in these reactions, a third band of radioactivity was found in the region of the gel expected for RNase trimer. Fig. 5 summarizes the overall experiment showing the kinetics of synthesis of dimer, trimer, and total polymer (dimer and trimer). The polymerization reaction proceeded without a detectable lag phase (see also Fig. 6 below). However, the rate of polymerization declined after 5–6 days, at about the same time that the rate of the

2 Several commercial sorbitol preparations were found to contain impurities which efficiently cross-linked and browned protein. These impurities were removed by reduction with NaBH4, and re-isolation of the sorbitol after desalting on mixed bed resin.
Glucosylation and Cross-linking of Protein

The glucosylation reaction itself began to decrease (see Fig. 2).

The experiments described thus far were carried out under experimental conditions significantly different from conditions found in vivo, i.e. 1 M glucose, 44 °C. These more extreme conditions were chosen initially for characterization of the RNase model system and in order to obtain higher yields of reaction products in shorter periods of time. The next series of experiments was carried out at lower glucose concentrations and at 37 °C in order to characterize the effects of nonenzymatic glucosylation of protein under more physiologically relevant conditions.

Pathway of the Cross-linking Reaction—To learn more about the mechanism of the cross-linking reaction, the rates of protein polymerization were studied as a function of both glucose and protein concentration. The kinetics of RNase cross-linking as a function of glucose concentration is shown in Fig. 6A. Notably, polymerization was readily detected even in 5 mM glucose, i.e. at physiological blood glucose concentration. In Fig. 6B, the initial rate of polymerization is plotted as a function of glucose concentration. The reaction kinetics approached first order in glucose at low sugar concentration, but the order declined with increasing glucose concentration. The mixed order of the reaction with respect to glucose may be explained by a multistep reaction scheme such as the following:

\[ \text{glucose} + \text{RNase} \xrightarrow{k_1'} \text{GlcRNase} \xrightarrow{k_2'} \text{RNase}_2. \]

Thus, at low glucose concentration, the rate of glucosylation \((k_1')\) may be rate limiting for polymerization; at higher glucose concentration, some later stage in the reaction may be rate limiting \((k_2')\) such as rearrangement or decomposition of the original glucose adduct or the dimerization process itself. Alternatively, at higher concentrations, glucose may compete with GlcRNase for active functional groups involved in protein dimerization.

The curves in Fig. 7A describe the kinetics of RNase polymerization as a function of protein concentration, and the plot in Fig. 7B indicates that the reaction is approximately first order with respect to protein. The fact that cross-linking was first order in protein suggested that the rate-limiting step was glucosylation or activation of one of the two protein molecules condensing to form dimer. To address this question, we proceeded to isolate Glc,RNase from glucosylation reactions and to evaluate its reactivity in the cross-linking reaction.

Fig. 3. SDS-PAGE of RNase glucosylation reactions after various times of incubation. RNase was incubated with glucose as described in Fig. 2. Aliquots were removed at indicated times for analysis by SDS-PAGE, as described under “Experimental Procedures;” gels are stained for protein with Coomassie blue. Molecular weight standards are phosphorylase \(a\) (94,000), bovine serum albumin (66,000), ovalbumin (43,000), and RNase A (13,700).

Fig. 4. Radioactivity profiles of SDS-PAGE lanes from \(^{125}\)I-RNase glucosylation mixtures. \(^{125}\)I-RNase (4 \(\times\) \(10^{6}\) cpm/mg) was glucosylated in 1 M glucose as described in Fig. 2. Gel lanes from SDS-PAGE of 50-\(\mu l\) aliquots taken at 0 (Fig. 3A) and 10 days (Fig. 3B) were sliced and counted for radioactivity as described under “Experimental Procedures.” Monomer, dimer, and trimer regions of the profile are indicated by \(M\), \(D\), and \(T\), respectively.

Fig. 5. Kinetics of RNase polymerization in 1 M glucose. Aliquots of the incubation mixture described in Fig. 4 were subjected to SDS-PAGE. Gel lanes were sliced and counted for radioactivity in the monomer, dimer, and trimer regions. Percentage of counts in each fraction was calculated by dividing radioactivity in that fraction by total radioactivity in all three fractions.
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We studied the cross-linking of nonradioactive GlcRNase concentration. cpm of ¹²⁵I-RNase was incubated in indicated millimolar concentrations of glucose at 37 °C. The polymer formed per day between day 0 and day 7 at each concentration was determined from the difference in the amount of polymerization shown in Fig. 6, order dependence on protein concentration. For this purpose, we established that, once protein glucosylation had occurred, the rate and extent of cross-linking reaction would involve condensation of glucosylated lysine on one RNase molecule with a free lysine residue on another. As discussed above, this scheme would also be consistent with the decline in the polymerization rate with increasing glucose concentration since, at high concentrations of sugar, the glucose may serve as an effective competitor for unglucosylated lysine residues. To test this pathway, we investigated the effectiveness of free lysine as a competitor for the polymerization reaction. As shown by the +lysine curve of Fig. 9, the inclusion of lysine in an incubation of GlcRNase with ¹²⁵I-RNase (absence of glucose) completely inhibited the cross-linking reaction. Thus, we propose that free lysine

![Graph A](https://via.placeholder.com/150)

**Fig. 6.** RNase polymerization as a function of glucose concentration. A, kinetics of polymerization. RNase (10 mg/ml, 5 x 10⁴ cpm of ¹²⁵I-RNase) was incubated in indicated millimolar concentrations of glucose at 37 °C. The 0 mM incubation represents a buffer control in which there was no detectable RNase polymer formed during the 21 days of the experiment. The amount of polymer (dimer + trimer) formed as a function of time was calculated after SDS-PAGE as described in Fig. 5. B, initial rate of the polymerization reaction as a function of glucose concentration. The initial rate of polymerization was determined from the difference in the amount of polymer formed per day between day 0 and day 7 at each concentration of glucose shown in A.

![Graph B](https://via.placeholder.com/150)

**Fig. 7.** RNase polymerization as a function of RNase concentration. A, kinetics of polymerization. RNase at the indicated concentrations (milligrams/ml; 8 x 10⁴ cpm of ¹²⁵I-RNase/ml) was incubated in 100 mM glucose at 37 °C. Per cent polymer formed as a function of time was calculated after SDS-PAGE as described in Fig. 5; milligrams of polymer were calculated by multiplying the per cent polymer times the amount of protein in each incubation. B, initial rate of the polymerization reaction as a function of RNase concentration. The initial rate was determined from the difference in the amount of polymer formed per day between day 0 and day 7 at each concentration of RNase shown in A. A least squares fit to the data yields a 1.1 order with respect to protein concentration.
petes with protein for the active cross-linking site on the glucosylated protein, and that the reaction between glucosylated and unglucosylated amino groups is the major pathway of protein cross-linking under physiological conditions in vitro.

**Cross-linking of Lysine to Glucosylated RNase: a Model for the Cross-linking Reaction**—The inhibition of the cross-linking reaction by lysine suggested that lysine might be reacting directly with glucosylated protein to form an analog of the cross-link structure between two proteins. To explore this possibility experimentally, we studied the reaction of Glc-RNase with [3H]lysine and N'-formyl-[3H]lysine. The experiments were carried out initially with radioactive lysine, and, when reaction of lysine with protein was observed, control reactions were conducted using N'-formyllysine to establish the reactivity of the ε-amino group. The incorporation of radioactive lysine and N'-formyllysine into protein was measured by chromatographing the reaction mixture on Sephadex G-25, and quantitating recovery of radioactivity at the void volume of the column. As shown in Fig. 10, both the free and α-blocked lysine reacted selectively with glucosylated RNase compared to native RNase, with approximately similar kinetics. SDS-PAGE of the void volume fraction from the 10-day time point, followed by measurement of radioactivity in the RNase band, showed that more than 90% of the 3H radioactivity applied to the gel was recovered in the RNase monomer region. Thus, the bond between [3H]lysine and glucosylated RNase was covalent and stable to electrophoresis under denaturing and reducing conditions. However, when either the lysine or N'-formyllysine derivative of glucosylated RNase was reduced with NaBH₄ and acid or base hydrolyzed, the primary radioactive product recovered by amino acid analysis was free lysine. These data indicate that the bond formed between lysine and glucosylated RNase is labile to hard acid or base hydrolysis. Thus far, we have also been unable to detect differences in radioactive or ninhydrin-reactive products isolated from glucosylated RNase monomer and dimer by acid or alkaline hydrolysis. Taken together, these results indicate that the cross-link structure between proteins is labile to hydrolysis in, strong acid or base. Alternative methods for labeling and isolating this cross-link structure are now being explored.

**DISCUSSION**

The studies described in this report may be distinguished from much previous research on the Maillard reaction in vitro which has emphasized reaction conditions substantially different from those found in vivo, e.g., solid phase reactions, high temperatures, and a range of pH values and solvents. In the present work, we have established a relatively simple model system, using the well characterized, soluble protein RNase A for studying the kinetics and products of nonenzymatic glucosylation and glucose-dependent cross-linking of protein under physiological conditions in vitro. By amino acid analysis, lysine residues were identified as the primary sites of glucosylation of RNase, in agreement with observations on the sites of glucosylation of numerous proteins in vivo (1, 2). Both the rate and extent of decrease in lysine residues paralleled the increase in radioactive glucose incorporation into the protein. However, after reduction and amino acid analysis, less than half of the modified lysine residues could be accounted for as HL + HL'. We have also observed this discrepancy between glucose incorporation and lysine loss in studies of the glucosylation of human albumin. The data suggest that acid-labile dehydration or rearrangement prod-

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**Fig. 8. Effect of glucose on the polymerization of Glc-RNase.** Glc-RNase was prepared as described under "Experimental Procedures." Two 1-ml aliquots of the protein solution were prepared, each containing 10 mg (5 x 10⁶ cpm) of protein. One was adjusted to 250 mM glucose concentration by the addition of solid glucose, and both were incubated at 37 °C. The amount of polymer formed as a function of time was calculated after SDS-PAGE as described in Fig. 5. A zero time value of 4% polymer reflects the fact that the reaction mixture contained some polymer generated during the initial glucosylation reaction.

**Fig. 9. Kinetics of Glc-RNase polymerization in the presence and absence of lysine.** Glc-RNase was prepared as described under "Experimental Procedures." Two reaction mixtures were then made, each containing 5 mg of Glc-RNase and 5 mg of [3H]-RNase (5 x 10⁶ cpm) in a final 1-ml volume. One mixture was adjusted to 100 mM lysine concentration and the other left unchanged. Both aliquots were then incubated at 37 °C and the amount of polymer formed as a function of time was calculated after SDS-PAGE as described in Fig. 5.
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Products are formed on the protein, subsequent to the initial glucosylation reaction, and suggest that measurements of the extent of glucosylation of proteins may be significantly underestimated by measurements of hexitol-lysine alone.

Kinetic analysis of glucose-dependent cross-linking of RNase has allowed us to draw some conclusions concerning the mechanism and biological significance of protein cross-linking by glucose. The observation of a first order relationship between protein concentration and cross-linking rate led to a series of experiments which established that, under physiological conditions, an isolatable but reactive glucose adduct is formed on the protein during nonenzymatic glucosylation. Thus, isolated glucosylated RNase continued to generate polymeric products or lysine adducts for 2-3 weeks after removal of free glucose from the incubation mixture (Figs. 8-10). These data suggest that short term increases in blood glucose in vivo may also exert a long term physiological impact by activating proteins for subsequent cross-linking reactions.

Studies with isolated glucosylated RNase also indicate that the primary mechanism for cross-linking involves the reaction between a glucosylated amino acid on one protein and an unmodified amino acid on the same or another protein. Thus, although dihydropyrazine compounds may be formed by condensation of two glucosylated amino acids (17) and are frequently isolated from Maillard-type reactions in vitro (5, 6), it is unnecessary to invoke these compounds as mediators of protein cross-linking under physiological conditions. Further, the fact that lysine forms a covalent adduct with glucosylated protein concomitant with its inhibition of protein polymerization (Figs. 9 and 10) also appears to exclude the commonly proposed cross-linking mechanism based on elimination of soluble, reactive dicarbonyl compounds from the protein (5, 6). It would be difficult to explain the condensation of free lysine with the protein (Fig. 10) by this mechanism. Thus, the large excess of free lysine in solution should not only trap the dicarbonyl compounds, but should also inhibit condensation of putative lysine-dicarbonyl adducts to protein, comparable to its efficiency in inhibiting protein-protein condensation in the same system. Overall, our results support the following type of reaction scheme for glucose-dependent cross-linking of protein under physiological conditions.

\[
\text{Glc} + \text{protein} \rightarrow \text{Glc-protein} \\
\rightarrow (\text{protein})_2 \\
\rightarrow \text{protein}
\]

The rate of cross-linking of RNase was observed to be markedly dependent on glucose concentration in the range 5-25 mM, which represents the range of blood glucose concentration from normoglycemia to severe diabetic hyperglycemia. Thus, protein cross-linking in vivo should also be affected by elevations in blood glucose in diabetes. There are, in fact, several studies which document decreased elasticity and solubility of collagens and lens crystallins in diabetes (18, 19), associated with increased levels of nonenzymatic glucosylation of these same proteins. However, the direct role of glucose in the in vivo cross-linking of these and other long lived proteins has not been established, in part because of difficulties in identifying the structures involved in the cross-link either in vivo or in vitro. Continued research with the RNase model system should be useful for characterizing the structure of glucose-protein adducts actually reactive in cross-linking, the structure of the cross-link, and the details of the pathway and mechanism of the reaction. Eventually, these studies in vitro should assist us in understanding the sequelae of nonenzymatic glucosylation of protein under physiological conditions, and assessing the significance of these reactions in vivo in diabetes.

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